The Role of the Autonomic Nervous System in Atherosclerosis

Targeting the Cholinergic Anti-inflammatory Pathway in Humans and Mice

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ABSTRACT

The autonomic nervous system (ANS) has been implicated in numerous atherosclerosis-induced cardiovascular disease, such as myocardial infarction and stroke. Although evidence suggests a relationship between autonomic dysfunction and atherosclerotic disease, the mediating mechanisms are still elusive. Considering the inflammatory pathophysiology of atherogenesis, we have investigated the role of nerve-driven immunity in this relationship, with focus on the alpha 7 nicotinic acetylcholine receptor (α7nAChR).

The link between ANS dysfunction, inflammation and prevalent disease was assessed in male subjects. The athero-protective effects of sympathetic inhibition and α7nAChR-signaling were investigated in atherosclerosis-prone mice, by β_1-blocker treatment with metoprolol, α7nAChR-stimulation with AZ6983, or by hematopoietic ablation of α7nAChR.

Our original contribution to knowledge includes data showing that inflammation could be a mediator in the association between dysfunction in the ANS and carotid atherosclerosis in humans, and that the athero-protective effects of metoprolol may include suppression of atherogenic cytokines. Further, for the first time, we show that α7nAChR-deficiency was associated with increased atherosclerosis, whereas α7nAChR-stimulation with AZ6983 reduced atherosclerosis and modulated both innate and adaptive immune responses. The α7nAChR was identified on immune cells in human carotid plaques, and stimulation by AZ6983 inhibited cytokine production in human blood, suggesting athero-protective effects of AZ6983 also in humans.

Taken together, our findings suggest that the balance between the sympathetic and parasympathetic branch of the ANS have an impact on atherosclerosis, and that inflammation is mediator. We propose that the α7nAChR is an interesting pharmacological target in this pathway.

Keywords: atherosclerosis, inflammation, autonomic dysfunction, ANS, alpha 7 nicotinic acetylcholine receptor, cytokines
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SAMMANFATTNING PÅ SVENSKA

Åderförfettning är en inflammatorisk sjukdom och den främsta orsaken till hjärt- och kärlsjukdomar. Åderförfettning startar ofta i tidig ålder och kan med tiden utvecklas till åderförfettningsplack som i allvarliga fall brister och leder till fatala komplikationer så som hjärtinfarkt och stroke. Rökning, höga kolesterolvärden, diabetes och högt blodtryck ökar risken för hjärt-kärlsjukdom, men studier visar även att förändringar i det autonoma nervsystemet kan vara en riskfaktor. Syftet med den här avhandlingen var att studera det autonoma nervsystemets roll i åderförfettning, med fokus på om inflammation kan vara en bidragande faktor.

Det autonoma nervsystemet reglerar kroppens icke viljestyrda funktioner så som andning, hjärtats rytm, kroppstemperatur, och delas in i det sympatiska nervsystemet, som är aktivt vid stress och kampsituationer, och det parasympatiska nervsystemet, som är aktivt vid vila. Tidigare humanstudier visar att det kan finnas en länk mellan dysfunktion i det autonoma nervsystemet och åderförfettning. Vi fann att orsaken kan vara att försämrad funktion av det autonoma nervsystemet leder till inflammation, som slutligen orsakar åderförfettning. Vi fann även att blockering av det sympatiska nervsystemet, genom metoprololbehandling, minskade både åderförfettning och nivå av cirkulerande inflammatoriska proteiner i möss. Tillsammans indikerar resultaten att inflammation påverkas av balansen mellan det sympatiska och det parasympatiska nervsystemet, som i sin tur har effekt på utvecklingen av åderförfettning.

Djurstudier har vid andra inflammatoriska sjukdomar visat på en skyddande effekt av att stimulera det parasympatiska nervsystemet, där alfa 7 nikotinacetylkolinreceptorn har visat sig vara en viktig del av mekanismen. Vi visar här att receptorn även fyller en funktion i åderförfettning. Tar man bort receptorn från benmärgsceller i möss så förvärras åderförfettningen, men stimulerar man receptorn med en agonist så sker förändringar i immunsystemet och sjukdomen minskar. Vi identifierade även receptorn på immunceller i åderförfettningsplack från människa, samt visade att behandling av humana blodceller med agonisten minskade produktionen av inflammatoriska proteiner. Detta visar att receptorn kan fylla en viktig funktion för åderförfettning även i människa.

Sammanfattningsvis antyder resultaten att balansen mellan det sympatiska och det parasympatiska nervsystemet har en påverkan på åderförfettning och att inflammation är en viktig mediator. Vi föreslår att alpha 7 nikotinacetylkolinreceptorn kan fylla en viktig funktion i åderförfettningsprocessen.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their corresponding Roman numerals.

I. **Ulleryd MA**, Prahl U, Börsbo J, Schmidt C, Nilsson S, Bergström GML, Johansson ME. The association between autonomic dysfunction, inflammation and atherosclerosis in men under investigation for carotid plaques. *Manuscript under review*.

II. **Ulleryd MA**, Bernberg E, Yang LJ, Bergström GML, Johansson ME. Metoprolol reduces proinflammatory cytokines and atherosclerosis in ApoE$^{-/-}$ mice. *BioMed Research International*. **2014**; 548783

III. Johansson ME, **Ulleryd MA**, Bernardi A, Lundberg AM, Andersson A, Folkersen L, Fogelstrand L, Islander U, Yan ZQ, Hansson GK. alpha7 Nicotinic acetylcholine receptor is expressed in human atherosclerosis and inhibits disease in mice. *Arteriosclerosis, thrombosis, and vascular biology*. **2014**; 34: 2632-2636

IV. **Ulleryd MA**, Panagaki D, Yang LJ, Michaëlsson E, Nilsson H, Johansson ME. The alpha nicotinic acetylcholine receptor (α7nAChR) agonist AZ6983 reduces atherosclerosis in ApoE$^{-/-}$ mice and reduces inflammatory cytokines in human blood. *Manuscript in preparation*.

CONTENT

ABBREVIATIONS

ACh	Acetylcholine
ANS	Autonomic nervous system
APOE	Apolipoprotein E
BiKE	The Biobank of Karolinska Endarterectomies
BMI	Body mass index
BMT	Bone marrow transplantation
BP	Blood pressure
BrdU	Bromodeoxyuridine
BRS	Baroreceptor sensitivity
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
CO2	Carbon dioxide
CRP	C-reactive protein
CVD	Cardiovascular disease
DAB	3,3´–Diaminobenzidine
DNA	Deoxyribonucleic acid
ECG	Electrocardiography
ELISA	Enzyme-linked immunosorbent assays
GROα	Growth-regulated oncogene-α
GTS-21	3-(2,4-dimethoxybenzylidene)-anabaseine
GUVASC	The Göteborg and Umeå Vascular study group
HDL	High density lipoprotein
HRV	Heart rate variability
ICAM-1	Intracellular adhesion molecule 1
IFNγ	Interferon gamma
IL-“X”	Interleukin “X”
JAK2-STAT3	Janus kinase-2 and signal transducer and activator of transcription 3
KO	Knockout

LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LN	Lymph node
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MI	Myocardial infarction
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-κb
oxLDL	Oxidized low density lipoprotein
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMT	Photomultiplier tube
PNS	Parasympathetic nervous system
qPCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Real-time polymerase chain reaction (reverse transcription)
SD	Standard deviation
SDNN	Standard deviation of r-r intervals
SEM	Standard error of the mean
SNS	Sympathetic nervous system
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor α
WBCC	White blood cell count
VCAM-1	Vascular cell adhesion molecule 1
WINGA	Western Region Initiative to Gather Information on Atherosclerosis
VLDL	Very low density lipoprotein
WT	Wild type
α7nAChR	Alpha 7 nicotinic acetylcholine receptor

1 INTRODUCTION

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death in the world, accounting for 32% of global mortality in 2012 (1). Although existing treatment methods are becoming more effective, the prevalence is still increasing (1). CVD encompasses a range of diseases that involve the heart and blood vessels. Many of these conditions, including stroke, peripheral artery disease and coronary artery disease (CAD), result from the formation of atherosclerotic lesions in the vessels. Together, stroke and CAD accounted for 78% of all cardiovascular deaths in 2008 (2), while non-fatal events can cause major sequelae, such as paralysis. The severe complications of atherosclerosis call for extensive research on the underlying risk factors, and on the mechanisms involved, for future prevention and pharmacological therapies.

1.2 Atherosclerosis

1.2.1 Overview

Atherosclerosis is a slowly developing disease that affects large and medium sized vessels, and is characterized by thickening of the endothelium-containing intima, the innermost layer of the arterial wall. Lesions are predominantly formed in vessel segments where blood flow is disrupted, *e.g.* near branch points and curvatures (3). Disease progression can start early in life (4), and strong evidence suggests that the process is initiated by the infiltration, retention and oxidation of low density lipoprotein (LDL), causing fatty streaks in the intima (5). Fatty streaks are not enough to cause any symptoms, however persisted inflammation in the vessels can turn fatty streaks into larger atherosclerotic plaques (6). Importantly, the lumen diameter can remain unaltered by outward remodeling of the vessel wall (7), not causing blood flow to be affected. Although this compensation is possible to a certain degree, larger lesions will eventually encroach upon the lumen and cause stenosis. The severity of the stenosis can also be dependent on shrinkage of local vessel segments (8). Vascular stenosis can be asymptomatic or cause symptomatic conditions, such as angina pectoris. Importantly, atherosclerosis is rarely fatal until a thrombotic occlusion appears, usually caused by plaque rupture, exposing pro-thrombotic content to the circulating blood (3). Depending on the location of the obstruction, an

occluded artery can cause life-threatening events, including stroke, myocardial infarction (MI) and heart failure.

In order to predict CVD outcomes and to develop new treatment methods, extensive efforts have been devoted to identifying the underlying risk factors for atherosclerosis. The major risk factors are smoking, diabetes, hyperlipidemia and hypertension (9). These are often attributed to secondary risk factors such as lifestyle and obesity, but also immutable parameters including age, heredity and gender. In addition, CVD has been associated with other conditions that implicate the autonomic nervous system, such as psychosocial stress (10, 11), and autonomic dysfunction (12-14).

