

Linköping Studies in Health Sciences Thesis No. 92

Angiotensin-Converting Enzyme

**Effects of Smoking and Other Risk Factors
for Cardiovascular Diseases**

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Linköping 2009

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During the course of the research underlying this thesis, Liza Ljungberg was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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Printed in Sweden by Linköpings Tryckeri AB, Linköping, Sweden, 2009

ISBN 978-91-7393-712-2

ISSN 1100-6013

*In science the credit goes to the
man who convinces the world,
not to the man to whom the idea
first occurs*

(William Osler 1849-1919)

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ABSTRACT

Cardiovascular diseases (CVDs) are the most common cause of death in Western countries. Smoking, hypertension, diabetes mellitus and hypercholesterolemia are considered as major risk factors. However, the underlying mechanisms by which these factors cause CVDs are not entirely clear. Angiotensin-converting enzyme (ACE) is a key enzyme in the renin-angiotensin-aldosterone system, converting angiotensin I to the vasoactive peptide angiotensin II. Besides being an important factor for normal regulation of blood pressure, ACE appears to be involved in the pathogenesis of atherosclerosis. Previous studies have shown an upregulation of ACE in atherosclerotic plaques. There is genetic polymorphism in the ACE gene (ACE I/D polymorphism) which is strongly connected to the levels of ACE in plasma, but has also been associated with higher risk for cardiovascular diseases. The aim of this thesis was to investigate ACE in vitro and in vivo, in relation to cardiovascular risk factors and CVDs. The results showed that nicotine and nicotine metabolites increase ACE activity in human endothelial cells in vitro. Smoking was associated with increased plasma ACE levels. This effect might be mediated by nicotine and nicotine metabolites. These results could explain one cellular mechanism by which smoking exerts negative effect on the vascular system. Extract of oral snuff inhibited ACE in human endothelial cells and in serum, whereas extract of cigarette smoke had no effect on endothelial ACE. If these results have any physiological relevance remains to be investigated. Cardiovascular risk factors and CVDs were associated with increased levels of ACE in plasma. No association between ACE D/D genotype and CVDs was found. Based on these results we suggest that an increased level of ACE, rather than ACE genotype, is associated with increased risk for CVDs.

SAMMANFATTNING

Hjärtkärlsjukdomar är den vanligaste dödsorsaken i industriländer. Åderförkalkning är den bakomliggande process som orsakar hjärtkärlsjukdomar. En mängd olika riskfaktorer har identifierats (rökning, högt blodtryck, diabetes etc.) men man har inte helt lyckats kartlägga mekanismerna för hur dessa riskfaktorer leder till förkalkning av kärlen. Renin-angiotensin-aldosteron-systemet är ett av de viktigaste systemen i kroppen vad gäller reglering av blodtryck och vätske- och saltbalans. Angiotensin-converting enzyme (ACE) är ett nyckelenzym i renin-angiotensin-aldosteron systemet och omvandlar angiotensin I till den aktiva peptiden angiotensin II. ACE är en central parameter för normal reglering av blodtryck, men man tror även att det är en viktig faktor för uppkomst av åderförkalkning. Tidigare studier har visat att individer med åderförkalkning har en ökad mängd ACE i det förkalkade området. Det finns en genetisk variation i genen för ACE som är starkt kopplad till mängd ACE i blodet. Ett antal studier har visat ett samband mellan denna genvariation och risken att drabbas av hjärtkärlsjukdomar, medan andra studier inte funnit detta samband. Syftet med denna avhandling var att undersöka ACE i relation till risk faktorer för hjärtkärlsjukdomar och förekomst av hjärtkärlsjukdomar. Resultaten visar att nikotin och levernedbrytningsprodukter av nikotin (nikotinmetaboliter) ökar aktiviteten av ACE i mänskliga endotelceller, den celltyp som täcker insidan av alla blodkärl. Dessutom visades att rökning resulterar i en ökad mängd ACE i blodet. Det är tänkbart att denna effekt orsakas av nikotin och nikotinmetaboliter och skulle kunna vara en mekanism för hur rökning ger upphov till hjärtkärlsjukdomar. Snus däremot minskade aktiviteten av ACE, medan cigarrettrök inte hade någon effekt. Dessutom visades att både riskfaktorer för hjärtkärlsjukdomar och förekomst av hjärtkärlsjukdomar resulterar i en ökad mängd ACE i blodet och vi föreslår att mängden ACE är en viktigare faktor än ACE-genvariant, vad gäller risken för att drabbas av hjärtkärlsjukdom.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their roman numbers. Some additional unpublished results are also included in the thesis.

- I. **Effect of Nicotine and Nicotine Metabolites on Angiotensin-Converting Enzyme in Human Endothelial Cells**, Liza U Ljungberg, Karin Persson, *Endothelium* 2008:15(5): 239-245

- II. **Is ACE Level, Rather than ACE Genotype, a Risk Factor for Cardiovascular Diseases?** Liza U Ljungberg, Urban Alehagen, Hanna M Björck, Toste Länne, Rachel DeBasso, Ulf Dahlström, Karin Persson (*in manus*)

ABBREVIATIONS

AU	Absorbance unit
ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
ACEi	Angiotensin-converting enzyme inhibitors
AD	Alzheimer's disease
Ang	Angiotensin
ARBs	Angiotensin II receptor blockers
AT ₁	Angiotensin II receptor type 1
AT ₂	Angiotensin II receptor type 2
AT ₃	Angiotensin II receptor type 3
AT ₄	Angiotensin II receptor type 4
BMI	Body mass index
CSE	Cigarette smoke extract
CVDs	Cardiovascular diseases
CYP2A6	Cytochrome P450 2A6
D/D	Deletion/deletion
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HUVECs	Human umbilical vein endothelial cells
IHD	Ischemic heart disease
I/I	Insertion/insertion
I/D	Insertion/deletion
MI	Myocardial infarction
NO	Nitric oxide
OSE	Oral snuff extract
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGI ₂	Prostacyclin
RAAS	Renin-angiotensin-aldosterone system

RAS	Renin-angiotensin system
ROS	Reactive oxygen species
RSD	Relative standard deviation
Rt	Retention time
SCB	Statistics Sweden (Statistiska centralbyrån)
T2D	Type 2 diabetes mellitus
U	Units
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

BACKGROUND

Cardiovascular diseases (CVDs) are the most common cause of death in Western countries. Smoking, hypertension, diabetes mellitus (DM) and hypercholesterolemia are considered major risk factors. However, the underlying mechanisms by which these factors cause CVDs are not entirely clear. The renin-angiotensin-aldosterone system (RAAS) is one of the most important systems regulating blood pressure and cardiovascular homeostasis and appears to have an important role for development of CVDs. This thesis focuses on angiotensin-converting enzyme (ACE), a key enzyme in RAS, and its connection to cardiovascular risk factors and CVDs.

The endothelium

The inside of all healthy blood vessels is covered by a monolayer of vascular endothelial cells. For a long time, the only known function of the endothelium was to serve as a barrier that separates the circulating blood from the vessel wall. Today, we know that the endothelium plays a critical role in the control of vascular function. Under physiological conditions the endothelium produces and releases a number of vasoactive substances including nitric oxide (NO), prostacyclin (PGI₂), endothelium-derived hyperpolarizing factor, thrombomodulin and tissue type plasminogen activator. Activation of endothelial cells, as can be induced by cytokines or vessel injury, results in production and release of procoagulant/prothrombotic factors such as platelet-activating factor and von Willebrand's factor. In addition, the endothelium expresses a number of adhesion molecules that are crucial for the transendothelial migration of leucocytes into an inflamed tissue. Endothelial cells also express ACE, which will be described in detail in the following sections. Endothelial dysfunction is considered a key factor in the development of atherosclerosis¹. The term endothelial dysfunction has no precise definition, but often refers to an impaired response to endothelium-dependent vasodilators². The underlying mechanisms causing endothelial dysfunction are not entirely clear, although production of reactive oxygen

species (ROS) most likely contributes to this phenomenon³. An impaired endothelial function results in reduced capacity to produce NO, increased endothelial permeability, enhanced platelet aggregation, and leucocyte adhesion as well as generation of chemokines, which contributes to a prothrombic state¹.

The renin-angiotensin-aldosterone system

RAAS is a powerful system regulating fluid-electrolyte balance and systemic blood pressure. Renin is a proteolytic enzyme synthesized, stored and secreted from the juxtaglomerular apparatus in the kidneys. The release of renin is triggered by a number of physiological stimuli, including PGI₂, decreased Na⁺ concentration in the distal tubule, reduced arterial pressure, renal sympathetic nerve activation and stimulation of β₁-receptors. Following secretion, renin acts upon the plasma protein angiotensinogen forming angiotensin I (ang I). Ang I has mild vasoconstrictor properties but not enough to cause significant functional changes. Ang I is further converted to angiotensin II (ang II) by ACE. Although ACE is the major catalyst for the conversion of ang I to ang II, other enzymes including tissue plasminogen factor, cathepsin G, tonin and chymase can also catalyze the conversion⁴.

