Angiotensin-converting enzyme in cardiovascular function and dysfunction

Liza Ljungberg

Division of Cardiovascular Medicine
Department of Medical and Health Sciences
Faculty of Health Sciences
Linköping University, Sweden

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Cover: 3D structure of the extracellular/soluble part of angiotensin-converting enzyme obtained from Entrez’s 3D structure database MMDB (ID84704).

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The scientist is not a person who gives the right answers, he is one who asks the right questions

Claude Lévi-Strauss
ABSTRACT

Angiotensin-converting enzyme (ACE) is a key enzyme in the renin-angiotensin system, converting angiotensin I to the vasoactive peptide angiotensin II, and degrading bradykinin. Angiotensin II is a multifunctional peptide, acting on a number of different tissues. A common genetic variation in the gene encoding ACE; ACE I/D polymorphism influences the level of ACE in the circulation, and has been linked to increased risk for cardiovascular disease. This thesis aimed to explore the connection between ACE and cardiovascular function and dysfunction.

The impact of nicotine and nicotine metabolites on ACE in cultured human endothelial cells was studied. Nicotine as well as nicotine metabolites induced increased ACE activity in cultured human endothelial cells. In elderly men higher ACE levels were seen in smokers compared to non-smokers. Furthermore, diabetes was associated with higher circulating ACE. Increased ACE level may represent a cellular mechanism which contributes to vascular damage.

Elderly men carrying the ACE D allele had higher abdominal aortic stiffness compared to men carrying the I/I genotype. Our data suggest that the mechanism by which the ACE D allele modulates aortic wall mechanics is independent of circulating ACE levels. Previous studies have indicated a link between the D allele and abdominal aortic aneurysm. Increased aortic stiffness suggests impaired vessel wall integrity, which combined with local hemodynamic and/or inflammatory factors may have a role in aneurysm formation.

Subjects with left ventricular dysfunction had higher levels of circulating ACE compared to those with normal left ventricular function, while there was no association between ACE and central hemodynamics. ACE might play a role in the pathogenesis of left ventricular dysfunction and our findings suggest a direct effect on the heart rather than affecting central blood pressure.
POPULÄRVETENSKAPLIG SAMMANFATTNING

Renin-angiotensin-aldosteron systemet är ett av kroppens viktigaste system för reglering av blodtryck. Angiotensin-converting enzyme (ACE) utgör en central del i renin-angiotensin-aldosteron systemet genom att omvandla den inaktiva peptiden angiotensin I till den betydligt mer aktiva peptiden angiotensin II. Angiotensin II har en rad olika effekter i olika vävnader och är involverad i regleringen av kardiovaskulära systemet, och sannolikt även i utvecklingen av hjärt-kärllsjukdom. Hos friska individer finns ACE lokalisert i det innersta cellagret (endotelet) i blodkärlens väggar, samt även fritt cirkulerande i blodet. Det finns en vanlig genetisk variation (polymorfi) i genen för ACE som brukar benämnas ACE I/D. Den styr till viss del hur mycket ACE som finns cirkulerande i blodet, även om det finns en stor variation även hos individer med samma genvariant. Tidigare studier har indikerat att ACE I/D polymorfin kan öka risken för hjärt-kärlsjukdom. I de fyra delarbetena som ligger till grund för denna avhandling studerades relationen mellan ACE och kardiovaskulär funktion samt sjukdom i kardiovaskulära systemet.