1.2.2 Inflammatory initiation of atherosclerotic lesions

The initiation and progression of atherosclerosis is complex and the mechanisms are still not fully understood. As previously described, atherosclerosis is initiated by the intimal infiltration and oxidation of LDL. Bioactive LDL induces the endothelium to express cell-adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1), and macrophage colony-stimulating factor (M-CSF) (15, 16). As reviewed by Hansson et al. circulating leukocytes, predominantly monocytes and T-cells adhere to VCAM-1 and enter the arterial intima, a process induced by cytokine production from vascular cells (17). Under the influence of M-CSF, monocytes differentiate into macrophages with the capability to accumulate oxidized LDL (oxLDL) through scavenger receptors (17). Macrophages containing intracellular lipids can transform into foamy structures, also known as foam cells (18), which are the major constituents of fatty streaks and early lesions (19). oxLDL also binds to Toll-like receptors (TLRs) on macrophages, promoting production of pro-inflammatory cytokines, such as TNFα (20). Antigen presenting cells, including macrophages, can activate T-cells by introducing oxLDL through major histocompatibility complex class II. Once activated, a main feature of atherosclerotic T-cells is the production of interferon gamma (IFNγ) (16), a pro-inflammatory cytokine that further activates macrophages and endothelial cells in the vessels (17). Continued inflammation accelerates the infiltration and activation of monocytes and T-cells, promoting fatty streaks to develop into more advanced lesions with a lipid-containing necrotic core, covered by a fibrous cap of collagen and smooth muscle cells (6). Lesions that are prone to cause thrombosis are commonly referred to as vulnerable plaques, and there are numerous factors that are considered to indicate such a phenotype, *e.g.* a thin fibrous cap, necrotic core, macrophage density and lesion size (3).

1.3 The autonomic nervous system

The autonomic nervous system (ANS) is the major system responsible for homeostasis and in general acts independently of voluntary control. The medulla oblongata and hypothalamus accounts for the central parts of the ANS, receiving information about the state of the body through afferent nerves, and sending compensatory signals through efferent nerves (21). The two branches of the ANS are the sympathetic (SNS) and parasympathetic nervous system (PNS), two pathways with frequently antagonistic responses (22). The major neurotransmitter in SNS is norepinephrine, which binds to the α- or β-subtype of adrenergic receptors (23). In the PNS, acetylcholine (ACh) is the primary neurotransmitter and binds to different subtypes of muscarinic and nicotinic receptors (23).

1.3.1 ANS in the cardiovascular system

The cardiovascular system includes the heart and blood vessels, which are innervated by both SNS and PNS. The ANS is responsible for maintaining an adequate supply of oxygenated blood to different tissues depending on the requirements (24). Sensory information about the systems homeostasis is mainly detected through two types of receptors. Baroreceptors are located in the heart and major blood vessels where they are activated by stretching of the vessel wall, deriving from changes in blood pressure. Chemoreceptors are mainly located in small clusters, also known as carotid bodies, close to the bifurcation of the carotid artery. Chemoreceptors detect the composition of circulating arterial blood, mainly the partial pressure of oxygen and carbon dioxide (24). Afferent sensory information is received by the nucleus of the solitary tract (NTS) in the medulla oblongata, via the vagus nerve, and routed to the hypothalamus and the reticular formation (24). In response to received information, efferent regulatory output is transmitted to ganglia of the specific motor pathways and conveys necessary changes in the cardiovascular system (24). For example, increased arterial and venous pressure inhibits sympathetic outflow, and activates parasympathetic outflow, resulting in reduced heart rate and vasodilation of peripheral vessels, ultimately leading to a reduction in blood pressure. In contrast, a decrease in blood pressure will have the opposite effects on SNS and PNS, ultimately leading to an increase in blood pressure. This reflex is mainly mediated by baroreceptors, and is only mildly influenced by chemoreceptor activity (24).

1.3.2 ANS in cardiovascular disease

Numerous clinical studies have shown a relationship between modulated autonomic function and CVD. Autonomic dysfunction is associated with an

elevated risk of cardiovascular events (14), increased mortality after MI (13, 25), atrial fibrillation (26), and progression of carotid atherosclerosis, independent of other traditional risk factors (12). Although the majority of these studies investigate the association between CVD and general ANS dysfunction, assessed by measuring heart rate variability using the standard deviation of RR-intervals (HRV SDNN), there are studies suggesting reduced parasympathetic activity as the main contributor (27).

Abnormalities can occur in either of the two branches of ANS. Numerous studies address the relationship between augmented activity of the SNS and increased risk for CVD. Psychosocial stress, such as lack of social support and depression, drives sympathetic activity (28), and is associated with cardiac mortality after MI (29). In addition, studies report that psychosocial stress prospectively could predict cardiovascular mortality and stroke (30). The effects of psychosocial stress have also been investigated in animal models, showing accelerated atherosclerosis in cynomolgus monkeys and mice living in an unstable environment (31-33). Studies have investigated the effects of targeting this route with pharmacological interventions. Inhibition of sympathetic drive using treatment with β-adrenergic antagonists (β-blockers), such as metoprolol, reduces mortality in patients with hypertension and heart failure (34, 35). Also, evidence suggests that β-blockers could have a direct protective effect on atherosclerosis (36-38).

It has been reported that the background level of parasympathetic activity significantly influences sympathetic control of heart rate. Studies in both animals and humans show that increased vagal activity may reduce sympathetic drive on the heart (39, 40). The effect of induced parasympathetic activity has been further studied in different animal models. Vagal stimulation was reported to be antiarrhythmic, and protected against ventricular fibrillation and sudden death after MI (41-43). In summary of the presented literature, evidence points to an increased risk for cardiovascular mortality in the presence of sympathetic drive, and a possible protective role for increased parasympathetic activity, yet the mediating mechanisms still remain unclear.

1.3.3 A mediating role for the immune system

The increased cardiovascular risk in the presence of increased sympathetic activity, and the corresponding protective effects of β-blockers, is commonly explained by their complex influence on traditional risk factors. Sympathetic drive induces a sustained increase in blood pressure (44), one of the major risk factors for CVD. However, studies in patients with hypertension and

high sympathetic tone shows that the increased risk of coronary artery disease can not be fully attributed to blood pressure elevation alone (45), also suggesting other links between ANS modulation and CVD, one of them being inflammation (46).

The concept of inflammation is central in atherosclerosis, and suggested to provide a mechanistic link between some of the traditional risk factors, such as obesity and smoking, and the progression of atherosclerosis (47). Interestingly, the conditions discussed in this thesis involving dysregulation of the ANS, not only affect CVD and atherosclerosis, but also inflammation. Numerous studies report that autonomic dysfunction associates with increased levels of inflammatory markers such as the cytokine IL-6, and general inflammatory markers C-reactive protein (CRP) and white blood cell count (WBCC) (48-51). IL-6 is upregulated in chronic inflammation and plays a central role in atherogenic pathways (52), while increased levels of WBCC and CRP can predict CVD (53-55), and are directly associated with atherosclerosis (56-60). Further, upregulation of the SNS in psychosocial stress is reported to be associated with increased proliferation of neutrophils and inflammatory monocytes in mice (61), as well as with increased activity of nuclear factor-κB (NF-κB) in animals and humans (62, 63). NF-κB activates multiple target genes that are associated with the development of atherosclerosis (64). Moreover, inhibition of NF-κB attenuates atherosclerosis in mice (65).

It should also be noted that sympathetic blockade has been reported to present anti-inflammatory effects. Treatment with β-blockers was associated with lower CRP in patients with CAD (66), and reduced levels of inflammatory cytokines in patients with dilated cardiomyopathy (67). This could possibly be added to the cardio-protective effects of β-blockers.

In addition to this concept, the last decade provided a number of studies describing an anti-inflammatory reflex in response to vagal activity, which will be discussed in the next chapter. The evidence for a nerve-driven effect on both inflammation and atherosclerosis demands further studies on the interrelationship between these factors and the causative direction of proven associations.

1.4 The cholinergic anti-inflammatory pathway

In the developing field of nerve-driven immunity, convincing data proposes an anti-inflammatory response to vagal stimulation, presenting a promising target for treatment of inflammatory disease. This route is called “the

cholinergic anti-inflammatory pathway" (68), and emerged in 2000 when Borovika et al. showed that acetylcholine attenuated the release of inflammatory cytokines in human macrophage cultures, and that TNFα production in liver and serum was inhibited by electrical stimulation of the vagus nerve in endotoxemic rats (69). Since then, electrical vagal nerve stimulation has repeatedly been used to study this route, showing anti-inflammatory responses in different experimental disease models, such as acute cerebral ischemia and reperfusion injuries, sepsis and colitis (70-72).

1.4.1 The alpha 7 nicotinic acetylcholine receptor

The alpha 7 nicotinic acetylcholine receptor (α7nAChR) is a ligand-gated ion channel forming a homomeric pentamer of α7 subunits, and is one of the most abundant nicotinic receptors in the brain (73). Apart from the neuronal circuit, α7nAChR have been identified in human immune cells, *e.g.* mononuclear leukocytes (74), B-lymphocytes (75), and basophils and mast cells (76).

Signaling through α7nAChR was early on proposed to play an important role in the mechanism of the cholinergic anti-inflammatory pathway (77). Studies showed that electrical stimulation of the vagus nerve inhibited TNFα production in wild type mice, however this was not achieved in mice lacking the α7nAChR (77). The α7nAChR-mediated inhibition of TNFα was further attributed to suppression of cytokine-producing macrophages in the spleen (77).

A role of the spleen

The spleen has been further implicated in the cholinergic anti-inflammatory pathway. Experiments have described that splenectomy in rodents abolished the inhibitory anti-inflammatory effects of vagus nerve stimulation (70). The vagal nerve is not directly linked to the spleen; instead it ends in the celiac-mesenteric ganglia where it synapses with the sympathetic splenic nerve (78). The splenic nerve is solely responsible for the neuronal input to the spleen (79, 80), and it has been proposed that cholinergic firing of the vagus nerve, ultimately triggers release of norepinephrine in the spleen (81, 82). However, the release of norepinephrine fails to explain the activation of α7nAChR on splenic macrophages. To assemble this pathway, norepinephrine has been proposed to activate β-adrenergic receptors on resident T-cells with ACh-producing properties (83). Locally synthesized ACh ultimately binds to α7nAChR on macrophages, inhibiting their production of TNFα (77). In addition, studies also revealed that the α7nAChR is essential for vagus nerve-mediated norepinephrine release in the spleen (82).