Ang II is considered the main effector peptide of the renin-angiotensin-system (RAS). Binding of ang II to specific receptors activates a number of different events in various tissues and cell types⁵. Four types of ang II receptors have been identified; AT₁-AT₄⁶. Most of the cardiovascular effects are generated through the AT₁ receptor⁶. The role of the AT₂ receptor is not as well characterized, but seems to counteract some of the processes mediated by the AT₁ receptor⁷. The function of the AT₃ receptor is unknown, whereas the AT₄ receptor has been identified in a wide range of tissues including adrenal gland, kidney, lung and heart and seems to have a role in learning and memory processing, modulation of glucose uptake into cells⁸ and release of plasminogen activator inhibitor I⁹.

Ang II plays an important role in blood pressure regulation by inducing arteriolar constriction, release of aldosterone from the adrenal medulla and

enhanced secretion of vasopressin, resulting in increased reabsorption of Na^+ and water in the kidneys. Ang II also induces several pathophysiological actions involved in the atherosclerotic process, such as proliferation and migration of smooth muscle cells, generation of ROS, lipid peroxidation and formation of foam cells^{4,10}. Furthermore, it seems as if ang II is involved in the vascular inflammatory response, by activating the recruitment of inflammatory cells to the injured arteries¹¹. In addition, ang II induces negative feedback inhibiting the release of renin from the juxtaglomerular cells.

The degradation of ang II by a number of different enzymes occurs only seconds after its formation, giving rise to other angiotensin peptides⁵. During the last decades, a number of new angiotensin peptides have been identified, and it has become evident that RAS is a more complex system than previously thought. Ang 2-8 acts through the ang II receptors AT_1 and AT_2 and generates similar physiological effects as ang II⁵. Ang 3-8 is involved in regulation of blood flow, neural development and learning and memory⁵. Ang 1-7 is produced from the precursor ang I and the conversion can be catalyzed by a number of endopeptidases, including neprilysin and prolyl-oligopeptidase¹². It is believed that ang 1-7 counteracts the effects caused by ang II¹². Ang 1-7 act through specific receptors (Mas receptors), but have also been reported to activate the AT_2 receptor¹³. Ang 1-7 have antihypertensive, antihypertrophic, antifibrotic and antithrombotic properties^{5,13}. In addition, ang 1-7 has been shown to be cardioprotective and it has been suggested that ang 1-7 has a role in the protective effect of ACE inhibitors (ACEi)¹².

Angiotensin converting enzyme

ACE (EC 3.45.15.1) was first discovered in the 1950s by Skeggs and colleagues, who discovered an enzyme which could convert ang I to ang II (called hypertensin I and hypertensin II at that time)¹⁴. This enzyme was called “converting enzyme“. A number of years later Yang and colleagues identified an enzyme in human blood, designated kininase II, which was able to degrade bradykinin¹⁵. Converting enzyme and kininase II was later

shown to be the same enzyme^{16,17} and today the enzyme is referred to as angiotensin-converting enzyme.

ACE is a dipeptidyl carboxypeptidase that exists in 2 isoforms. Somatic ACE is expressed in various tissues and cell types including the cardiovascular system, kidneys, intestine, adrenal gland, liver, uterus etc¹⁸, whereas testicular ACE only can be found in sperm cells¹⁹. Both isoforms have a hydrophobic trans-membrane domain and a short cytoplasmic fragment²⁰. Somatic ACE has two homologous domains with 2 catalytic sites and Zn²⁺ binding regions^{20,21}, whereas testicular ACE only has one extracellular domain, and subsequently only one catalytic site²². A few years ago, a homologue of ACE (ACE2) was identified in humans^{23,24}. ACE2 catalyzes the degradation of ang I to ang 1-9 and the conversion of ang II to Ang 1-7. The distribution of ACE2 is more restricted compared to ACE and has been identified in the human heart, kidney and testis^{23,24}.

In the vascular system, ACE exists circulating in the blood and bound to the membrane of different cell types, including vascular endothelial cells²⁵. ACE is located on the luminal side of the endothelium with the C-terminal anchored to the plasma membrane²⁰. Circulating ACE originates mainly from endothelial cells and has been released into the blood after proteolytic cleavage of the anchor^{26,27}. ACE has also been identified in vascular smooth muscle cells²⁸, T-lymphocytes²⁹ and monocytes/macrophages^{30,31}. Due to the high vascularisation, the lungs are the major site for production of circulating ACE. Besides being an important enzyme for the conversion of ang I to ang II and degradation of bradykinin, ACE acts on a number of other natural substrates, including enkephalin, neurotensin and substance P³².

Regulation of ACE

There is a large inter-individual variation in the amount of circulating ACE³³. Approximately 20-50% of the variation can be accounted for by the ACE I/D polymorphism³³⁻³⁵, as described in detail in the next section. However, serum ACE activity seems to be relatively stable when measured in the same individual at different occasions³⁶.

ACEi alter the activity and expression of ACE in vivo and in vitro. The direct effect of ACEi treatment is a decreased serum ACE activity^{37,38}. However, a number of studies have shown that ACEi induce an increased expression and activity of ACE in cultured endothelial cells^{39,40}, cultured human macrophages⁴⁰, t-lymphocytes⁴¹ and in human serum^{38,42,43}, probably due to a compensatory synthesis of the enzyme. Furthermore, rats treated with ACEi showed increased ACE expression in several tissues^{38,44}, however no effect can be seen on testis ACE⁴⁴. In addition, treatment with angiotensin II receptor blockers (ARBs) or renin inhibitors have no effect on the expression of ACE⁴⁴.

Results from our laboratory and others have showed that NO regulates ACE⁴⁵⁻⁴⁹. NO-releasing compounds inhibit ACE in a dose dependent manner and NO released from the endothelium reduces the conversion of ang I to ang II⁴⁵. In addition, it has been shown that shear stress reduces ACE activity⁴⁶. However, other results indicate that NO regulates ACE under static conditions but has no effect under shear stress⁴⁷. Results from our laboratory showed that NO-donors inhibit ACE in cultured human endothelial cells⁴⁹ and that NO generated from glyceryl-trinitrate inhibits ACE in human serum in vivo⁴⁸.

Statins are lipid lowering drugs, effective in treatment and prevention of CVDs. Atorvastatin has been reported to inhibit induction of ACE by vascular endothelial growth factor (VEGF) in endothelial cells⁵⁰. Upregulation of ACE in differentiating human macrophages is also reduced by atorvastatin⁵¹. Unpublished results from our lab however indicate that simvastatin and pravastatin increase ACE activity in human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner.

In addition, thyroid hormone, glucocorticoids and steroids have been shown to induce an increased ACE activity in cultured endothelial cells from different origins^{30,52,53}, whereas estradiol reduces the expression of ACE in rat kidneys, lungs and aorta⁵⁴, suggesting that ACE is under hormonal control. However, no association was found between hormonal parameters and the level of ACE in plasma⁵⁵. Other substances that have been shown to regulate ACE are; atrial natriuretic peptide⁵⁶, cyclic adenosine monophosphate^{56,57}, extract of *Panax ginseng*⁵⁸, phorbol ester^{59,60}, platelet activating factor⁶¹, tea flavanoids⁶² and VEGF⁶³.

Genetics

The gene encoding ACE is located on chromosome 17q23. This gene encodes both ACE isoforms, but has two different promoters resulting in different mRNAs⁶⁴. There is a genetic variation within the gene, a 278 base pair insertion/deletion (I/D) polymorphism, resulting in three different ACE genotypes: I/I, I/D and D/D⁶⁵. As the polymorphism is located in an intron, it will not affect the structure of the enzyme, However, the polymorphism is strongly connected to the level of ACE in plasma, where I/I, I/D and D/D have low, medium and high levels respectively^{33,34}. In addition, the expression of ACE in T-lymphocytes²⁹ and in human cardiac tissue⁶⁶ is also influenced by the ACE I/D polymorphism, suggesting that tissue ACE and circulating ACE are under similar genetic control⁶⁷. Furthermore, increased conversion of ang I to ang II have been reported in carriers of the D/D genotype compared to I/I⁶⁸. The genotype frequencies for ACE are different in different ethnic groups⁶⁹⁻⁷¹. In most studies, the frequency of the D/D genotype in Caucasian and African American populations varies from 25% to 30%, whereas in Asian populations less than 20% carry the D/D genotype⁶⁹⁻⁷¹.

Circulating and tissue RAS

RAS was previously thought to be an endocrine system. However, over the last 10-15 years it has become evident that RAS also exists locally in many tissues⁷². All components of RAS including renin, angiotensinogen, ang I, ang II and ACE, have been identified in a number of different tissues⁷². Only about 10% of ACE in humans exists circulating in the plasma, the remaining can be found bound to the membrane in different cells and tissues^{73,74}. If the circulating level of ACE reflects the level of ACE in tissues is still unknown. It has been suggested that circulating RAS is important for short-term regulation of cardiovascular homeostasis, whereas local RAS plays a central role in the long-term regulation of homeostasis and appears to have a role in cardiovascular disease⁷². Renin is the rate limiting step in the circulating RAS, whereas the role of renin in tissue RAS has not been clarified.

Outside-in-signalling

Quite recently the “outside-in-signalling concept” was proposed by Ingrid Fleming’s group⁷⁵⁻⁷⁷. Using cultured endothelial cells, they were able to show that ACEi and bradykinin induce an intracellular signalling pathway in endothelial cells. This pathway involves phosphorylation of casein kinase 2, c-Jun N-terminal kinase and MAP kinase kinase and generates altered gene expression of ACE and cyclooxygenase-2⁷⁵⁻⁷⁷. They suggest that signalling mediated by ACEi and bradykinin may be an important physiological mechanism^{75,78}. Activation of this pathway by ACEi may account for some of the beneficial effects these drugs have on the cardiovascular system⁷⁴.