Det är välkänt att rökning har negativa effekter på hjärta och kärl, och nikotin verkar kunna påverka flera processer som är involverade i uppkomst av hjärt-kärlsjukdom. När nikotin tas upp i blodet omvandlas (metaboliseras) det framförallt i levern och ett antal nya metaboliter bildas. Trots att metaboliterna förkommer i hög koncentration i blodet hos tobaksnyttjande individer, är deras påverkan på hjärta och kärl relativt okänd. I delarbete I användes mänskliga endotelceller isolerade från blodkärl i navelsträngar, för att studera hur nikotin och levermetaboliter av nikotin påverkar ACE. En ökad aktivitet av ACE, och därmed ökad bildning av angiotensin II skulle kunna vara en cellulär mekanism som leder till ökad risk för hjärt-kärlkomplikationer. Både nikotin och flera av nikotinmetaboliterna visade sig ökar ACE aktiviteten. Nikotin och dess metaboliter hade olika stor effekt på ACE i celler från olika navelsträngar. Det är i dagsläget oklart vad denna individuella skillnad beror på, men våra resultat tyder på att det verkar vara oberoende av ACE I/D polymorfin.

Ett flertal riskfaktorer för hjärt-kärlsjukdom har identifierats (t.ex. rökning och diabetes), men man har inte lyckats kartlägga de bakomliggande mekanismerna för uppkomsten av hjärt-kärlsjukdom. I delarbete II studerades sambandet mellan ACE I/D polymorfin, mängd ACE i blodet, kardiovaskulära riskfaktorer samt förekomst av hjärt-kärlsjukdom i en population av 672 äldre män och
kvinnor. Hos män fanns ett samband mellan antalet riskfaktorer och mängd ACE i blodet. Rökning och diabetes var de riskfaktorer som visade sig ha störst betydelse för mängden ACE i blodet. Det är tänkbara att en ökad mängd ACE är en av de cellulära mekanismer som leder till ökad risk för kardiovaskulära komplikationer hos rökare och diabetiker.

I 406 individer av populationen som studerades i delarbete 2 undersöktes även mekaniska egenskaper (styrhet/elasticitet) i den stora kroppspulsådern i buken (bukaorta). Detta område är speciellt ofta drabbat av sjuklig vidgning med ett pulsåderbråck som resultat. De bakomliggande mekanismerna för utveckling av pulsåderbråck är okända, men man tror att det finns genetiska variationer som ger en ökad benägenhet att utveckla pulsåderbråck. ACE I/D polymorfin är en av de gen-variationer som pekats ut som en möjlig kandidat. I det tredje delarbetet studerades kopplingen mellan ACE och mekaniska egenskaper i bukaorta. Män som var genetiska bärare av ACE D varianten visades ha en högre styrhet i bukaorta, medan inget sådant samband kunde återfinnas hos kvinnor. Inget samband mellan mängd ACE i blodet och styrhet i bukaorta kunde identifieras, varken hos kvinnor eller hos män. En förändrad vägmekanik indikerar en störd funktion i aortaväggen som tillsammans med andra faktorer skulle kunna ge en ökad risk för utveckling av pulsåderbråck hos män som är bärare av D varianten av ACE genen.