α7nAChR in experimental disease models

Interest regarding α7nAChR in inflammation has emerged in a number of different experimental studies using pharmacological agonists to investigate possible effects on immune related disease. 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) is partially selective for α7nAChR, and one of the most frequently used agonists. Treatment with GTS-21 has been associated with improved outcomes in pancreatitis (84), sepsis (85), and ischemia-reperfusion models (86). In addition, the α7nAChR agonist ARR-17779 attenuates arthritis in mice (87). Whether these effects are mediated through activation of α7nAChR expressed on the celiac-mesenteric ganglia, or directly via receptor-activation on immune cells, is not yet determined. The beneficial effect of α7nAChR-stimulation in different inflammatory conditions raises the question of whether treatment could also improve outcomes in atherosclerotic disease. A recent study showed that treatment with the α7nAChR agonist PNU-2822987 reduced TNFα, IL-1β, and IL-6 in the heart and aorta of spontaneously hypertensive rats (88). Interestingly, these cytokines are involved in the pathophysiology of atherosclerosis (16).

The detailed intracellular-mechanisms of α7nAChR-signaling are still far from understood. *In vitro* studies have indicated that the anti-inflammatory properties could be mediated by inhibition of the transcription factor, NF-*κ*B (89, 90). Moreover, *in vivo* models have implicated the Janus kinase-2 and signal transducer and activator of transcription 3 (JAK2-STAT3) pathway (91), further supported by studies showing that GTS-21 inhibition of cytokine production in human whole blood is dependent on JAK2-STAT3 (92).

2 AIM

The overall aim of this thesis was to investigate the role of the autonomic nervous system in the development of atherosclerosis. Our particular focus has been to test the hypothesis that inflammation is a mediator in this relationship, and that the cholinergic anti-inflammatory pathway is an important regulator in this context.

In this thesis, we specifically aimed to investigate:

- If autonomic dysfunction is related to atherosclerosis due to an independent association with inflammation (Paper I)

- If the athero-protective effect of sympathetic blockade with metoprolol involves inhibition of atherogenic cytokine production in mice (Paper II)

- If α7nAChR-deficiency increases atherosclerosis, and if stimulation of α7nAChR decreases atherosclerosis (Paper III and IV)

- If α7nAChR is localized in human carotid tissues and have a functional role in human blood cells (Paper III and IV)

3 METHODOLOGICAL CONSIDERATIONS

3.1 Study populations and biopsies

All studies on human subjects were conformed according to the ethical guidelines of the 1975 Declaration of Helsinki, and reviewed and approved by regional ethical boards. All subjects gave written informed consent to participate in the studies.

3.1.1 Patients under investigation for carotid atherosclerosis (Paper I)

Male patients (n=124, ≥40 years), under investigation for carotid atherosclerosis were enrolled from Western Region Initiative to Gather Information on Atherosclerosis (WINGA, http://wlab.gu.se/bergstrom/winga) to further investigate autonomic function and inflammatory status. WINGA provides records of patients undergoing diagnostic carotid ultrasound examinations due to minor stroke- or TIA-suspected symptoms within the Gothenburg region in Sweden. Subjects with rheumatoid disease, WBCC above $30x10^9$ cells/L, CRP above 10 mg/L, or unsuccessful assessment of autonomic function were excluded.

3.1.2 Carotid plaque specimens for histology (Paper III)

Patients with symptomatic and severe carotid plaques can be treated by endarterectomy, a surgical procedure to remove atherosclerotic material from the aortic wall. Several clinical trials concluded that the degree of stenosis is relevant for risk assessment in these conditions, and endarterectomy is suggested to patients with a stenosis degree of more than 80% (93). Atherosclerotic carotid specimens from this type of surgeries were provided to us from the Göteborg and Umeå Vascular study group (GUVASC, http://wlab.gu.se/bergstrom/guvasc) for histology (n=10).

3.1.3 Gene profiling in human plaques (Paper III)

The Biobank of Karolinska Endarterectomies (BiKE) at Karolinska University Hospital, Stockholm, Sweden, was used for analysis of gene expression in atherosclerotic specimens (n=107). BiKE contains information on RNA, DNA, *in situ* and *in vitro* analysis of atherosclerotic specimens, clinically removed based on the same criteria as described above. In addition,

the BiKE-database also provides plentiful clinical information, such as medications and anthropometric data.

3.1.4 Blood samples from healthy subjects (Paper IV)

Blood samples for *in vitro* stimulations were collected from healthy donors of mixed age and gender (n=12), from the blood bank, Droppen, at the Sahlgrenska University Hospital in Gothenburg. All subjects were non-smokers.

3.2 Assessment of autonomic function (Paper I)

Paper I assessed the relationship between autonomic function, inflammation and atherosclerosis by measuring the cardiac response to input from the autonomic nervous system (ANS). In the absence of sympathetic or parasympathetic input, the sinus node has an intrinsic rate of depolarization, producing an intrinsic heart rate (94). In reality, heart rate is dynamically affected by input from the ANS, determining the real heart rate (95). Heart rate variability (HRV) and baroreceptor sensitivity (BRS) are well established, and the most frequently used methods, to assess the dynamic autonomic function in regulating cardiac frequency.

3.2.1 Heart rate variability

HRV is the physiological variation in heartbeat intervals and can be assessed by a number of different methods. HRV measurements in the time domain evaluates the QRS complex intervals from continuous electrocardiography (ECG) recordings, ranging from 5 minutes to 24 hours (96). In Paper I, HRV was assessed using short-term recordings of 20 minutes in supine position, and analyzed by calculating the standard deviation of RR-intervals (SDNN). HRV-measurements can reflect different types of input from the autonomic nervous system. However, SDNN is commonly used as a marker for both sympathetic (SNS) and parasympathetic activity (PNS) (96).

3.2.2 Baroreceptor sensitivity

The arterial baroreflex is the physiological response of heart rate and vascular resistance after acute changes in blood pressure (BP). Baroreceptors are constantly providing the central nervous system with information on changes in BP, dynamically modulating the ANS to minimize the variations (97, 98). A rise in BP increases PNS-activity and decreases SNS-activity, resulting in decreased heart rate, contractility and peripheral vascular resistance. In

contrast, reduced BP will have the opposite effects. In paper I, function of the baroreflex was evaluated by measuring spontaneous BRS in the time domain. Spontaneous BRS is defined as the change in heart rate in response to spontaneous changes in BP. BRS was assessed with the sequence method, where three consecutive heart beats with the RR-interval following a change in BP defines a sequence (99). Each sequence was analyzed by linear regression, and the average was calculated. The response time between the PNS and the SNS is significantly different, with an almost immediate response for the PNS and a slower response for the SNS (100). Thus, this fast beat-to-beat measure of BRS is regarded as marker of parasympathetic function (100).

3.3 Clinical parameters (Paper I)

To establish whether a factor independently can predict an outcome, it is necessary to adjust for other possible co-founders. In Paper I, we aimed to investigate if there was an independent association between autonomic function and inflammation, and between inflammation and carotid atherosclerosis. Thus, traditional risk factors for both inflammation and atherosclerosis were adjusted for, including prevalent CVD, diabetes, smoking, body mass index, hypertension, systolic blood pressure, cholesterol-lowering medicine, age and antihypertensive medicine.

3.4 Animal models of atherosclerosis (Papers II-IV)

Medical research uses mice extensively because of their many advantages, including quick reproduction, small size, easy maintenance, low costs, and well-characterized genetic background. However, mice do not spontaneously develop atherosclerotic lesions, and need to be genetically modified before they can be used in experimental models. The reason for this is probably that, in contrast to humans, circulating cholesterol in mice is high in LDL and low in HDL (101-103). By deletion of specific genes the lipid profile can be altered, providing strains that are prone to developing atherosclerosis. Mice are convenient to use in studies of diseases with slow progression, due to their short life span. In humans, atherosclerosis is a disease with a slow development, starting early in life and eventually leading to clinical events late in life. In mouse models, severe atherosclerosis can be developed in a couple of weeks instead of decades. One of the limitations with mice is that the small size of the animal prevents extensive collection of tissue and sample volumes.

3.4.1 Atherosclerosis-susceptible mouse strains

In atherosclerosis studies, two of the most commonly used mouse models are the apolipoprotein E-deficient ($ApoE^{-/-}$) mouse and the low-density lipoprotein receptor deficient ($LDLr^{-/-}$) mouse. Both models have been used in this thesis.

$ApoE^{-/-}$ Mice (Papers II and IV)

In 1992, the $ApoE^{-/-}$ mouse was the first genetically modified strain to be introduced in our research field (104). Apolipoprotein E (APOE) is a cholesterol and lipid carrier protein, essential for lipid metabolism (105). APOE-deficiency provides a mouse model with spontaneous hypercholesterolemia, especially in remnants of VLDL and chylomicrons(104, 106), and atherosclerosis (101, 104, 106). A drawback with the $ApoE^{-/-}$ mouse is that the lipid profile is different from humans, where most of the plasma cholesterol is transported as LDL. Importantly, initiation and progress of atherosclerosis with fatty streaks developing into advanced plaques with a fibrous cap appears to resemble the morphological features seen in human atherosclerosis (107). Since the primary focus of this thesis was to characterize the development of atherosclerotic lesions, $ApoE^{-/-}$ mice were used in Paper II and IV.

$LDLr^{-/-}$ Mice (Paper III)

In contrast to $ApoE^{-/-}$ mice, cholesterol-enriched food is necessary for $LDLr^{-/-}$ mice to develop hypercholesterolemia and atherosclerosis. The LDLr is expressed on liver cells and is important for clearing the blood of lipoprotein particles, by binding to APOE. Unlike $ApoE^{-/-}$ mice, $LDLr^{-/-}$ mice display a lipid profile that is more similar to humans, where cholesterol is mainly confined to the LDL fraction (108). The morphology of early stage atherosclerosis is considered to resemble the human counterpart, however late stage atherosclerosis has not been fully described (109). $LDLr^{-/-}$ mice are preferable in bone marrow transplantation (BMT) studies, which will be further discussed in a subsequent chapter. Thus, $LDLr^{-/-}$ mice were used in Paper III.

3.4.2 Diet induced hypercholesterolemia (Papers II-IV)

$LDLr^{-/-}$ mice has to be fed a cholesterol-enriched or high fat diet in order to develop atherosclerosis, and even though $ApoE^{-/-}$ mice spontaneously develop plaques, this process can be further accelerated by such food manipulations (106). The cholesterol-enriched diet used in Paper III contains

15% fat and 1.25% cholesterol, whereas the high fat diet in Paper II and IV contains 21% fat and 0.15% cholesterol.

3.4.3 α7nAChR deficient mice (Paper III)

Different methods are used to investigate the physiological role of a protein in mice. One of the common techniques is to over- or under-express the protein of interest and compare the physiological response to control mice. To investigate the role of a protein in the development of atherosclerotic lesions the expression needs to be modified in atherosclerosis-prone mice. In this thesis, the functional role of the α7nAChR was investigated in under-expressed $LDLr^{-/-}$ mice.