ACE in diseases

ACE has been extensively studied in the context of cardiovascular homeostasis, and several studies have suggested that ACE is an important factor in CVDs^{72,79,80}. However, ACE seems to be involved in development of other diseases as well^{81,82}.

Cardiovascular diseases

In 1992, Cambien and colleagues reported an association between ACE D/D genotype and myocardial infarction (MI), especially in patients considered to be at low risk⁷⁹. Since then, extensive research has been performed examining the association between ACE genotype and cardiovascular diseases. Besides MI, the D/D genotype has been associated with a number of different cardiovascular complications, including coronary artery spasm, coronary artery disease⁸³, coronary artery calcification⁸⁴, heart failure⁸⁵, hypertension^{86,87}, left ventricular hypertrophy⁸⁸⁻⁹⁰ and stent restenosis⁹¹⁻⁹³. However, several studies have failed to show any association⁹⁴⁻⁹⁶. A large meta-analysis including a total of 32715 individuals reported that small studies often found an association between D/D genotype and hypertension, ischemic heart disease (IHD) and ischemic cerebrovascular disease, whereas a larger study including more than 10000 subjects could not find this association⁹⁷. Today, there is no consensus regarding the importance of ACE genotype for CVDs. A feedback mechanism has been suggested in healthy individuals neutralising genetically enhanced ACE levels in D/D carriers⁹⁸.

A number of studies have shown upregulation of ACE in different vascular diseases. Atherosclerosis is the underlying process causing many different CVDs. Accumulation of ACE has been reported both in early and advanced atherosclerotic lesions^{99,100} and in the aortic wall in hypertensive rats¹⁰¹. In the atherosclerotic plaque, ACE is mainly localized to regions where the infiltration of inflammatory cells is high¹⁰⁰. Furthermore, increased ACE levels in plasma and in the atherosclerotic plaque have been associated with increased risk for stent restenosis^{91,92}. The expression of ACE in the normal healthy heart is relatively low, with the majority found in the endothelium of arteries and arterioles¹⁰².

Upregulation of cardiac ACE has been shown in hypertrophized hearts¹⁰³, in experimental heart failure¹⁰⁴, and both cardiac and circulating ACE are enhanced in patients who have suffered a MI^{35,105}. In addition, ang II has been shown to induce protein synthesis in the intact heart¹⁰⁶ and is considered an important factor contributing to the development of cardiac hypertrophy⁷³.

Other diseases

DM is considered a major risk factor for CVDs. A number of studies have demonstrated that patients with DM have increased levels of circulating ACE compared to healthy controls^{81,107}. In addition, elevated serum ACE levels have been shown in DM patients with cardiovascular complications¹⁰⁷ or diabetic nephropathy¹⁰⁸. Furthermore, a correlation was reported between serum ACE levels and glycosylated hemoglobine¹⁰⁹, which reflects the average blood glucose concentration over a prolonged period of time. A few studies indicated a possible association between ACE D/D genotype and type 2 DM (T2D)¹¹⁰ and the D/D genotype have also been associated with both diabetic and non-diabetic renal disease^{111,112}. In addition, carriers of D/D genotype have been associated with increased sensitivity to insulin¹¹².

Several studies have reported an association between the ACE I/D polymorphism and Alzheimer's disease (AD)^{82,113}. It has been reported that ACE levels are increased in hippocampal, parahippocampal and temporal cortex, but decreased in cerebrospinal-fluids in patients with AD¹¹³. Furthermore, a few in vitro studies have reported that ACE can degrade β -amyloid^{114,115}, a protein which is important in AD, indicating a possible role for ACE in development of AD and that a high ACE level could be protective against AD.

Sarcoidosis is a multisystem granulomatous disease with different clinical manifestations. The pathogenesis of the disease is unknown although both genetic and environmental factors seem to be important^{116,117}. Most people with sarcoidosis have elevated levels of ACE in serum^{116,118} and

determination of serum ACE is used as a tool for diagnosis and to monitor the disease¹¹⁷.

Pharmacological inhibition of RAS

ACEi are first-line therapy for patients with hypertension and heart failure. A number of large clinical trials have been conducted evaluating the cardioprotective effects of ACEi; EUROPA¹¹⁹, GISSI-3¹²⁰ and HOPE¹²¹. These studies provide convincing evidence that ACEi decrease overall mortality and reduce the incidence of MI, stroke and heart failure. In most people, ACEi lower mean, diastolic and systolic blood pressure but only a small part of the beneficial effects of ACEi seems to be due to a reduction in blood pressure¹²¹.

ARBs are a quite recent developed class of drugs that block the effect of ang II at the receptor level and thereby inhibit the biological effects of ang II¹²². ARBs can be used for treatment of hypertension where the patients are intolerant to ACEi therapy. In addition to the blood pressure lowering properties, ARBs also provide cardiovascular protection¹²³.

Tobacco

Tobacco consumption

According to statistics from the World Health Organization (WHO), more than 5 million people die every year from lung cancer, CVDs and other diseases as a consequence of tobacco use¹²⁴. Since cigarettes were introduced on the market in the beginning of the 20th century, the global consumption has been rising continuously, and today over 15 billion cigarettes are smoked worldwide every day¹²⁵. In 1980 more than 30% of the adults in Sweden were smokers, but the cigarette consumption in Sweden has declined since then, and in 2006 14% of the adult population were smokers¹²⁶. In developing countries, however, the use of tobacco still increases¹²⁵. Oral tobacco is used in different forms throughout the world¹²⁵. In Sweden, oral snuff (snus) has been used for about 200 years, and today, about 20% of the adult Swedish men, and 2-3% of the women are daily snuff users¹²⁷.

Nicotine and nicotine metabolites

Nicotine is a naturally occurring alkaloid found in many plants, e.g. *Nicotiana tabacum* (Solanaceae). It is absorbed through the oral cavity, skin, lung and gastrointestinal tract¹²⁸. The rate of absorption is a pH dependent process; increased pH enhances the absorption of nicotine¹²⁸. Following absorption, nicotine is metabolized mainly in the liver, to a number of metabolites. There are several individual differences in the pattern of nicotine metabolism, but the pattern appears to be consistent for an individual at different times¹²⁹. On average, 70-80% of the nicotine is metabolized to cotinine¹³⁰, about 4% is converted to nicotine-1'-N-oxide and 0,4% to nornicotine¹²⁹. Cotinine is further metabolized to cotinine-N-oxide, norcotinine and trans-3'-hydroxycotinine, among others¹²⁹. For most people, trans-3'-hydroxycotinine is the most abundant metabolite in urine, accounting for 38% of the metabolites¹²⁹. The cytochrome P450 2A6 (CYP2A6) isoenzymes play a central role in the metabolism of nicotine¹³¹. There is a genetic polymorphism in human CYP2A6 gene, which to some extent may explain the differences in nicotine

metabolism¹³². The half-life of nicotine is approximately 2-3 hours^{129,133} and plasma nicotine concentration in smokers usually range between 20-40 ng/ml (0.12-0.25 μ M)^{129,133}. The nicotine metabolites have considerable longer half-lives compared to nicotine, on average 16-17 hours^{130,134}. Because of the long elimination time, levels of nicotine metabolites tend to accumulate throughout the day¹³⁰ resulting in high plasma concentrations for cotinine and trans-3'-hydroxycotinine in tobacco users^{130,135}.

Cardiovascular effects of tobacco use

There is a strong association between cigarette smoking and risk for atherosclerosis and other CVDs¹³⁶⁻¹³⁸. Cigarette smoke is a complex mixture of chemical substances, containing not only nicotine, but also a number of potentially cardiotoxic substances¹³⁹. The underlying mechanisms by which smoking induce CVDs are not entirely clear, but are most likely multi-factorial. Use of oral snuff, where nicotine is absorbed through the oral mucosa, and nicotine infusion have been shown to cause endothelial dysfunction^{140,141}. In addition, a large-scale study has shown that long-term use of snuff is associated with an increased risk of fatal MI¹⁴². The role for nicotine in the development of CVDs in tobacco users is unknown.

Nicotine gum and patches are commonly used as smoking cessation therapy, and nicotine has been evaluated as a therapeutic agent for ulcerative colitis, AD and Parkinson¹⁴³. Nicotine affects vascular biology in many ways and some of the mechanisms are well characterized. By activating the sympathetic nervous system, nicotine induces increased heart rate, myocardial contraction, vasoconstriction in the skin and coronary blood vessels and induces adrenal and neural release of catecholamines¹⁴³. Using heart rate as a marker of sympathetic neural activation, it has been reported that the sympathetic nervous system is activated 24 hours a day, during regular smoking¹⁴⁴. Results from studies in cholesterol-fed rabbits have indicated that treatment with nicotine accelerates the development of atherosclerosis^{145,146}. However, the relevance of these findings to human atherogenesis has been questioned¹⁴³.