Ett högt blodtryck är en välkänd riskfaktor för att utveckla hjärt-kärlsjukdom. Vanligen mäts blodtrycket i överarmen, men på senare år har det centrala hjärtvägskärlens blodtryck i aorta kommit mer i fokus, eftersom detta tryck visat vara mer kraftfullt relaterat till utveckling av hjärt-kärlsjukdom. I delarbete IV studerades sambandet mellan ACE, centralt blodtryck och hjärtfunktion på äldre män och kvinnor i en delpopulation av individerna i delarbete II. De med nedsatt väsnertkammarsfunktion i hjärtat hade högre mängd ACE i blodet jämfört med individer med en normal hjärtfunktion. Däremot fanns inget samband mellan mängd ACE och blodtryck. ACE I/D polymorfin hade ingen effekt på varken hjärtfunktion eller blodtryck. ACE skulle kunna ha en direkt påverkan på hjärtat snarare än indirekt via påverkan på blodtrycket vid nedsatt väsnertkammarsfunktion.
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<table>
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<tr>
<td>AAA</td>
<td>Abdominal aortic aneurysm</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ACEi</td>
<td>Angiotensin-converting enzyme inhibitors</td>
</tr>
<tr>
<td>AIx</td>
<td>Augmentation index</td>
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<td>AIx HR75</td>
<td>AIx adjusted to a heart rate of 75 beats/minute</td>
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<tr>
<td>Ang I</td>
<td>Angiotensin I</td>
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<td>Ang II</td>
<td>Angiotensin II</td>
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<td>ARBs</td>
<td>Angiotensin II receptor blockers</td>
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<td>AT1</td>
<td>Angiotensin II receptor 1</td>
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<tr>
<td>AT2</td>
<td>Angiotensin II receptor 2</td>
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<tr>
<td>CC</td>
<td>Compliance coefficient</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>D</td>
<td>Deletion</td>
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<tr>
<td>DC</td>
<td>Distensibility coefficient</td>
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<tr>
<td>D/D</td>
<td>Deletion/deletion</td>
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<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>I</td>
<td>Insertion</td>
</tr>
<tr>
<td>I/D</td>
<td>Insertion/deletion</td>
</tr>
<tr>
<td>I/I</td>
<td>Insertion/insertion</td>
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<tr>
<td>IMT</td>
<td>Intima-media thickness</td>
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<tr>
<td>LV</td>
<td>Left ventricular</td>
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<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PWV</td>
<td>Pulse wave velocity</td>
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<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
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<tr>
<td>RAS</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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This thesis is based on the following papers, which will be referred to by their roman numbers.

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. The association between circulating angiotensin-converting enzyme and cardiovascular risk in the elderly – a cross sectional study

III. Impaired abdominal aortic wall integrity in elderly men carrying the angiotensin-converting enzyme D allele
   Liza U Ljungberg, Rachel De Basso, Urban Alehagen, Hanna M Björck, Karin Persson, Ulf Dahlström, Toste Länne (submitted manuscript)

IV. Circulating angiotensin-converting enzyme levels are associated with left ventricular dysfunction, but not with central aortic blood pressure, aortic augmentation or pulse pressure amplification
   Liza U Ljungberg, Urban Alehagen, Rachel De Basso, Karin Persson, Ulf Dahlström, Toste Länne (submitted manuscript)
The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) is a powerful system regulating fluid-electrolyte balance and systemic blood pressure. A schematic overview of RAAS is shown in Figure 1.

Angiotensinogen is constitutively produced and released from the liver into the circulation where it is converted to angiotensin I (ang I) by renin. Renin is a proteolytic enzyme synthesized, stored and released primarily from the juxtaglomerular apparatus in the kidneys in response to decreased blood pressure, low sodium concentration and increased sympathetic activity. Ang I has mild vasoconstrictor properties but not enough to cause significant physiological effects. Ang I is further converted to angiotensin II (ang II) by angiotensin-converting enzyme (ACE). Although ACE is the major catalyst for this conversion, other enzymes are also able to generate ang II e.g. chymase [2], cathepsin [3] and tonin [4]. Ang II induces negative feedback inhibiting the release of renin, thereby acting in a self-regulatory manner. The renin-angiotensin system (RAS) was originally considered as an endocrine system. However, during the last decades it
has become evident that all components of RAS (i.e. renin, angiotensinogen, ang I, ang II, ACE and angiotensin receptors), are present in a number of different tissues, including the vessel wall, the heart, and the kidneys [5]. Ang II is considered the main effector peptide of RAS. Ang II induces its effects through binding to specific receptors [6]. Most of the physiological effects are generated through the AT1 receptor [6], while the AT2 receptor is not as well characterized, but may counteract some of the processes mediated by the AT1 receptor [7, 8].