Bone marrow transplantation (Paper III)

Bone marrow transplantation (BMT) transfers the hematopoietic genotype from a donor to a recipient. The transferred genotype will only be adopted in bone marrow-derived cells, and the original genotype remains unaltered in all other cells. Since the atherogenic effect of LDLr-deficiency derives from its expression on liver cells, $LDLr^{-/-}$ mice are well suited for BMT, with the advantage that the donor does not need to be $LDLr^{-/-}$ for the mouse to develop atherosclerosis. After BMT in $LDLr^{-/-}$ mice, it takes about 8-12 weeks for the white blood cell count return to normal (110). It is reported that $LDLr^{-/-}$ mice after BMT display larger plaques in the aortic root, but smaller plaques in the thoracic aorta, compared to $LDLr^{-/-}$ mice unexposed to BMT (110). This is important to remember when comparing lesion morphology with studies using other techniques for protein depletion. In Paper III, recipient bone marrow was depleted with 2 doses of irradiation, then received bone marrow from $\alpha7nAChR^{-/-}$ mice or wild type mice (WT), and recovered for 4 weeks before being fed a cholesterol-enriched diet.

Double-knockout mice (Paper III)

A common method to create a mouse with the characteristics of two or more transgenic models is by cross breeding. These mice will eventually express the combined genotype, which can be further maintained in a breeding facility. In Paper III, an atherosclerosis-prone mouse with α7nAChR deficiency was used by cross breeding homozygous (-/-) LDLr mice with heterozygous (+/-) α7nAChR mice. An $LDLr^{-/-}/\alpha7nAChR^{+/-}$ breeding generates offspring where all mice are $LDLr^{-/-}$ but can be either homozygous, heterozygous or wild type (+/+) for α7nAChR. The great advantage with a heterozygous breeding is that you will have littermate controls for your double knockout (KO) mice, avoiding the risk of genetic drift, thus providing more reliable comparisons than when using WT mice from a separate

breeding (111). However, the method generates extra burden and lower yield than maintaining separate breeding for KO- and WT -mice.

3.5 Drug administration (Papers II and IV)

Reliable drug administration is crucial to ensure appropriate concentrations and the desired response throughout the experimental period. The preferred route of administration is dependent on many factors, *e.g.* pharmacokinetic properties of the substance, site of action, and duration of the treatment period. Animal experiments on atherosclerosis usually expand over weeks to months. The duration of these experiments makes administration techniques with low researcher interventions favorable, such as implanted minipumps or administration via food and drinking water. Injections are invasive, and usually need to be repeated at a high frequency to maintain a steady concentration. Besides being time consuming, and causing repeated disturbance to the animals, there is also a risk of injecting the drug into the wrong compartment. Independent of drug administration methods, successful treatment is preferably verified by drug concentration measurements.

3.5.1 Osmotic minipumps (Papers II and IV)

In Paper I and IV (8-week study), metoprolol or α7nAChR-agonist were administered by subcutaneous implantation of osmotic minipumps on the back of the mouse, through a small incision on the neck. The operation was performed during 5-10 minutes of anesthesia with isoflurane, and buprenorphine for postoperative analgesia. Osmotic minipumps have the benefit of continuously delivering an even amount of drug so that steady state concentrations can be achieved. Even though the method is invasive, no further interventions are necessary after the implantation. A disadvantage with osmotic minipumps is the short duration of 4-6 weeks, necessitating replacement of the pumps during long-term experiments. In this thesis, minipumps were replaced once during the experiments.

3.5.2 Drug-admixed food method (Paper IV)

In Paper IV, the drug-admixed food method was used to give the α7nAChR-agonist in the 12-week study. Drug-admixed food is a non-invasive method with the advantage of not disturbing the animals during the treatment period. The drug can be mixed into the appropriate diet and fed to the mice using ordinary routines. A disadvantage with drug-admixed food is that the appropriate dose is calculated based on the estimated food intake, and any deviation in consumption can affect the desired effect, or cause variations in exposure between the animals. This can be controlled for during the

experiment by measuring food consumption, but requires mice to be in separate cages. This type of control was used in Paper IV.

3.6 Physical stressor (Paper II)

In Paper II, the appropriate dose of metoprolol was verified by a dose-response study on the heart rate effects after air-jet induced stress. Mice were placed in a special cage where they were repeatedly exposed to jet streams of compressed air for randomized time and recovery periods (2-10 min) for a total duration of 2 hours. This method has previously been shown to effectively increase both heart rate and blood pressure (112).

3.7 Electrocardiography in mice (Paper II)

Anesthesia is associated with depressed autonomic control and basal cardiac function, *i.e.* reduced heart rate (113). Therefore, heart rate is preferably measured in conscious mice during cardiovascular experiments, and telemetry has become the gold standard in blood pressure measurements (114). In paper II, ECG with radiotelemetry transmitters was used to measure conscious heart rate in mice. Transmitters were implanted in the abdomen and ECG electrodes were placed under the skin. Recordings were conducted in freely moving mice by detectors placed under the cage. An advantage with radiotelemetry, in comparison to non-invasive methods is that no restrain of the animals is needed, and there is no disturbance from researchers during the measurements which provides reliable data (115). Disadvantages are the demand for surgical skills and expensive equipment.

3.8 Histology

Histology is an essential technique to examine the morphology of a specimen, or the distribution and localization of proteins and cells in a tissue. The examination is conventionally performed under a microscope, usually after a preservative treatment, and enhances visualization of structures using different staining techniques.

3.8.1 Atherosclerosis quantifications (Papers II-IV)

The assessment of atherosclerosis is usually determined by either *en face* quantification in the aorta, or cross-sectional quantification in the aortic root. Both methods are based on the staining of lipid deposits in the plaques, providing a better definition of the lesions. The degree of atherosclerosis in different parts of the aorta have been reported to show a strong correlation in

some studies (116), while other studies indicate only a weak correlation (102). If possible, quantifications in different compartments can be of value. In order to compensate for differences in vessel size, the amount of plaque is usually normalized to the total area of the aorta, or to the circumference of the aortic root.

En face quantification (Paper II and IV)

To perform *en face* quantification lipids are stained on the intimal surface of an open vessel. This method only gives a 2-dimensional assessment of a 3-dimensional structure and no information on height, composition or developmental stage. In Paper II and IV, *en face* quantifications were performed in longitudinally opened thoracic aortas, and stained with Sudan IV for lipids.

Cross-sectional quantification (Papers II-IV)

A cross-sectional quantification is conventionally conducted in serial sections of a vessel. This technique allows for more extensive and volumetric measurements on the severity of atherosclerosis, including the height of the plaque and how much of the vessel lumen is covered by lesions. Another advantage is the possibility to investigate the morphology, developmental stage, and immunological composition of the plaques using different staining techniques. In Paper II-IV, cross-sections from 6 (Papers II and IV) or 8 (Paper III) different levels, 100-800 μm from the aortic sinus, were stained with Oil Red O for lipids.

3.8.2 Immunohistochemistry (Papers II-IV)

The first immunohistochemistry study was reported in 1942 (117), and has now become one of the most frequently used methods to identify and quantify proteins in tissue. Immunohistochemistry involves the use of biochemical and immunological techniques to visualize a protein through the binding of a target-specific antigen to a labeled antibody. The choice of antibody is essential, and they are produced to be either polyclonal or monoclonal. Both types have their separate advantages and disadvantages (118, 119). Polyclonal antibodies detect multiple epitopes on the antigen with the advantage of a stronger signal due to multiple binding sites. Affinity for multiple epitopes also provides these antibodies with higher tolerance for changes in the antigens. However, polyclonal antibodies are in general less specific due to cross-reactivity and can have a large batch-to-batch variability. Monoclonal antibodies are aimed at a single epitope on the antigen with the advantage of increased specificity, and low background. However, monoclonal antibodies are sensitive to chemical influence to the

epitope, for example caused by fixation. In the direct method, the primary antibody is already labeled and can be detected in a microscope without further processing. The indirect method involves the use of a secondary antibody, directed against the IgG of the animal species in which the primary antibody was produced. This allows for the flexibility of using different detection methods using the same primary antibody. The secondary antibody can be labeled with a fluorochrome, or an enzyme that can be further oxidized by a substrate to produce colorimetric stainings.

Immunoperoxidase (Papers II-IV)

In this thesis, immunoperoxidase staining has been used to characterize the atherosclerotic plaque composition, or receptor localization in humans (Paper III) and mice (Paper II-IV). A biotinylated secondary antibody was added to the specimens after incubation with the primary antibody, followed by Vectastain ABC reagent (Vector Laboratories). Vectastain ABC reagent forms an Avidin/Biotin enzyme complex with the biotin on the secondary antibody, and can be detected in a brightfield microscope after a reaction with an organic substrate. In this thesis, either 3,3´–Diaminobenzidine (DAB), or NovaRED (Vector Laboratories) was used for colorimetrical detection. Depending on the type of marker, the target was quantified by either counting the total number of positive stained cells, or by a software filter that measures the stained area. An advantage with peroxidase stainings is the possibility to get a structured overview of the tissue, thus providing the location of the target to recognizable areas and structures.

Immunofluorescence (Paper III)

Immunofluorescence allows for the detection of several antibodies in the same specimen. Primary antibodies are labeled with different fluorochromes, either direct or indirect as described earlier, and can be detected in a light microscope with different filters. Since the secondary antibodies must bind specifically to their respective target, primary antibodies from different host species are recommended for indirect labeling. In this thesis, the investigation of α7nAChR expression on different immune cells was accomplished using double staining with indirect labeling in human plaques. The images captured of each target were merged together, and possible co-localization between a certain cell type and the receptor was determined. This is also the great advantage with immunofluorescence, the technique allow for investigating co-localization between different targets.