In male squirrel monkeys, long-term administration of nicotine has been reported to affect lipid metabolism; generating an atherogenic lipoprotein profile with increased levels of low-density lipoprotein and decreased high density lipoprotein/total cholesterol ratio¹⁴⁷. In healthy non-smokers, however, oral nicotine administration for 2 weeks did not affect the lipid profile¹⁴⁸. Furthermore, a few studies have suggested that nicotine increases platelet activity¹⁴⁹, however, most studies report no effect of nicotine on platelet function^{150,151}. In endothelial cells, nicotine has been shown to induce changes in various atherosclerosis-related genes, including nitric oxide synthase¹⁵², platelet-derived growth factor¹⁵³, basic fibroblastic growth factor¹⁵⁴ and VEGF. In addition, nicotine has also been reported to cause morphological changes in endothelial cells¹⁵⁵, increased endothelial cell death¹⁵⁶ and enhanced trans-endothelial transport of macromolecules¹⁵⁷. A number of studies have also demonstrated that nicotine can cause acute endothelial dysfunction in animal models^{152,158,159} and humans^{141,160}. There are quite limited data on the effect of nicotine on ACE. A few early in vivo studies have been performed suggesting that the acute effect of smoking and nicotine infusion is an increased plasma ACE activity^{161,162}, whereas long-term use generates the opposite response^{163,164}.

AIM

The aim of this thesis was to investigate ACE in relation to cardiovascular risk factors and CVDs.

In paper I, we studied the effect of nicotine and nicotine metabolites on ACE in vitro. We also examined if the effect of nicotine and nicotine metabolites is influenced by ACE genotype.

In paper II, we investigated variations in plasma ACE level in order to elucidate the associations between circulating ACE levels, ACE genotype, known cardiovascular risk factors and CVDs in an elderly population.

MATERIALS AND METHODS

Materials and methods have been described in detail in paper I and II. This section will therefore only include a summary of some methods and some methodological considerations.

Culture of endothelial cells, paper I

Most of the results in paper I are based on experiments performed in HUVECs. The method for isolation and cultivation of HUVECs was first described by Jaffe and colleagues¹⁶⁵. Umbilical cords are by far the most used source to obtain human endothelial cells. As the umbilical cords are readily available and have unbranched vessels of decent size, they are suitable for isolation of endothelial cells.

Umbilical cords were collected from the delivery ward at Linköping University Hospital, Sweden after normal vaginal deliveries without complications. Informed consent was obtained from the mothers. The umbilical vein was treated with collagenase to make the endothelial cells detach from the underlying extracellular matrix. The collagenase solution containing endothelial cells was collected and endothelial cells were seeded in culture flasks. The extracellular matrix to which endothelial cells are attached in the blood vessels is a complex network consisting of a number of macromolecules such as collagen, elastin, fibronectin, laminine and proteoglycans. Endothelial cells that are grown on uncoated plastic have low spontaneous proliferation and relatively high apoptosis, whereas growth on surfaces of collagen, fibronectin or gelatin is more optimal¹⁶⁶. In this thesis HUVECs were grown on surfaces consisting of 0.2% gelatin. HUVECs were cultured in cell culture medium containing endothelial cell growth factor, heparin, HEPES, insulin, nonessential amino acids, oxalacetic acid, penicillin, streptomycin, and 17% heat-inactivated fetal bovine serum. Since HUVECs are primary culture cells they have limited life span and culturing for more than 2-3 passages results in a reduced proliferation rate. In addition, it has also been reported that the expression

of ACE in HUVECs is reduced by cultivation⁵⁹ and HUVECs were thus used at the first or second passage.

Study population, paper II

The study population used in paper II was based on 672 individuals (321 men, 351 women) aged 69-87. In 1999, all inhabitants aged 64-82 in Kinda municipality, Sweden, (n=1130) were invited to take part in a longitudinal study investigating the prevalence of heart failure. A total of 876 individuals agreed to participate. A few years later (2003-2005), all participants were re-invited to take part in a follow-up study. A total of 123 individuals had died before the follow-up study started and 675 of the remaining population decided to participate. Two individuals were excluded from the study due to difficulties obtaining a blood sample and one individual was excluded due to hepatitis infection, resulting in a final study population of 672 individuals. All data included in this study are based on the follow-up study.

Data sampling, patient history and definitions, paper II

A cardiologist performed a physical examination of all participants. Blood pressure was determined, with subjects in the supine position after at least 30 min rest, using a sphygmomanometer. Mean value from three consecutive measurements was calculated, and according to clinical routine, adjusted to the nearest 5 mmHg. Blood samples were drawn after overnight fasting and plasma was prepared by centrifugation. Blood and plasma were stored at -70°C pending analysis. Height and weight were recorded, body mass index (BMI) calculated and fasting plasma glucose concentrations determined. Patient history concerning cardiovascular risk factors, CVDs, and medications were recorded by the examining cardiologist. DM was defined as fasting plasma glucose $>7\text{mmol L}^{-1}$ or diagnosis of DM with ongoing treatment. Dyspnea was defined as a subjective sensation of difficulty in breathing. Individuals diagnosed and treated for hypertension, or a blood pressure $>160/95$ mmHg, were

defined as hypertensive. IHD was defined as history of angina pectoris, treatment of angina pectoris and/or history of MI. Heredity for CVDs was defined as mother, father or siblings having a history of hypertension, stroke or MI. Individuals who stated that they smoked were considered to be smokers.

Measurement of ACE activity and level

We used two different methods in order to determine ACE activity (Units, U) and ACE level (ng/ml). ACE activity was measured using a radioenzymatic assay (ACE-direct REA, Bühlmann Laboratories, Schönenbuch, Switzerland). The principle for the assay is based on the cleavage of the synthetic substrate ³H-hippuryl-glycyl-glycine into ³H-hippuric acid and glycyl-glycine dipeptide. This conversion is catalyzed by ACE. Adding of HCl stops the enzymatic reaction and the scintillation cocktail separates the ³H-hippuric acid from the unreacted substrate. The amount of ³H-hippuric acid is measured using a beta-counter and can be used to calculate ACE activity. One unit ACE is defined as the amount of enzyme required to produce 1 μmol hippuric acid per minute and liter.

The level of ACE in was measured using enzyme-linked immunosorbent assay (ELISA) (Quantikine, Human ACE Immunoassay, R&D Systems, Minneapolis, USA). The principle for the assay is as follows; monoclonal antibodies specific for ACE are coated on the bottom of a microplate. When serum, plasma or cell lysate are added to the wells, ACE will bind the antibodies. After washing away unbound substances a polyclonal antibody directed against ACE is added followed by an enzyme, which will be linked to the polyclonal antibody. Thereafter, a substrate, which will be converted to a coloured compound, is added. The amount of colour, which is proportional to the amount of ACE in the samples, is measured using a spectrophotometer.

In order to determine whether the activity correlates to the level of the enzyme, both ACE activity and level was analysed in serum samples from 21 individuals and in HUVECs from 15 individuals. HUVECs were sub-cultured in 96-well plates for analysis of ACE activity and in 24-well

plates for analysis of ACE level. A previous study has shown a strong correlation between ACE activity and ACE level in plasma, determined by similar methods⁵⁵. As shown in Figure 1, we found a strong correlation between ACE activity and level in both HUVECs and serum.

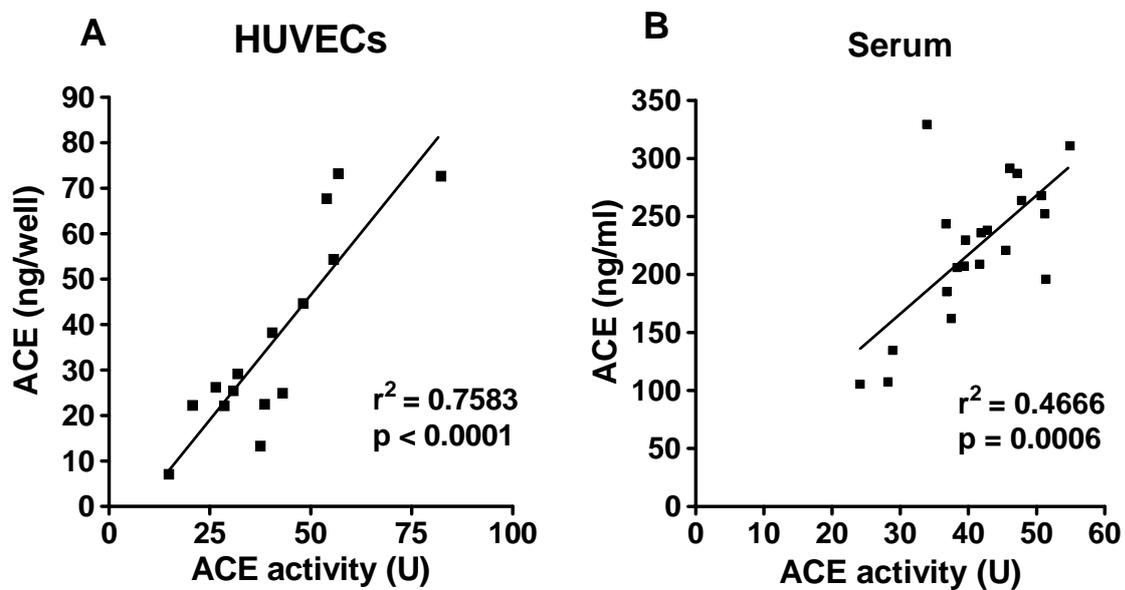


Figure 1. Correlation between ACE activity and ACE level in A) HUVECs ($r^2 = 0.7583$, $p < 0.0001$) and B) serum from healthy individuals ($r^2 = 0.4666$, $p = 0.0006$).