Ang II is a multifunctional peptide, acting on a number of different tissues (Figure 1). Ang II stimulates release of aldosterone from the adrenal gland and generates constriction of renal arterioles thereby increasing salt and water retention in the kidneys. In the brain, ang II is involved in regulation of salt-and fluid homeostasis by influencing the autonomic nervous system [9], vasopressin release [10] as well as thirst and salt appetite [11]. In the blood vessels, ang II act as a powerful vasoconstrictor, and is involved in vascular wall remodeling through induction of smooth muscle cell growth [12, 13], up-regulation of growth factors [14] and by affecting the synthesis of extracellular matrix proteins [15, 16]. In the heart, ang II has inotropic [17, 18] and hypertrophic effects [19] and promotes cardiac fibrosis [20]. In addition, ang II seems to be involved in inflammation by inducing up-regulation of endothelial adhesion molecules [21-23], proinflammatory cytokines [24, 25], and production of reactive oxygen species [25-27].

Degradation of ang II into other peptides occurs rapidly after its formation [28]. A number of angiotensin peptides have been identified during the last decades, and named according to number of amino acids [29]. Angiotensin 1-7 has attracted the most attention as it has been shown to be pharmacologically active [30-32], and as it counteract some of the effects caused by ang II [33]. The conversion of angiotensin 1-9 into angiotensin 1-7 [34], as well as degradation of angiotensin 1-7 to angiotensin 1-5 is catalysed by ACE [35].
Angiotensin-converting enzyme

Structure, location and substrates

ACE is a 170 kDa Zn$^{2+}$ metallopeptidase existing in two isoforms. Somatic ACE is expressed in various tissues and cell types including the cardiovascular system, kidneys, intestine, adrenal glands, liver, uterus etc [36]. Testicular ACE, on the other hand, can only be found in germinal cells in the testes [37]. In the cardiovascular system, ACE exists both in a soluble form in the blood and bound to the cell membrane of different cell types [38-40]. High levels of ACE can be found in vascular endothelial cells [38], but has also been identified in e.g. T-lymphocytes [39], macrophages [40], in cardiac tissue [41], and in the vessel wall [42]. In endothelial cells, ACE is located on the luminal side. The C-terminal part is anchored to the plasma membrane [43], with a hydrophobic trans-membrane domain and a short cytoplasmic fragment (Figure 2). The extracellular part consists of two homologous domains, with two catalytic sites and Zn$^{2+}$ binding regions [43, 44].

![Image of ACE](image.png)

**Figure 2.** ACE located on the luminal side of vascular endothelium. ACE has two homologous domains with two active sites. The N-terminal part is released into the circulation following cleavage of the C-terminal domain by secretase.

Circulating ACE originates mainly from endothelial cells and is released into the blood after proteolytic cleavage of the anchor [45, 46]. One of the major sources for production of ACE is the lungs, due to its high vascularisation. If the circulating level of ACE reflects the level of ACE in tissues is still unknown.
Besides catalyzing the conversion of ang I to ang II, ACE also acts on a number of other natural substrates e.g. bradykinin [47], enkephalin [48], neurotensin [49] and substance P [49].

The ACE gene

The gene encoding ACE is located on chromosome 17q23. This gene encodes both ACE isoforms, but has two different promoters resulting in different mRNAs [50]. There is a common genetic variation within the gene, consisting of a 287 bp insertion/deletion (I/D) polymorphism located in intron 16 [51] (Figure 3). This polymorphism generates three different ACE genotypes: I/I, I/D and D/D.

![Figure 3. Schematic illustration of the 287 bp insertion/deletion polymorphism located in intron 16 within the ACE gene.](image)

As the ACE I/D polymorphism is located within an intron it will not affect the structure of the enzyme. However, there is an association between the ACE I/D polymorphism and circulating levels of ACE, where I/I, I/D and D/D genotypes have low, medium and high levels, respectively [51]. The ACE I/D polymorphism has been shown to account for approximately 20-50% of the variation in plasma ACE [51, 52]. The influence of the ACE I/D polymorphism on ACE level does not seem to be restricted to the circulation as similar patterns have been detected in e.g. T-lymphocytes [39] and cardiac tissue [41], suggesting that tissue ACE and circulating ACE are under similar genetic control.