3.9 RT-qPCR (Paper III and IV)

The polymerase chain reaction (PCR) was first developed in the 1980s (120), and was a revolutionary method used to examine gene expression. In traditional PCR, DNA is amplified and detected in an end-point analysis. Since then, the development of real-time PCR (qPCR) allows quantification of the product, not only at the end of amplification, but after each cycle. qPCR is considered to be a reliable method with the advantages of high sensitivity and precise measurements (121). In Papers III and IV, the expression of messenger RNA (mRNA) for different atherosclerosis-associated markers was analyzed with qPCR using reverse transcription (RT-qPCR). In RT-qPCR, mRNA is used as the starting material. RNA is further transcribed into complementary DNA (cDNA) by the enzyme, reverse transcriptase. In the following PCR-reaction, cDNA is degenerated into a single-stranded molecule by heating to 95 °C, and allows primers to be incorporated when lowering the temperature. Primers for the mRNA of interest provide a specific amplification of the corresponding sequence of cDNA by again raising the temperature to around 70°C, and can be quantified by a bound fluorescent dye. This process can be repeated, and for each amplification cycle, the amount of cDNA doubles together with the fluorescent signal. An important consideration in running RT-qPCR is to validate the mRNA quality and efficiency of the reverse-transcription reaction, for both target genes and also for the reference genes (122). Reference genes are also used to normalize the obtained data (123). The choice of reference gene is usually determined by including multiple housekeeping genes when setting up a new experimental assay. The housekeeping genes with the most stable mRNA expression are used for normalization. In this thesis, the stability of a number of reference genes was evaluated for each study and specimen, and the applicable one chosen accordingly.

3.10 Microarray technology (Paper III)

Although RT-qPCR is a reliable and convenient method to measure mRNA for a limited number of targets, large gene expression profiles are better accomplished using other methods. To investigate cellular pathways and complex interactions, the characterization of thousands of targets is sometimes necessary. Microarray technology is one way to accomplish this, by measuring mRNA levels of many genes at the same time (124). Microarray technology is based on chips that can be labeled with a large amount of probes, making them sufficient to perform genome-wide mRNA profiling (124). In Paper III, we analyzed the BiKE-database (described

above) for the expression pattern of Chrna7 mRNA in human carotid plaques. The genome-wide mRNA expression profile in BiKE has been obtained using the Affymetrix's GeneChips technology.

3.11 Flow cytometry (Paper III and IV)

In Paper III and IV, cell populations in the spleen were analyzed with laser-based flow cytometry, a powerful method to characterize individual cells in a single cell suspension by their different properties. With high-throughput, each cell in the suspension passes through a flow cell where physical properties such as size and granularity are measured by their exposure to laser beams. The reflection of the laser beams scatters differently depending on cell properties. Scattered light is detected by photodiodes and digitally converted for further computer processing. The full capacity of flow cytometry manifests when this method is combined with fluorescence-labeled antibodies. Antibodies directed at different cell targets allow for customized panels with the appropriate read-out for the experiment. Multiple fluorochromes can be detected simultaneously, only limited by the number of different lasers and fluorescence detectors installed in the system. Evoked fluorescence is routed via a system of beam-splitters, usually dichroic mirrors, and wavelength-specific filters before detection in the correct photomultiplier tube (PMT). The system uses a single PMT for each spectral wavelength, and when the emitted photons strike the corresponding PMT, the signal is weak. Importantly, the photons are converted into electrons that are multiplied in the PMT and further amplified into a voltage pulse. The size of the voltage pulse corresponds to the number of detected photons and can be stored and analyzed in a computer system after digital conversion. In Paper III, cells were examined using a flow cytometer (FACSCantoA, BD Biosciences) with 2 lasers. Flow cytometry has the advantages of cell-by-cell characterization of antigen expression and physical properties in large cell-populations with high-throughput, at a reasonable cost. This technique can be used for any tissue where single-cell suspension can be achieved. A disadvantage is that the need for single-cell suspension complicates the analysis of "sticky" cells. It should also be mentioned that a fluorophore used in flow cytometry emits photons of multiple wavelengths. This can cause a spillover effect of photons from the designated PMT into a detector with filters in the nearby wavelength range. To overcome the problem of unwanted signals, compensation controls for the included fluorophores are used to correct for the amount of spill over.

3.12 Proliferation assay (Paper III)

A commonly used assay for measuring cell proliferation is the thymidine incorporation assay, where the radioactive nucleoside ^{3}H-thymidine is incorporated into new strands of DNA. Cultured cells are usually triggered with a mitogen to induce proliferative properties. The amount of radioactivity in a cell culture corresponds to the degree of cell division and is commonly assessed by measuring β-particles with a scintillation counter. In Paper III, splenocyte proliferation was investigated after stimulation with the T-cell mitogen, concanavalin A. Thymidine was added to cultured cells 48 hours after concanavalin A stimulation, and incubated for another 18 hours at 37°C, 5% CO_2, before measuring radiation. Precaution should always be used when working with radioactive molecules. ^{3}H-Thymidine can be toxic and inflict chromosomal changes and cell death (125). Without proper facilities or manageable handling and waste of radioactive compounds, the thymidine analog bromodeoxyuridine (BrdU) can be used as an alternative (126). Instead of measuring radioactivity, immunohistochemistry or flow cytometry can be used to detect BrdU. However, in immunohistochemistry the signal needs to be amplified, which can cause unreliable data (127).

3.13 *Ex vivo* treatment of human blood (Paper IV)

Ex vivo stimulations of whole blood is an effective method to examine cytokine production in response to treatment with different compounds. In Paper IV, human blood from healthy donors was stimulated with lipopolysaccharide (LPS) and treated with an α7nAChR-agonist. After 4 h of incubation in 37 °C, samples were centrifuged and serum was analyzed for inflammatory cytokines. In addition to whole-blood assays, *in vitro* stimulations are commonly performed in peripheral blood mononuclear cells (PBMC). However, the isolation of PBMCs is more labor-intensive and requires larger sample volumes. In addition, studies show that experiments in whole blood provide higher cell viability, compared to PBMCs (128), and that cells in a serum-free environment react differently compared to cells in serum-supplemented medium (129). The use of whole blood for *in vitro* experiments have the advantages of being a low cost method, reflect the circulating environment, and mimicking the *in vivo* response (130).

3.14 Biochemical analysis in mice

3.14.1 Systemic drug exposure (Paper II and IV)

Verification of drug exposure is important in all pharmacological studies. Depending on the method of administration, the reliability of the exposure varies. Subjects or animals failing to receive the desired concentration should be excluded from the study. In this thesis, systemic concentration of metoprolol in serum (Paper II), and of α7nAChR-agonist in whole blood or plasma (Paper IV), was measured with liquid chromatography-mass spectrometry (LC-MS). An advantage of LC-MS is its high sensitivity and the possibility of analyzing tiny amounts of a substance, allowing for non-terminal measurements where the sample volume is limited, such as in Paper IV.

3.14.2 Total cholesterol (Paper II-IV)

In this thesis, total cholesterol was assessed colorimetrically in serum (Paper II-IV) or in plasma (Paper IV, 12 week study), using the enzymatic colometric kit according to manufacturers protocol (RANDOX Laboratories Ltd., Crumin, UK) and subsequent detection in a spectrophotometer.

3.14.3 Cytokines

The measurement of cytokines is frequent in studies where inflammation is central. Enzyme-linked immunosorbent assays (ELISA) are widely used and enables reliable and sensitive measurement of the target (131). Although the method is reasonably cheap and effective, it is limited to measuring one cytokine per sample. Since atherosclerosis is a complex process, involving numerous cytokines (16), single measurements are not always satisfying. Repeated measurements with ELISA are expensive, time consuming and demands large amount of sample volume. Under these circumstances, multiplex assays are a convenient option that allow to measure numerous cytokines at the same time, saving money, time and sample volume.

TNF-α, IL-1β, and IL-6 (Paper III and IV)

Human serum levels of TNFα, IL-1β, and IL-6 (Paper IV), and supernatant levels of TNFα from the mouse splenocyte proliferation assay (paper III), were measured with colorimetric ELISA. In this thesis, human samples were analyzed using ELISA MAX™ (eBioscience, Inc. CA, US), and mouse samples using an ELISA kit (RandD Systems, MN, USA) according to manufacturer's protocol. A spectrophotometer was used to further detect and calculate sample concentrations after enzymatic reactions.

Th1/Th2 cytokines (Paper II)

In paper II, a mouse Multiplex ELISA (Meso Scale Discovery, MD, US) was used to measure Th1 (IL-1β, IL-2, IL-12, IFNγ, TNFα, and CXCL1) and Th2 (IL-4, IL-5 and IL-10) cytokines, according to the manufacturer's protocol.

3.15 Statistics

In this thesis, all data was assessed for normality using Shapiro-Wilk´s normality test and the appropriate statistical method chosen accordingly. In some analysis, skewed data was logarithmically or arcsine transformed to achieve normal distribution. In all cases, untransformed data was presented for better comparison with other studies. Univariate associations were analyzed with Pearson´s correlation (Paper I and III). Atherosclerotic lesion areas in different levels of aortic root were analyzed with repeated measurement two-way ANOVA (Paper III and IV).

In paper I, comparisons between patient categories were analyzed with Student´s t-test for continuous variables and Chi-square test for categorical variables. For mediation analysis, multiple regressions including 2 predictors were used. Independent associations were investigated using multiple regressions with stepwise adjustment (selection of $p<0.05$) for other clinical predictors. In paper II, 24-hour heart rate was analyzed with repeated measurement ANOVA, followed by Dunnet's post hoc test. In paper III, flow cytometry and proliferation experiments were conducted and terminated at two different time-points. Thus, this data was analyzed using two-way ANOVA, with experiment as a co-variate, and presented as estimated marginal means ± SEM. Mean lesion area in the aortic root was analyzed using Student's t-test. In Paper IV, mean lesion area in aortic root and thoracic aorta was analyzed using Students t-test. Stimulations in human samples were conducted by stimulating blood from the same subject with different stimuli and internal controls. Thus, this data was analyzed with repeated measurement one-way ANOVA followed by Holm's sequential Bonferroni correction. Gene expression was analyzed with Mann Whitney-U test, including Holm's sequential Bonferroni. For data not already mentioned in this section, Mann Whitney U-test was used for statistics.

In this thesis, if nothing else was discussed, all human data was expressed as mean ± SD, and all mouse data as mean ± SEM. $P<0.05$ was considered as statistically significant in all analysis except for Th1/Th2 cytokines in Paper II, where the level of significance was considered at $P<0.01$, to reduce the risk of mass significance.

4 RESULTS AND DISCUSSION

4.1 The link between autonomic dysfunction, inflammation and atherosclerosis (Paper I)

Studies prior to this thesis have reported that autonomic dysfunction associates with both inflammation (48-51), and CVD (13, 14). Further, numerous studies describe low-grade inflammation as a risk factor for CVD (53-55). Surprisingly, few studies have investigated if autonomic dysfunction is directly related to CVD, or if inflammation could mediate this association, and to our knowledge, never with carotid atherosclerosis as the primary endpoint. In paper I, we studied these conditions in men under investigation for carotid atherosclerosis by assessing two markers of autonomic function (BRS and HRV SDNN), and two markers of inflammation (WBCC and CRP).