ACE genotyping

The first method for determination of ACE-genotype was described in 1990 by Rigat et. al. A few years later Shanmugam and colleagues reported that this method resulted in mistyping of a number of subjects, where carriers of the I/D genotype were identified as D/D carriers¹⁶⁷. As many as 10-49% of subjects previously identified as D/D carriers have been retyped as I/D carriers using improved methods¹⁶⁸⁻¹⁷⁰. To avoid mistyping, we used a modified multiplexed polymerase chain reaction (PCR) method previously described¹⁷¹. In this method, three primers were used, which allows detection of a 237 bp fragment for the deletion allele, and two fragments, 155 bp and 525 bp, for the insertion allele (Figure 2). The amplified DNA was separated by gel electrophoresis and visualized by UV-light. ACE genotype was determined based on the number and length of the fragments. If there were the least uncertainty about the result, the samples were reanalysed.

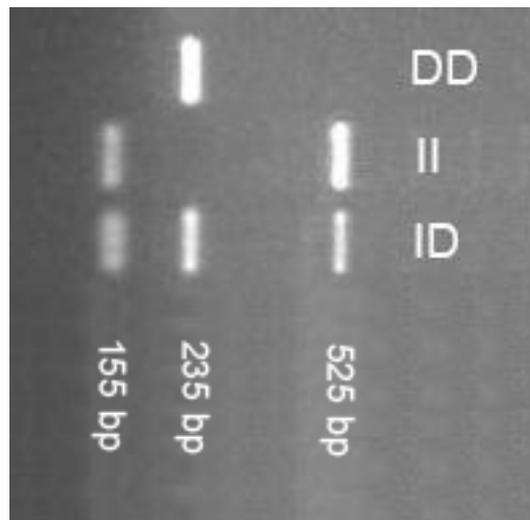


Figure 2. PCR products separated by gelelectrophoresis and visualised by UV-light. The D/D genotype results in one band at 235 bp, I/I genotype two bands at 155 and 525 bp and the I/D genotype generates all three bands.

Tobacco extracts

In addition to the experiments described in paper I and II, a few pilot-experiments have been performed studying the effect of oral snuff extract (OSE) and cigarette smoke extract (CSE) on ACE activity in human serum and/or in HUVECs.

OSE was prepared using a modified method first described by Petro and colleagues¹⁷². Ten grams of oral snuff (Ettan Lös, Swedish Match, Stockholm, Sweden) were mixed with 100 ml of phosphate-buffered saline (PBS). The mixture was incubated for 2 hours at 37°C followed by centrifugation for 10 min at 450 g. The supernatant was re-centrifuged for 1 hour at 13000 g. The remaining solution was sterilized using a 0.2 µm filter and pH was adjusted to 7.4. OSE was stored in aliquots at -70°C. The filtered solution was considered to be 100%.

CSE was prepared as described by Su et al.¹⁷³ with a few modifications. Camel cigarettes (R. J. Reynolds Tobacco Company, Winston-Salem, NC, USA) were combusted using the equipment shown in Figure 3. Smoke from two cigarettes was drawn through 10 ml PBS, pre-warmed to 37°C using water suction at a constant flow. Each cigarette was smoked for 5 min ±30 sec. The solution was sterilized using a 0.2 µm filter and the filtered solution was considered to be 100%. CSE was prepared 30 min prior to use.

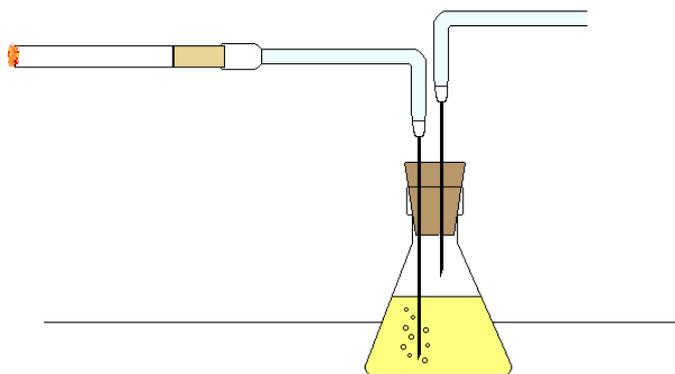


Figure 3. Illustration of the equipment used for preparation of cigarette smoke extract. Smoke from two cigarettes was drawn through 10 ml pre-warmed phosphate-buffered saline using water suction at a constant flow.

Analysis of nicotine content in tobacco extract

In order to compare effects of nicotine and tobacco extracts, nicotine content in the extracts were analysed. High-performance liquid chromatography (HPLC) was used to separate nicotine from other constituents and nicotine content was quantified using a UV detector. Chromatography is a physical separation method where the components in a mixture are distributed between two phases, one is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction. In HPLC the mobile phase is a liquid, delivered under high pressure to ensure constant flow and reproducible chromatography. The stationary phase is packed onto a column capable of withstanding high pressures. Samples are injected into a stream of mobile phase which is driven by a pump through the column. Due to interactions with the stationary phase, the substance of interest passes slowly through the column before reaching the detector. The retention time refers to the time it takes for a substance to pass through the column, and results in an absorption peak at the detector. Quantification of a specific substance is possible if the substance is separated from other substances absorbing UV at the same wavelength. The area and/or height of the chromatographic peak will be proportional to the amount of substance injected into the column.

Our system consisted of a P680 HPLC pump from Dionex (Sunnyvale, CA, USA), and a Gina 50 autosampler and a photo-diode array UV-detector UVD340U from Gynkotec (Germinger, Germany). The column was an X-bridge C18 3 μm , 3x100 mm from Waters (Milford, MA, USA). Samples were separated using a mobile phase consisting of 5:95 (v/v) acetonitrile:ammonium formiate 10 mM, pH 4.2, at flow rate of 500 $\mu\text{l}/\text{min}$. Each sample was injected into the HPLC system in a volume of 20 μl and nicotine was detected at a wavelength of 260 nm. The run time for each sample was 4 min and the retention time was 2.3 min (Figure 4). A standard curve was constructed using 25, 50, 100 and 250 μM of nicotine. CSE was diluted 1:1-1:5 in mobile phase, while OSE was diluted 1:25. The between-day relative standard deviation (RSD) and precision were

$\leq 8\%$ and 98-101%, respectively (n=4 for 2 different concentrations) and the within-day RSD and precision were $\leq 9\%$ and 96-103%, respectively (n=4 for 2 different concentrations).

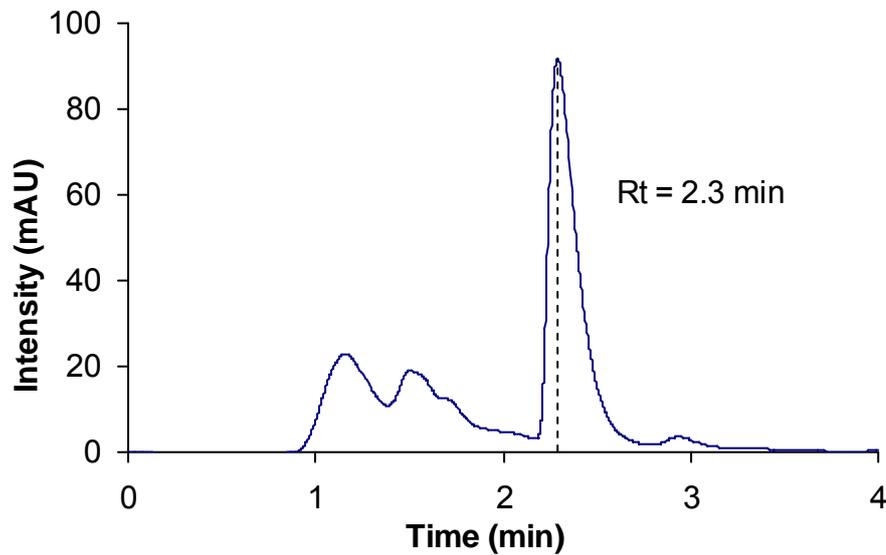


Figure 4. Typical chromatogram for the HPLC method used for quantification of nicotine in tobacco extracts. AU = absorbance unit, Rt = retention time

Statistical methods

In paper I, one-way ANOVA for repeated measures followed by Dunnett's post-hoc test was used. Statistical analyses were performed using GraphPad prism version 4 (GraphPad Software Inc., San Diego, CA, USA).

In paper II, a number of different statistical methods were used. One-way ANOVA, followed by Bonferroni post-hoc test was used to compare mean values for continuous data between the three genotypes or between groups based on number of risk factors. Chi-square test was used for distinct variables. Correlation analysis was performed in order to study the relationship of ACE level (ng/ml) and ACE activity (U). The influence of cardiovascular risk factors and CVDs on plasma ACE level was analyzed using multi-way ANOVA, where main effects and 2-way interactions were analysed. Interactions that were statistically significant ($p < 0.05$) were included in the final analysis. Effect of cardiovascular medications on plasma ACE was analysed in a separate model using multi-way ANOVA, where only main effects were analysed. Statistical analyses were performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

For unpublished results regarding the effect of tobacco extract on ACE, one-way ANOVA for repeated measures followed by Dunnett's post-hoc test was used. Statistical analyses were performed using GraphPad prism version 4 (GraphPad Software Inc., San Diego, CA, USA). Data were presented as mean value \pm SEM

Results are presented as mean values, unless otherwise stated. P-values < 0.05 were considered statistical significant. Statistical significance was described with: * = $p < 0.05$ and ** = $p < 0.01$ and *** = $p < 0.001$

RESULTS & DISCUSSION

Effect of tobacco use on ACE, paper I & II

There is no doubt about the negative impact smoking has on the cardiovascular system. The effect of smokeless tobacco however, (e.g oral snuff) is more controversial. We studied the effect of nicotine, nicotine metabolites, smoking and oral snuff on ACE in order to elucidate the role of ACE in development of CVDs in smokers and to shed light on the possible impact of oral snuff on the cardiovascular system.