In an initial analysis we found that subjects with prevalent CVD, defined as stroke or MI, exhibited reduced BRS, increased levels of WBCC and augmented carotid atherosclerosis compared to subjects with no history of CVD (Table 1). There were no differences in HRV or CRP (Table 1). Although HRV and CRP did not prove a difference between the groups, this data indicates that autonomic function is reduced, and inflammation is increased in subjects with prevalent CVD, prompting us to further investigate the associations between these conditions. Since rupture of atherosclerotic plaques is the major cause of MI and stroke (17), and our data showed an increased burden of carotid atherosclerosis in subjects with prevalent CVD, we chose to use this as our cardiovascular endpoint in subsequent analysis. In our study, prevalent CVD was regarded to be present in subjects with a history of stroke or MI. The relevance for carotid atherosclerosis in MI could be debated. Importantly, it has been shown that the presence of carotid atherosclerosis is directly correlated with the extent of coronary atherosclerosis (132), and carotid bruits is an important predictor for MI and myocardial mortality (133).

Table 1. Characteristics of population and comparison between subjects with or without prevalent CVD

Variable	All	No history of CVD	Prevalent CVD	*P**
Carotid plaque area, mm^2	58.3 ± 51.6	44.1 ± 40.4	73.0 ± 57.9	<0.001
CRP, mg/L	1.81 ± 1.7	1.72 ± 1.61	1.91 ± 1.77	0.3
WBCC, cells 10^9/L	6.1 ± 1.6	5.8 ± 1.6	6.4 ± 1.7	0.040
HRV SDNN, ms	44.3 ± 18.4	45.9 ± 17.9	42.7 ± 18.9	0.3
BRS ms/mm Hg	11.1 ± 6.8	12.8 ± 7.9	9.5 ± 5.1	0.022

Values expressed as means ± SD. CRP: C-reactive protein, WBCC: White blood cell count, HRV: Heart rate variability, SDNN: Standard deviation of RR interval, BRS: Baroreceptor sensitivity.

We investigated possible predictors for carotid plaque area using bivariate correlations. Carotid plaque area was positively correlated with WBCC and CRP, and inversely correlated with BRS (Figure 1A). No significant association between HRV SDNN and carotid plaque area was found (r=-0.16, *P*=0.081). For the inflammatory markers, CRP was inversely correlated with BRS (r=-0.23, *P*=0.009), and WBCC was inversely correlated with both BRS (r=-0.29, *P*=0.001) and HRV SDNN (r=-0.22, *P*=0.014). Taken together, this data suggest that both autonomic dysfunction and inflammation associates with atherosclerosis in this population. We can only speculate why only BRS, and not HRV SDNN, inversely associates with atherosclerosis. One explanation could be that HRV SDNN is regarded as a general marker for autonomic function (96), whereas BRS is regarded as a marker of parasympathetic function (100), potentially reflecting different aspects of the ANS. If the parasympathetic branch of the ANS is the major contributor to the relationship between ANS-dysfunction and atherosclerosis, HRV SDNN may be too blunt to detect such association in this population. In addition to the data presented in this section, other predictors for atherosclerosis and inflammation were also assessed with bivariate correlations (Table 2, Paper I).

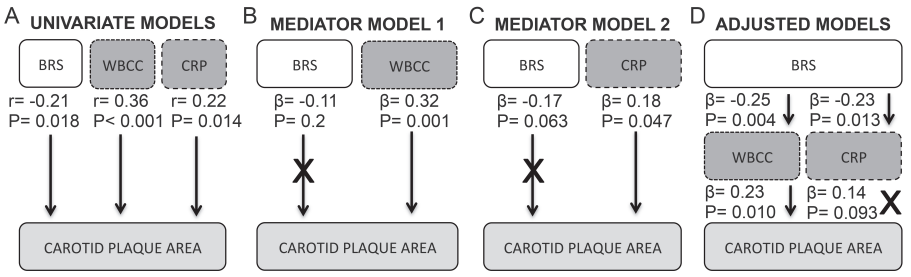

Figure 1. *Relationships between autonomic function, inflammation and carotid plaque area.* r-values in the univariate models are Pearson correlation coefficients (A) and β-values in mediator and adjusted models are standardized regression coefficients (B,C,D). Mediator models are multiple linear regressions including one variable of autonomic function and one variable of inflammation (B, C). Adjusted models are multiple linear regressions, adjusted for prevalent CVD, diabetes, smoking, BMI, hypertension, systolic blood pressure, cholesterol-lowering medicine, age and antihypertensive medicine (D). CRP: C-reactive protein, WBCC: White blood cell count, HRV: Heart rate variability, SDNN: Standard deviation of RR-interval, BRS: Baroreceptor sensitivity.

To investigate whether the association between BRS and carotid plaque area is direct or mediated via inflammation, 2-predictor multiple linear regression models were used to adjust for the inflammatory markers. When adjusted for either WBCC, or CRP, the association between BRS and carotid plaque area was attenuated (Figure 1B and C). WBCC and CRP still remained significantly associated with carotid atherosclerosis after adjustment for BRS (Figure 1B and C). This indicates that inflammation, at least partly, could be mediating the effects of autonomic dysfunction on atherosclerosis. However, the role of CRP as a mediator should be cautiously interpreted since the attenuation was weak. Also, even though this data propose the internal order of this pathway to be autonomic dysfunction-inflammation-carotid atherosclerosis, the causal direction of this link cannot be determined in our study. There are reports, both supporting and opposing, that the presence of carotid atherosclerosis could have an impact on BRS (134, 135). However, these studies are rarely adjusting for inflammation, a predictor of aortic stiffness (136, 137).

Given the suggested relationship between BRS, variables for inflammation and atherosclerosis, the independency of theses associations were investigated by adjusting for other potential confounders. After full adjustment for age, medication, prevalent CVD, smoking, BMI, diabetes, dyslipidemia and systolic blood pressure, WBCC and CRP was still independently inversely associated with BRS (Figure 1D). Further, carotid plaque area still remained independently associated with WBCC, whereas

CRP did not remain significantly associated in the fully adjusted model (Figure 1D). In summary, we demonstrate that autonomic function is associated with atherosclerosis and that inflammation could be mediating this relationship. The retrospective nature of this study is a limitation, and prospective studies are necessary to determine the casual direction between these associations.

4.2 Sympathetic blockade reduces pro-inflammatory cytokines and atherosclerosis in mice (Paper II)

Increased sympathetic activity is associated with cardiovascular disease (138). Some of the risk factors for cardiovascular mortality, such as hypertension, can be controlled by the inhibition of sympathetic activity using β-blockers. The use of β-blockade is also suggested to have a direct effect on the development of atherosclerosis, however the underlying mechanisms are still not fully understood. In Paper II, we investigated the effects of treatment with the β_1-adrenoceptor antagonist, metoprolol, on high fat diet-induced atherosclerosis in $ApoE^{-/-}$ mice. These mice were grouped under normal conditions, in the absence of generated stress conditions.

11 weeks of treatment with metoprolol significantly reduced atherosclerosis in the thoracic aorta, compared to controls (Figure 2A). In the aortic root, a similar effect was discovered, however this did not reach the level of significance (Figure 2B). Histology of the aortic root revealed a lower composition of macrophage content in the lesions after metoprolol treatment, compared to controls (Figure 2E, Paper II). Further, metoprolol reduced the serum levels of the pro-inflammatory cytokines TNFα and CXCL1 (Table 1, Paper II), and had no effects on total cholesterol in serum (control 12.3 ± 0.5 versus metoprolol 11.6 ± 0.4 mmol/L).

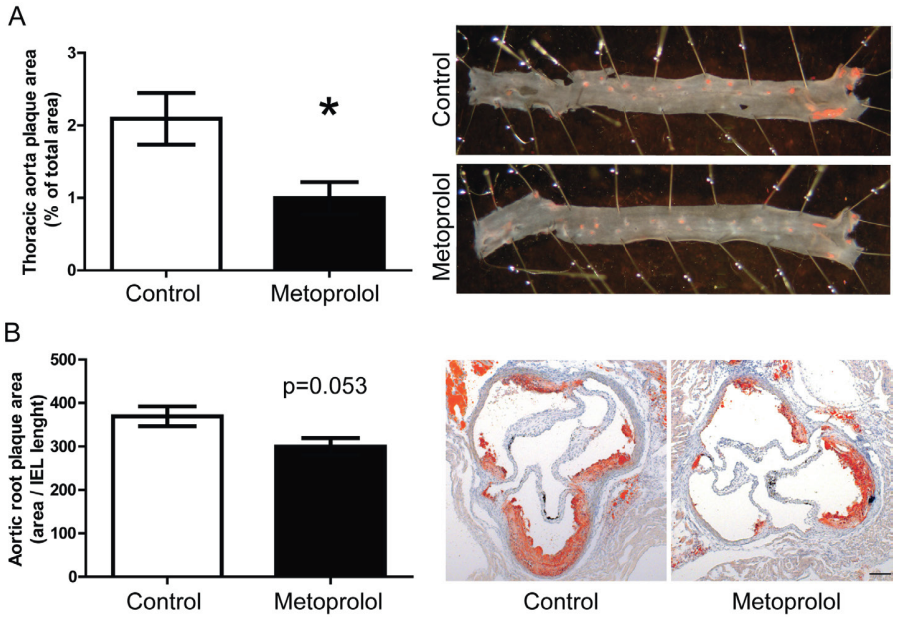

Figure 2. *Metoprolol reduced atherosclerosis in ApoE$^{-/-}$ mice.* The effect of 8 weeks of sympathetic inhibition with the β_1-antagonist metoprolol, on atherosclerosis in the thoracic aorta (A), and in the aortic root (B), compared to controls. Data expressed as mean ± SEM *P<0.05

Our data on reduced atherosclerosis from β-blockade supports previous studies in comparable models, showing an athero-protective effect of metoprolol (139). Numerous factors could contribute to the beneficial effects of the treatment. We did not find any influence on serum total cholesterol levels, indicating other mechanisms for the athero-protective effects. We did not differentiate between different lipoproteins, thus alterations in the lipid profile could be present. In addition, metoprolol reduced the serum levels of pro-inflammatory cytokines TNFα and CXCL1. These cytokines are involved in the pathogenesis of atherosclerosis and attenuation of their presence could play a role in inhibiting the disease.