Effect of nicotine and nicotine metabolites on ACE, paper I

The effect of nicotine and nicotine metabolites on ACE in human endothelial cells and in human serum was examined in paper I. We used the five most abundant metabolites found in plasma from tobacco users; cotinine, cotinine-N-oxide, nicotine-1'-N-oxide, norcotinine, and trans-3'-hydroxycotinine^{129,130}. Nicotine and nicotine metabolites were used in concentrations ranging from 0.1 to 10 μ M, which is comparable to the levels observed in plasma in smokers^{129,133}.

Results showed that nicotine and nicotine metabolites increased both activity and expression of ACE in HUVECs. The effect on ACE activity was quite clear and most of the metabolites induced a dose-dependent increase. The expression of ACE was not affected to the same extent. Cotinine however had no effect on either ACE activity or expression. Thus, it appears as if nicotine and nicotine metabolites increase ACE activity by affecting both synthesis and activity of the enzyme. Pre-treatment of HUVECs with a protein synthesis inhibitor further confirmed this conclusion. If the increase in ACE activity was due to an increased expression of the enzyme solely, incubation with a protein synthesis inhibitor would abolish the effect of the drugs. However, nicotine could still induce an elevation in ACE activity, although the effect was slightly reduced at the highest concentrations.

HUVECs were incubated with nicotine and nicotine metabolites for 10 min up to 24 hours. In a few experiments ACE activity was increased already after 10 min and in some experiments the effect sustained for up to 24 hours. Although in most experiments an incubation time of 30 to 60 min had the greatest effect on both ACE activity and expression.

Next we were interested in examining the effect of nicotine on circulating ACE. Therefore, ACE activity was analyzed in serum from three healthy volunteers after 1 hour incubation with nicotine using the same concentrations as for HUVECs. The results showed no effect of nicotine on serum ACE. Thus, it appears as if nicotine can regulate ACE only when it is attached to the cell membrane.

A few previous studies have examined the effect of nicotine on ACE. Saijonma and colleagues examined the effect of nicotine alone (in concentrations similar to those in our study) and together with vascular endothelial growth factor on ACE in HUVECs¹⁷⁴. They used real-time PCR to quantify ACE mRNA and an inhibitor-binding assay to determine ACE activity. The results showed no effect of nicotine alone after 4, 18 or 24 hours incubation, but together with VEGF nicotine potentiated the VEGF-induced ACE up-regulation. These results are inconsistent compared to the results from our study. Different methods and incubation times may be reasons for these discrepancies. Zhang and co-workers studied the effect of nicotine on ACE in cultured human coronary artery endothelial cells showing that incubation with nicotine for 24 hours induced an increased expression of ACE mRNA¹⁷⁵. Although a different cell type was used, the results are in agreement with the findings from our study. However, in contrast to Zhang et al. our results showed an immediate effect, as ACE was affected already after 1 hour.

In addition, Sugiyama et al showed an increased serum ACE activity in dogs 30-60 min after intravenous administration of nicotine¹⁶², which is in agreement with our results.

Cardiovascular effects of nicotine have been studied extensively, but there are quite limited data regarding the effects of nicotine metabolites. As plasma concentrations of some of the nicotine metabolites (cotinine and

trans-3'-hydroxycotinine) are higher than nicotine, we believe that the effects of the metabolites are important and should be addressed more often.

Effect of tobacco extract on ACE activity in HUVEC

After studying the effect of nicotine and nicotine metabolites on ACE, we were interested in examining whether entire tobacco products have similar effects (unpublished results).

The results showed a dose-dependent inhibition of ACE after 1 hour incubation with OSE (Figure 5A & B). This effect could be seen both in HUVECs and in human serum. No effect was however seen in HUVECs after incubation with CSE (Figure 5C). The effect of CSE on ACE in serum was not studied. The nicotine content in 1, 5 and 10% OSE was 45, 225 and 450 μM respectively. CSE was used in concentrations of 0.1, 1 och 10%, which corresponds to a nicotine content of 0.043, 0.43 and 4.3 μM respectively.

The nicotine concentrations used in paper I were 0.1, 1 and 10 μM , which is a bit lower compared to the OSE experiments, but quite similar to the concentrations used in the CSE experiments. Consequently, nicotine concentrations used in the OSE experiments were higher than it usually is in plasma from tobacco users (0.12-0.25 μM)^{129,133}. However, it is not unlikely that at least the lowest concentration (45 μM) can be obtained locally in the capillaries surrounding the oral cavity.

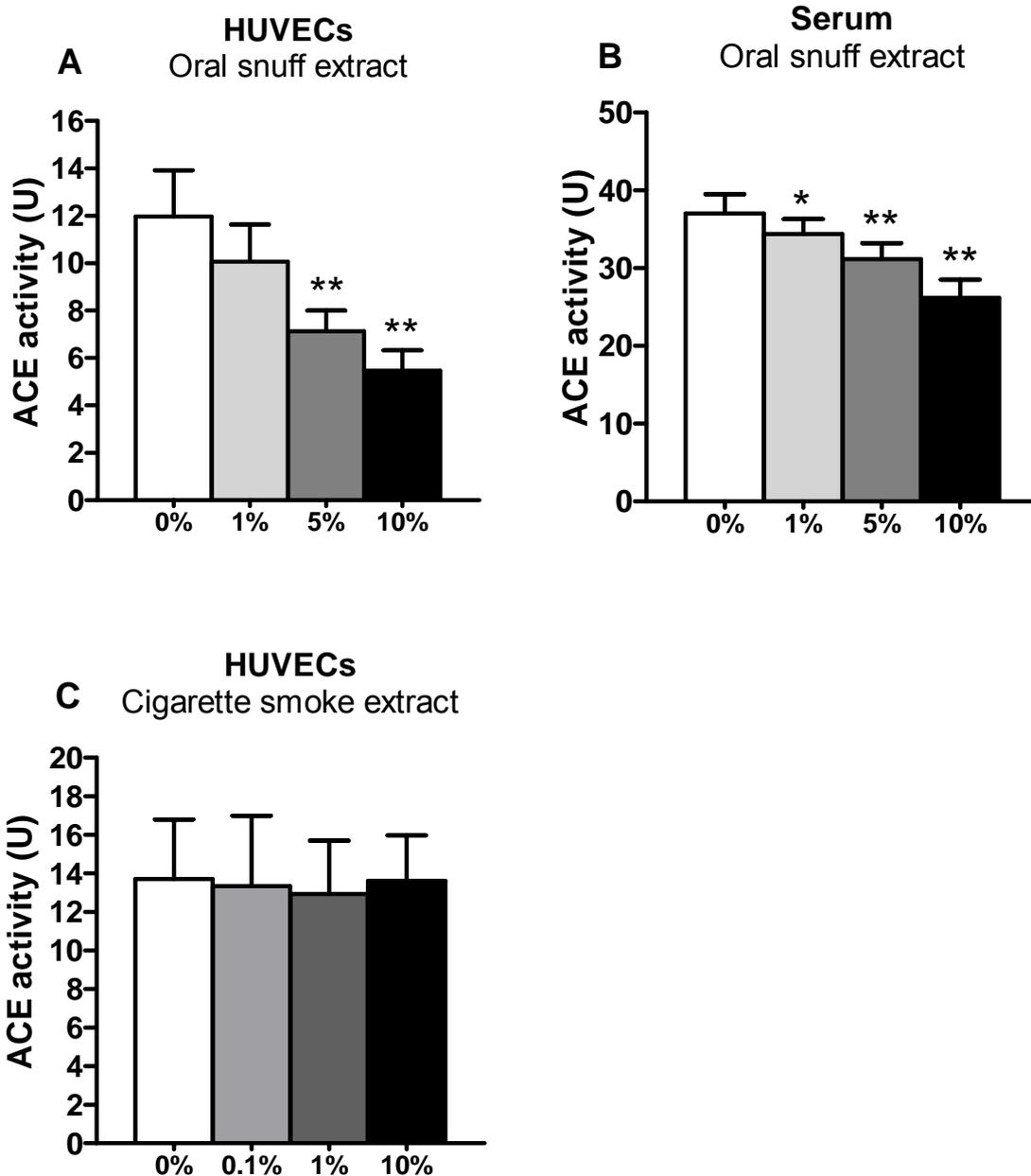


Figure 5. Effect of tobacco extract on ACE activity. ACE activity in (A) HUVECs (n=3) and (B) serum (n=4) after 1 hour treatment with OSE in different concentrations. (C) ACE activity in HUVECs (n=3) after 1 hour treatment with CSE in different concentrations. Values are mean \pm SEM. Statistical significance was calculated using one-way ANOVA for repeated measures. * $p < 0.05$, ** $p < 0.01$

One could argue that the high nicotine levels in the OSE experiments probably were cytotoxic, as shown previously¹⁵⁶, and that a decreased ACE activity reflects a reduced viability rather than an actual effect on the enzyme. This is however contradicted by the fact that OSE also inhibited ACE in serum, where no cells are present. In addition, no visual signs of toxicity could be observed when looking at HUVECs in a light microscopy.