The chemokine CXCL1 corresponds to growth-regulated oncogene-α (GROα) in humans, and is involved in chemotaxis of leukocytes. CXCL1 and its receptor CXCR2 are both present in atherosclerotic lesions, where they have an important role in the adhesion of monocytes and accumulation of macrophages (140-142). Interestingly, we previously showed that sympathetic activation by psychosocial stress increased the plasma levels of CXCR1 and increased atherosclerosis (31). TNFα contributes to atherogenesis in numerous ways, including the induction of vascular cell

adhesion molecules VCAM-1 and ICAM-1, and reactive oxygen species (143). Importantly, inhibition of TNFα reduced atherosclerosis in ApoE$^{-/-}$ mice (144, 145) Adhesion molecules are involved in the recruitment of monocytes into the vessel wall, and are commonly upregulated in atherosclerosis-prone areas (146). On the notion that TNFα and CXCL1 can be involved in the vascular recruitment of monocytes, reduced cytokine levels after metoprolol treatment could explain the lower frequency of macrophage content in the lesions of the aortic root. In the initiation and progression of atherosclerosis, the infiltration of monocytes and their subsequent differentiation into accumulating macrophages are essential (147). We can only speculate as to why β-blockade reduces pro-inflammatory cytokines. One possible explanation could be modulation of ANS. Studies have reported that metoprolol treatment both reduces sympathetic activity and increases parasympathetic activity (148-150). Interestingly, vagal stimulation has been associated with reduced production of TNFα in endotoxemic rats (69), a pathway known as the cholinergic anti-inflammatory pathway.

Other athero-protective mechanisms could be present in this study. Hypertension is one of the major risk factors for atherosclerosis, and is commonly treated with β-blockers. On the other hand, lowering of blood pressure seems to have little impact on atherosclerosis in normotensive mice (151). Unfortunately, this study cannot provide any data on this subject. Importantly, another study using a similar dose of metoprolol showed that treatment did not cause any changes in blood pressure (139). In conclusion, metoprolol reduces atherosclerosis in ApoE$^{-/-}$ mice, possibly by reducing pro-inflammatory cytokines. Further, the impact on cytokine production could potentially be mediated via reduced sympathetic or increased parasympathetic activity.

4.3 The role of α7nAChR in atherosclerosis (Paper III and IV)

Intrigued by the findings in Paper I and II, displaying a relationship between modulation of ANS, inflammation and atherosclerosis, Paper III and IV aim to investigate the role of the α7nAChR in atherogenesis. α7nAChR has been implicated as a key component in the cholinergic anti-inflammatory pathway, where activation of the receptor has been associated with improved outcome in a number of different inflammatory experimental models (84-87). However, the role of α7nAChR in atherosclerosis has been poorly investigated.

4.3.1 α7nAChR in mice

The role of α7nAChR on atherosclerosis in mice was approached using two different models. In Paper III, we investigated the atherogenic response in absence of hematopoietic α7nAChR in LDLr$^{-/-}$ mice, whereas the main focus in Paper IV was the potential effects on atherosclerosis in response to pharmacological stimulation of α7nAChR by treatment with the selective agonist AZ6983 for 8 or 12 weeks.

Development of atherosclerosis

In Paper II, mice reconstituted with bone marrow from α7nAChR$^{-/-}$ mice displayed a 74% increase in atherosclerosis, in the aortic root, compared to recipients of wild type bone marrow, after 8 weeks on a cholesterol-rich diet (Figure 3).

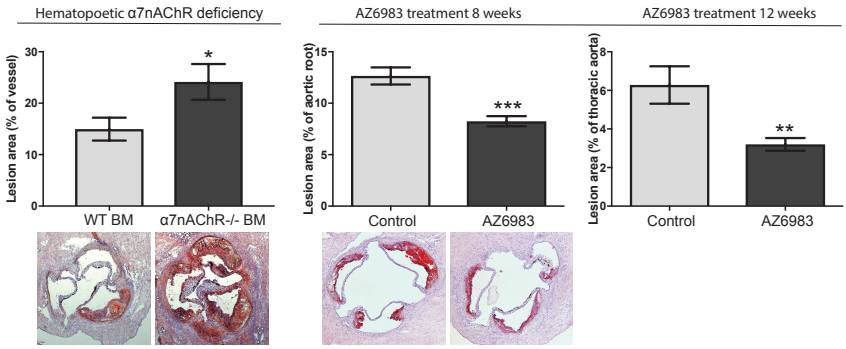

Figure 3. *The role of the α7nAChR in the development of atherosclerosis*. Atherosclerosis in the aortic root after reconstitution with bone marrow from α7nAChR$^{-/-}$ mice (left), after α7nAChR-stimulation with the selective agonist AZ6983 for 8 weeks (middle), or in the thoracic aorta after AZ6983-treatment for 12 weeks (right), compared to control mice. Data expressed as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

This was the first study presenting data on the implication of α7nAChR in atherosclerosis. Surprisingly, in a following study by Kooijman el al., also using the bone marrow transplantation approach, atherosclerosis did not differ between the groups, despite a systemic increase in leukocytes and elevated levels of pro-atherogenic cytokines (152). In addition, a recent study by Lee at al. reported unchanged lesion size after 8 weeks, and to our surprise, opposite effects after 14 weeks (153). We can only speculate on the cause of the controversial outcomes on atherogenesis. An obvious difference between our study and those by Kooijman et al. and Lee et al. is the use of male mice in ours, and female mice in theirs (152, 153). However, any

possible gender effect on α7nAChR-functionality in atherosclerosis needs to be further investigated.

The beneficial effects of the α7nAChR in atherosclerosis is supported in Paper IV, where we demonstrate that pharmacological activation of the α7nAChR using the selective agonist AZ6983 inhibits progression of the disease, in two separate studies. Mice treated with AZ6983, exhibited 37% less atherosclerosis in the aortic root after 8 weeks (Figure 3), and 49% less atherosclerosis in the thoracic aorta after 12 weeks (Figure 3), compared to controls. This is in line with a recent study by Hashimoto et al., which showed that the selective α7nAChR agonist AR-R17779 suppressed atherosclerosis in an angiotensin II-induced disease model (154). Hashimoto et al. proposed that the anti-atherogenic effects of AR-R17779 could possibly be derived from reduced blood pressure or attenuated serum lipid levels (154). In addition, expression of mRNA for IL-1β, IL-6 and NADPH oxidase 2 were decreased in the aortas of treated mice, suggesting a possible impact on local inflammation (154). In both our studies, 4 weeks of treatment with AZ6983 did not affect serum total cholesterol. At 8 weeks cholesterol was affected in opposite directions, and at 12 weeks no differences were detected between the groups (Table 1, Paper IV). Thus, changes in total cholesterol cannot account for the atherogenic effects of AZ6983, suggesting other mechanisms are involved. We did not perform blood pressure measurement and cannot rule out any possible anti-hypertensive effects of the drug on atherosclerosis.

Immune cells and inflammatory responses

Analysis of aortic mRNA in Paper III showed that α7nAChR$^{-/-}$ transplanted mice exhibited increased IFNγ mRNA (Figure 2C, Paper III). IFNγ is produced by T-cells and are involved in a number of pro-atherogenic immune responses (155). This could possibly indicate that aggravated local inflammation in the vessels may contribute to the increase in atherosclerosis.

Since the spleen has been implicated as an important organ in the cholinergic anti-inflammatory pathway (156), splenic mRNA was analyzed with RT-qPCR. To our surprise, α7nAChR$^{-/-}$ transplanted mice displayed increased FoxP3 mRNA in the spleen (Supplemental Figure 2A, Paper III), compared to controls. FoxP3 is expressed by regulatory T-cells that are regarded as anti-inflammatory. However, there are reports on the presence of FoxP3^{+} T-cells with pro-inflammatory properties, in the spleen, atherosclerotic aortas and mesenteric lymph nodes (157). Importantly, in a separate experiment where splenocyte subsets were compared between α7nAChR$^{-/-}$ and WT mice, flow cytometry did not detect any differences in the frequency of FoxP3^{+}

cells between the groups (data not shown). Thus, the functional relevance of increased splenic FoxP3 mRNA in the BMT-study should be interpreted with caution. Interestingly, Kooijman et al. also reported increased systemic immune-activity in α7nAChR$^{-/-}$ transplanted mice, including increased numbers of leukocytes in multiple compartments, elevated CRP and TNFα mRNA in peritoneal cells, as well as increased TNFα in the spleen, compared to controls (152). Taken together, these studies suggest aggravated pro-inflammatory activity of the immune system in absence of hematopoietic α7nAChR. Further, these immune responses might be present both systemically and locally in the vessels.

In Paper III, the immune-regulating role of α7nAChR in lymphoid organs was only investigated briefly. Thus, in Paper IV we addressed this subject more thoroughly. Treatment with the selective α7nAChR agonist AZ6983 reduced splenic mRNA of CD86, CD4, CD8 and IFNγ (Table 2). Further, AZ6983 reduced the frequency of CD3$^+$ T-cells, whereas CD11b$^+$ monocyte frequency was increased (Table 2). Of CD11$^+$ monocytes, AZ6983 treatment increased the frequency of Ly6Chigh monocytes and decreased the frequency of Ly6Clow monocytes (Table 2). To further investigate the immune response in proximity to the aorta, aortic lymph nodes were analyzed with RT-qPCR. AZ6983 treatment increased IL-1β mRNA, and reduced CD86 mRNA, compared to controls.

Table 2. Lymphoid immune responses after treatment with the α7nAChR-agonist AZ6983

	Marker	Spleen	Aortic LN
mRNA levels	CD4	↓	↔
	CD8	↓	↔
	CD86	↓	↓
	IL-1β	↔	↑
	IFNγ	↓	↔
Cell populations	CD11b$^+$ monocytes	↑	na
	CD3$^+$ lymphocytes	↓	na
	CD11b$^+$ Ly6Chigh monocytes	↑	na
	CD11b$^+$ Ly6Clow monocytes	↓	na

Selected data on immune related responses in spleen, or aortic lymph nodes, reported in Paper IV. Up and down arrows represent significant changes in mRNA or cell population frequencies, and their direction, after 8 weeks of treatment with the α7nAChR-agonist AZ6983, compared to control mice. na: not assessed, LN: lymph nodes.