One previous study has reported that cigarette smoke induces a release of ACE from cultured endothelial cells¹⁷⁶. Alterations in the total amount or activity of the enzyme were unfortunately not examined. Instead, they argued that the release of ACE reflects endothelial cell injury as a consequence of exposure to cigarette smoke. The effect of oral snuff or other smokeless tobacco products on ACE has not been addressed previously, and it is unknown if our findings can be applied in vivo. Oral snuff has previously been shown to cause endothelial dysfunction¹⁴⁰ and it has been reported that oral snuff users face a higher risk of dying in CVDs compared to non-users¹⁴². Although there may be an inhibitory effect of oral snuff on ACE, the net effect on the cardiovascular system seems to be negative^{140,142}.

Tobacco contains numerous chemical compounds and most tobacco also contains a number of additives. It is unknown which of these compounds exerts the inhibitory effect on ACE, but it appears as if this compound is present in snuff, but not in cigarette smoke, as no effect was seen by CSE. In addition, the inhibitory effect of OSE seems to exceed the stimulatory effect of nicotine.

Effect of smoking on plasma ACE, paper II

Smoking was one of the risk factors for CVDs examined in relation to plasma ACE in paper II. Subjects were classified as smokers or non-smokers based on their own statements. Sixty three individuals stated that they were smokers (9%), but nineteen of those were not included as they were treated with ACEi. Approximately 5-11% of the Swedish population older than 65 years are according to Statistics Sweden (SCB), daily smokers¹⁷⁷, which is similar to the number in our study.

We found that smoking was associated with increased levels of ACE in plasma. It is possible that this effect is mediated, at least to some extent, by nicotine and nicotine metabolites. In addition, we found an interaction between smoking and IHD, where smokers with IHD had higher plasma ACE levels compared to non-smokers.

Previous studies have shown that the acute effect of smoking is an increased serum ACE activity^{161,162}. Reports regarding the effect of long-term use have however been inconsistent^{163,164,178}. Two small studies including healthy volunteers showed that smokers have a decreased ACE activity compared to non-smokers^{163,164}. These studies did not adjust for ACE-genotype, which might have influenced the outcome. In contrast, a large population based study reported that smokers have higher plasma ACE activity compared to non/former smokers¹⁷⁸. This difference was significantly higher among D/D and I/D carriers, whereas only a tendency was seen among carriers of the I/I genotype. In addition, a higher risk for coronary heart disease and CVDs in smokers was seen among carriers of the D/D genotype compared to I/I¹⁷⁹.

Thus, results from our and previous studies^{163,164,178,179} indicate that tobacco use in some way interfere with RAS, but it appears as if the association is rather complex. It is possible that genetic factors and/or presence of CVDs are of importance for how tobacco use influences ACE, but such associations remain to be investigated in future studies.

Individual variations, paper I & II

Because of the limited life span of HUVECs and reduced expression of ACE by cultivation⁵⁹, all experiments were performed in the first or second passage. Each experiment was performed using HUVECs obtained from different individuals. The basal activity and expression of ACE in untreated endothelial cells varies a lot between HUVECs from different individuals. In our experiments, the basal ACE activity and expression varied between 5-82 U and 10-41 ng/well respectively. As all comparisons were made between treated and untreated cells from the same individual, this variation should not interfere with the results. However, when mean values were calculated this variation generated a relatively large spread. It is likely that at least part of the individual variation is due to the ACE I/D polymorphism. ACE levels in cardiac tissue and T-lymphocytes are under influence of the ACE I/D polymorphism^{29,66}, but this association has to our knowledge not been studied in endothelial cells.

The influence of ACE genotype on ACE level in plasma was studied in paper II and the results confirmed previous findings³³⁻³⁵ showing that I/I, I/D and D/D carriers have low, medium and high plasma levels respectively. However there were large variations in plasma ACE among individuals with the same genotype (Figure 6) and several I/I carriers had higher plasma ACE level than the majority of the D/D carriers. In our study, approximately 15% of the variation in plasma ACE could be explained by the ACE I/D polymorphism, which is lower compared to previous studies showing that 20-50% of the variation can be accounted for by the ACE genotype³³⁻³⁵. This was not very unexpected though, since our study population was more heterogeneous (elderly men and women with relatively high prevalence of different diseases) with more possible interfering factors.

When examining the effect of nicotine and nicotine metabolites on ACE in HUVECs, an individual variation in response to the drugs was observed. We hypothesized that this variation could be due to genetic variations in the ACE gene. Thus, ACE genotype was analyzed in cell lysate from HUVECs and the relationship between ACE activity and genotype was elucidated. We also examined whether the effect of the drugs were influenced of basal ACE activity. However, no such associations were found and the explanation for the variation remains unknown.

It appears as if there are other factors than the ACE I/D polymorphism that influence the level of ACE. Several genetic variations, other than the ACE I/D polymorphism, have been identified in the ACE gene, and some of them might affect the activity of the enzyme¹⁸⁰⁻¹⁸². Other factors such as hormonal status, diet, salt intake and physical activity could perhaps explain some of the variations, but such factors remains to be investigated in future studies.

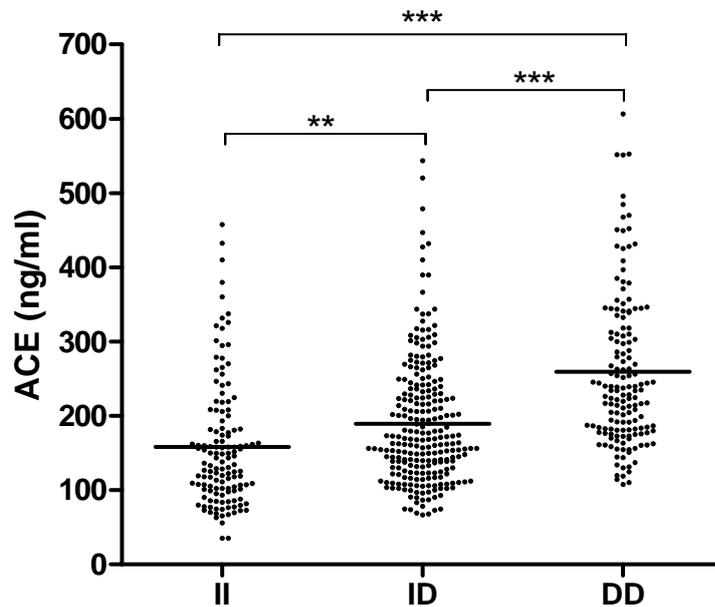


Figure 6. Plasma ACE levels (ng/ml) according to ACE I/D polymorphism. Individuals receiving ACEi are excluded. n=531 (I/I 134, I/D 243, D/D 154), ** p<0.01, *** p<0.001. I=insertion, D= Deletion

Population based study of ACE in relation to cardiovascular medications, risk factors and diseases, paper II

In paper II, ACE genotype and ACE level in relation to cardiovascular medications, risk factors and diseases, was studied. The population consisted of 675 men and women aged 69-87 (mean age 78.2 years). An elderly population, where the prevalence of CVDs is relatively high makes it possible to discover accurate associations.

ACE inhibitors

A total of 141 subjects (21%) received ACEi. We demonstrated that ACEi-therapy is associated with increased plasma ACE levels. Mean plasma ACE level for individuals receiving ACEi was 325.4 ng/ml compared to 200.7 ng/ml for subjects who were not treated with ACEi. These results are in accordance with previous findings^{40,42,44,183} and we suggest that this is due to a compensatory increase in synthesis of the enzyme. As a consequence of such increase, patients treated with ACEi could experience a rebound effect if the treatment is interrupted or changed. Furthermore, it can be speculated that adjustment of the dose after a few weeks of treatment could perhaps improve the effect of the medication.

In less than 25% of individuals receiving monotherapy as treatment for hypertension, the therapy is able to normalize the blood pressure¹⁸⁴. Treatment with ACEi seems to have a more pronounced effect, concerning blood pressure, in carriers of the I/I genotype than in D/D carriers¹⁸⁵. It is possible that individuals with high ACE levels need higher doses of ACEi to lower their blood pressure. Determination of ACE level in hypertensive patients might be a useful tool to find the best blood pressure lowering treatment.

As treatment with ACEi clearly affects the level of ACE we chose to exclude those individuals from further data analyses concerning ACE level.

ACE genotype and CVDs

The first report regarding an association between ACE I/D polymorphism and risk for MI was published in 1992 by Cambien et al.⁷⁹. This finding generated huge scientific interest and have yielded extensive research regarding a potential association between the ACE I/D polymorphism and different diseases. A recent review reported that more than 100 different conditions have been investigated in relation to the ACE I/D polymorphism, with CVDs being most commonly studied¹⁸⁶. The findings by Cambien et al⁷⁹. have been confirmed in a number of small studies, whereas a large-scale study failed to find any association⁹⁶.