Treatment with AZ6983 reduced splenic mRNA for IFNγ, T-cell markers CD4 and CD8, and CD86. In addition, flow cytometry showed that AZ6983 reduced the frequency of CD3+ T-cells. CD86 is expressed by antigen presenting cells and is important for T-cell activation (158). Since these markers are all related to T-cells, this suggests that AZ6983 suppresses T-cell activity in the spleen, an effect that could contribute to the athero-protective effects of the treatment. To our surprise, AZ6983 increased the frequency of CD11b+ monocytes and skewed the phenotype into a more pro-atherogenic Ly6high subpopulation (159). This is in disparity with our finding of reduced atherosclerosis. It is hard to find an explanation for this, however cell-frequencies are in relation to the number of counted cells, and cannot give information on whether the total number of Ly6Chigh monocytes is increased after treatment. Moreover, it has been suggested that the spleen can have an acute reservoir function for monocytes. Myocardial infarction triggers the release of monocytes from the spleen into the circulation, reducing the numbers in the spleen and increasing the numbers in the blood stream (160). It is interesting to further speculate that treatment with AZ6983 could retain pro-atherogenic monocytes in the spleen, reducing their presence in the blood stream and the subsequent recruitment into the vessels. Unfortunately, we did not measure any circulating immune cells and this needs to be further investigated.

In accordance with the spleen, CD86 mRNA in aortic lymph nodes was decreased after treatment with AZ6983, suggesting reduced activation of T-cells. Further, AZ6983 dramatically increased IL-1β mRNA. This finding was notable since IL-1β mainly has a pro-inflammatory role in atherosclerosis (161-163), even though it has been suggested that IL-1β can improve plaque stability (164). This needs to be further investigated. This study is limited in describing where the interaction between AZ6983 and the α7nAChR occurs. The lymphoid immune responses may indicate a direct or indirect interaction with immune cells in lymphoid organs. The expression of α7nAChR on splenic macrophages has already been reported as a regulator of inflammation (77), and there might also be a role for other α7nAChR-expressing immune cells.

In summary, this data provides information on an athero-protective role for α7nAChR in mice. Hematopoietic α7nAChR deficiency accelerates atherosclerosis, whereas stimulation of the receptor with the selective agonist AZ6983 attenuates disease progression. Further, α7nAChR presence and activation seems closely linked to modulation of both adaptive and innate immune responses in the spleen, as well as in closer proximity to aortic lesions. We suggest that these immune-modulating effects, at least partly,

could be mediating the beneficial effects of α7nAChR-signaling in atherosclerosis. However, the exact mechanisms need to be further investigated.

4.3.2 α7nAChR in humans

Apart from its neuronal expression, the α7nAChR has been identified in a number of different human immune cells (74-76). Since we showed an anti-atherogenic and immune-modulating role for α7nAChR in mice, we wanted to investigate if α7nAChR-expressing immune cells are present in human atherosclerotic plaques. Further, we wanted to evaluate if circulating immune cells respond to α7nAChR-stimulation.

α7nAChR expression in carotid lesions (Paper II)

Peroxidase staining revealed that α7nAChR was expressed in inflamed regions of human carotid plaques (Figure 4 A). Further, double stainings showed that the expression of α7nAChR co-localized with both $CD68^+$ (Figure 4 B) and $CD163^+$ macrophages (Figure 4 C), and $CD3^+$ T-cells (Figure 4 D). The expression of α7nAChR on $CD163^+$ macrophages was confirmed with analysis of mRNA patterns from the global gene expression array database of BIKE. mRNA for *Chrna7,* the coding gene for α7nAChR, and CD163 were significantly correlated (r=0.282, *P*=0.0033).

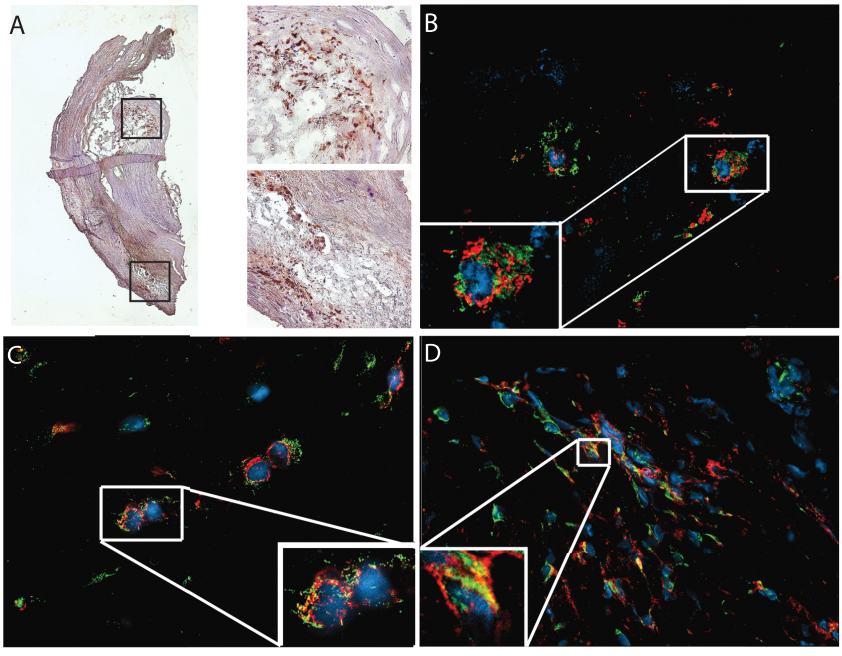

Figure 4. *The expression of α7nAChR in human atherosclerotic plaques*. Representative immunoperoxidase staining of the α7nAChR in human carotid plaques (A). Immunofluorescent double stainings showing co-localization of α7nAChR in green (B, C, D), with macrophage markers CD68 (B) and CD163 (C), and T-cell marker CD3 (D), in red. Nuclei are stained in blue with 4',6-diamidino-2-phenylindole.

This suggests that human atherosclerotic plaques contain α7nAChR-expressing immune cells. At least partly, these α7nAChR^{+} immune cells comprise T-cells and macrophages. Since α7nAChR co-localized with CD163, a marker for M2 polarized macrophages, we speculate that the receptor is expressed by this subset, known to exhibit anti-inflammatory properties (165, 166). We find this interesting, since it indicates that treatment with α7nAChR-agonists may promote local interaction with immune cells in the vessels, and needs to be further investigated.

α7nAChR activation by AZ6983 attenuates cytokine response in human blood

AZ6983 attenuated the response of pro-inflammatory cytokines TNFα, IL-1β and IL-6 in LPS-challenged blood from healthy donors (Figure 5). The inhibitory effects were significant at AZ6983 concentrations of 10 and 100 µmol/L for TNFα, and 100 µmol/L for IL-1β and Il-6 (Figure 5).

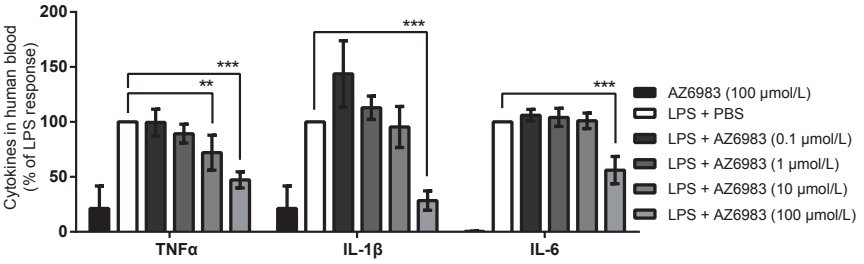

Figure 5. *AZ6983 inhibits pro-inflammatory cytokine production in human blood ex vivo.* The production of pro-inflammatory cytokines TNFα, IL-1β and IL-6 in lipopolysaccharide (10 ng/ml) challenged blood from healthy subject in presence of PBS or 0.1, 1, 10 or 100 µmol/L of the α7nAChR agonist AZ6983. Bars represent % response of LPS stimulations and are expressed as mean ± SEM. PBS: Phosphate-buffered saline, LPS: Lipopolysaccharide. **P*<0.05, ***P*<0.01, ****P*<0.001.

Here we show that AZ6983 effectively reduces production of pro-inflammatory cytokines in LPS-challenged human blood. The effect of AZ6983 is similar to previous studies on the selective α7nAChR agonist GTS-2 and the broad cholinergic agonist nicotine, where treatment reduced pro-inflammatory cytokines in comparable models (167, 168). Compared to GTS-21 and nicotine, the suppressive effects of AZ6983 are present at lower concentrations, suggesting AZ6983 to be more potent (167, 168). Since TNFα, IL-1β and IL-6 all are associated with the pathophysiology of atherosclerosis (16), this could indicate that AZ6983 also might have a potent effect on inhibiting lesion development in humans.

5 SUMMARY AND CONCLUSION

In this thesis, we investigated the link between the ANS, inflammation and atherosclerosis. We show that there is an association between autonomic dysfunction, measured by BRS, and carotid atherosclerosis in men. However, this association was attenuated when adjusting for the inflammatory marker WBCC. Thus, it appears that inflammation could be mediating this relationship. This was further supported by our novel demonstration of an independent association between autonomic dysfunction and WBCC, and between WBCC and carotid atherosclerosis. Since BRS is regarded as a parasympathetic marker, dysfunction in this branch of the ANS may be of relevance for disease progression.

We also found evidence of a link between sympathovagal balance, inflammation and disease progression by suppressing sympathetic activity in hypercholesterolemic mice. Treatment with the β_1-adrenoceptor antagonist, metoprolol, attenuated atherosclerosis and reduced serum levels of the pro-inflammatory cytokines TNFα and CXCL1. Since these cytokines play a part in the pathophysiology of atherosclerosis, suppressed inflammation in response to reduced sympathetic drive or increased parasympathetic activity, may contribute to the athero-protective effects. While the sympathetic nervous system has gained a lot of attention in the cardiovascular field, the parasympathetic nervous system has been demonstrated to have an immune-modulating role in other fields. Others have shown that vagal stimulation reduces pro-inflammatory cytokines via activation of the α7nAChR, improving outcomes in experimental models of other inflammatory diseases. This prompted us to investigate the role of α7nAChR in atherosclerosis.

For the first time, we reveal an athero-protective role for the α7nAChR. α7nAChR-deficiency increased IFNγ mRNA in aorta and accelerated atherosclerosis, whereas stimulation with the novel agonist AZ6983 modulated immune responses in lymphoid organs, and attenuated atherosclerosis. Further, the α7nAChR was identified on T-cells and macrophages in human carotid plaques, and stimulation of α7nAChR in human blood effectively inhibited production of pro-inflammatory cytokines.

Taken together, our findings suggest that the balance between the two branches of ANS could have an impact on atherosclerosis, and that inflammation may mediate this response. Although underlying mechanisms are elusive, signaling via the α7nAChR could be part of this pathway. Pharmacological targeting of the α7nAChR using AZ6983 could be a future intervention for atherosclerosis, and should be further investigated.

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