We found no association between the D/D genotype and CVDs, instead we found a higher prevalence of IHD among carriers of the I/I genotype ($p=0.009$). I/I carriers were slightly older compared to D/D carriers (78.4 years for I/I compared to 77.5 for D/D), but this difference could not entirely explain the higher prevalence of IHD for I/I carriers ($p=0.043$ after correction for age). In agreement with most previous studies^{187,188}, no association between ACE genotype and blood pressure was found. In addition, we could not find any differences in BMI, plasma glucose, medications or other diseases between the genotypes.

If the D allele is in fact associated with CVDs and increased cardiovascular mortality, a selection bias before inclusion may have occurred with subsequent underestimation of the importance of the genotype. The risk for such bias is obviously higher when studying an elderly population. However, such selection would be reflected in the frequency of the genotypes, in favour of the I/I genotype. There are to our knowledge no reports of such selection.

Taken together, the value of the D/D genotype as predictor for CVDs is controversial and has been questioned repeatedly^{95,97}. If there is an association it still does not seem to be a very strong predictor for CVDs.

ACE level and cardiovascular risk factors and CVDs

We reported an increased plasma ACE level in individuals with IHD (defined as history of MI or angina pectoris). Cambien and colleagues have previously reported increased plasma ACE levels in patients who survived a MI³⁵. They found higher ACE levels in patients younger than 55 years, but no difference was seen among older subjects. Our results indicated however that this association is present among older subjects as well.

There are several differences between our study and the study by Cambien et al. Firstly, the study designs were different; the study by Cambien et al was a case-control study whereas our study was a population based cross-sectional study. Secondly, only men between 25-64 years were included in the Cambien study compared to both men and women at higher age (69-87 years) in our study. Thirdly, different outcomes were studied; Cambien et al included patients who survived a MI, whereas our study examined individuals with IHD. Although there are a number of differences, both studies indicate that cardiac complications generate an increased plasma ACE level.

Hypertension was associated with increased ACE levels. A possible explanation for such increase could be that the drugs used to lower the pressure are the ones that induce the increase. However, since all individuals receiving ACEi were excluded and as treatment with beta receptor blockers showed no effect, such explanation seems unlikely. Hypertension was defined as diagnosis with ongoing treatment or a blood pressure >160/95 mmHg. We chose these blood pressure cut-offs as blood pressure was measured on one single occasion and since most of the hypertensive individuals probably received their diagnosis based on this cut-offs. Using 140/90 mmHg as cut-offs would define almost 90% of the population as hypertensive. A quick analysis showed no difference between hypertensive and normotensive individuals when using the lower cut-offs. In addition, no association was found between ACE level and mean, diastolic or systolic blood pressure.

We also reported that ACE levels increased with increasing number of cardiovascular risk factors. Only risk factors that showed a statistical significant effect or a tendency to affect plasma ACE levels were included, i.e. DM, heredity for CVDs, hypertension and smoking.

The connection between these risk factors, atherosclerosis and CVDs is well established, however, the role of ACE is not that clear.

As we see it, there are two possible scenarios; 1) risk factors induce an upregulation of ACE which in turn leads to the development of an atherosclerotic plaque, or 2) risk factors induce a development of atherosclerosis through mechanisms independent of ACE, and the atherosclerotic process itself leads to increased expression of ACE. However, a combination of these scenarios is perhaps the most probable explanation. In addition, the underlying mechanism causing atherosclerosis is most certainly multi-factorial, where ACE only is one of several important factors.

ACE level and diabetes

Cardiovascular complications are common in patients with DM, but the underlying mechanisms for such complications are not entirely clear. We reported an increase in plasma ACE level in individuals with DM ($p=0.05$). These findings are in agreement with several previous studies^{81,107,109}, and may be explained by increased prevalence of CVDs in the DM population¹⁸⁹. However, no previous study examining ACE level in relation to DM has considered the influence of ACE genotype. In addition, our study does not support an association between ACE genotype and risk for DM previously reported¹¹⁰.

A number of studies have reported protective effect of RAS inhibitors against T2D¹⁹⁰⁻¹⁹². A previous meta-analysis including more than 70000 subjects showed a 23% relative risk reduction for onset of T2D in subjects treated with ACEi¹⁹³. A similar beneficial effect was seen in subjects treated with ARBs. Thus, targeting RAS might be a successful strategy to prevent onset of T2D in “risk individuals”. The mechanism explaining the preventive effects of RAS inhibition on onset of T2D seems to be complex¹⁹³ and the role of RAS for developing T2D remains unknown.

Is ACE genotype or phenotype, a risk factor for CVDs?

Our study does not support an association between D/D genotype and CVDs. Instead we found an association between plasma ACE level and cardiovascular risk factors and CVDs. We suggest that an increased level of ACE, rather than ACE genotype, is associated with increased risk for CVDs. Although CVDs seem to be associated with increased plasma ACE level, a high ACE level might not necessarily be associated with cardiovascular complications. It has been suggested that genetically enhanced ACE levels might be neutralized by other regulatory factors⁹⁸ and disruptions in such balance might favour the atherosclerotic process. Hence, there is a need for more studies investigating ACE genotype and phenotype simultaneously and more knowledge about the regulation of ACE is needed in order understand its role in human disease.

Limitations

Umbilical cords, which were used as source to obtain endothelial cells, are a unique type of tissue that only exists under a limited time period. If HUVECs behave in the same manner as endothelial cells from other tissues have not been clarified. However, umbilical cords are by far the most used source to obtain human endothelial cells.

Uptake of nicotine from cigarettes or other forms of tobacco is a quite well studied process. However, to what extent other substances in tobacco are absorbed into the circulation is not as well documented. The physiological relevance of our findings regarding the inhibitory effect of OSE on ACE activity remains thus unknown.

Furthermore, we found that cardiovascular risk factors and CVDs are associated with increased levels of ACE in plasma. If such increase is a cause or an effect of the disease remains unknown.

In addition, whether plasma ACE level reflects the level of ACE in tissues has not yet been clarified

MAIN FINDINGS & CONCLUSIONS

- Nicotine and nicotine metabolites increase ACE activity in endothelial cells by affecting both synthesis and activity of the enzyme. There are however individual variations in the response. The explanation for such variation remains unknown, but seems to be independent of ACE genotype
- Smoking is associated with increased plasma ACE levels. This effect might be mediated by nicotine and nicotine metabolites. Upregulation of ACE could be one mechanism by which smoking exerts negative effects on the vascular system.
- OSE inhibits ACE activity in human endothelial cells and serum. The substance/substances responsible for such effect remain unknown, but do not appear to exist in CSE. If these results have any physiological relevance remains to be investigated.
- IHD, hypertension, DM and heredity for CVDs are associated with increased plasma ACE levels. This study does not support an association between ACE D/D polymorphism and CVDs, but suggests that a high level of ACE in the circulation is a risk factor for CVDs.
- ACEi therapy induces an upregulation of ACE in plasma, and such treatment needs to be considered in studies examining ACE.

TACK

Jag skulle vilja rikta ett stort tack till alla er som på något sätt bidragit till denna avhandling. Speciellt skulle jag vilja tacka...

Min handledare, **Karin Persson**, för att du givit mig möjlighet att bedriva forskning och för att du introducerat mig till ett spännande område. Du har gett mig bra stöd och uppmuntran, men även förtroende och utrymme att arbeta självständigt. Det har varit väldigt lärorikt!

Min biträdande handledare, **Rolf Andersson** för bra synpunkter på både delarbeten och avhandlingen.

Medförfattarna till delarbetena i denna avhandling, speciellt tack till...

Hanna Björck för ett givande samarbete, för dina insatser under arbetet med delarbete 2 och för att du läst och haft bra synpunkter på avhandlingens innehåll. Du är dessutom en mycket bra vän och en utmärkt festfixare!

Toste Länne och **Urban Alehagen** för många givande diskussioner och bra synpunkter under arbetet med delarbete 2.

Henrik Gréen för engagemang och ovärderlig hjälp vid analys av nikotin i tobaksextrakten. Det var både kul och lärorikt!

Alla medarbetare på farmakologen, speciellt...

Andreas Eriksson, för alla uttömmande, men inte alltid så vetenskapliga diskussioner kring bl.a. Don Juan, cynism och små marginaler. Dessutom antar du alltid en utmaning trots att du oftast förlorar.

Ann-Charlotte Svensson Holm för alla trevliga promenader, jobbiga träningspass och för att du är en mycket bra vän.

Caroline Skoglund för att du alltid är snäll och hjälpsam, och för alla fiffiga presenttips.

Mina vänner från studietiden på Medicinsk Biologi, för alla trevliga middagar, fester, resor och annat under och efter studietiden.

Mina barndomsvänner från Småland, även om det inte blir lika ofta nuförtiden är det alltid lika kul när vi ses.

Min familj, **mamma**, **pappa** och **Erika** för att ni tror på mig och stöttar mig i alla lägen.

Andy för all kärlek, stöd och uppmuntran.

Finansiärerna av denna avhandling: Cardiovascular Inflammatory Research Center vid Linköpings Universitet (CIRC), Elanora Demeroutis Fond för Kardiovaskulär forskning vid Universitetssjukhuset, Linköping, Hälsobonden, Linköping, Rådet för Medicinsk Tobaksforskning (Swedish Match Northern Europe AB), Östergötlands läns landsting (ÖLL) och Vetenskapsrådet.

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