Given the fact that the ACE I/D polymorphism is located in a non-coding region, it is unlikely that this is the functional variant responsible for the differences in
circulating ACE. A strong genetic linkage has been shown in the chromosomal region in which the ACE gene is located [53, 54], suggesting that the ACE I/D polymorphism may represent a marker for another genetic polymorphism involved in the regulation of ACE level. However, the exact identity or location of such functional polymorphism is still unknown.

Although, the ACE I/D polymorphism influences the level of circulating ACE, no differences in circulating ang II levels have been detected [55, 56]. However, the pressor response to ang I [55, 57], as well as the generation of ang II after intravenous infusion of ang I [55] has been shown to be higher in D/D carriers compared to carriers of the I/I genotype.

The ACE I/D polymorphism has been extensively studied in relation to cardiovascular disease (CVD). The first study reporting an association between the ACE I/D polymorphism and CVD was published in 1992 by Cambien and colleagues [58]. They found that the ACE D/D genotype was more frequent among subjects who had suffered a myocardial infarction compared to healthy subjects, especially in a subgroup of patients considered to be at low risk [58]. Some studies have confirmed an association between the D/D genotype and myocardial infarction [59, 60], while others failed to identify any association [61, 62]. Besides myocardial infarction, the D/D genotype has been associated with e.g. coronary artery disease [63, 64], hypertension [65, 66] and heart failure [67]. Today, there is no consensus regarding the importance of the ACE genotype for CVD. The D/D genotype alone might not be a risk factor for CVD, but together with other factors it may increase the risk for cardiovascular complications and may be of particular importance in specific subgroups.

**Regulation of ACE**

There is a large inter-individual variation in the amount of circulating ACE, partly due to the ACE I/D polymorphism [51]. However, ACE level is very stable when measured in the same individual at different occasions [68]. ACE may be regulated at the transcriptional level, affecting the synthesis of the enzyme. Studies have shown that the expression of ACE is affected by endogenous as well as exogenous factors e.g. vascular endothelial growth factor [69], estradiol [70], glucocorticoids [71, 72], thyroid hormones [72], and low-salt diet [73]. Alternatively, ACE may be regulated at the protein level, changing the enzymatic activity without altering the expression. ACE inhibitors (ACEi) are commonly used blood pressure lowering drugs and bind to the active sites of ACE, thereby
inhibiting ACE activity [74, 75]. However, a number of studies have shown that long term treatment with ACEi induces an increased expression of ACE in endothelial cells [76, 77], as well as in the circulation [52, 78]. Furthermore, nitric oxide, which is produced and released from vascular endothelium, has been shown to reduce ACE activity [79, 80], and has been suggested as an competitive inhibitor of ACE [79]. Alternative mechanisms for regulation of ACE might involve clearance of ACE from the circulation, as well as release and shedding of the enzyme from ACE containing cells [45, 81].
Nicotine and nicotine metabolites

Nicotine is a naturally occurring alkaloid found in many plants e.g. *Nicotiana tabacum*. It is absorbed through the oral cavity, skin, lung and gastrointestinal tract [82]. Following absorption, nicotine is metabolised to a number of metabolites, mainly in the liver (Figure 4). As the enzymes involved in the metabolic pathway of nicotine are highly polymorphic, there are individual differences in the metabolic pattern of nicotine [83]. However, the pattern appears to be consistent for in one individual over time [84].

![Nicotine Metabolism Diagram](image.png)

**Figure 4. Overview of the nicotine metabolism and its main metabolites.**

On average, 70-80% of the nicotine is metabolised to cotinine [85], about 4% is converted to nicotine-1’-N-oxide and 0.4% to nornicotine [85]. Cotinine is further metabolised to cotinine-N-oxide, norcotinine and trans-3´-hydroxycotinine, among others [84]. For most people, trans-3´-hydroxycotinine is the most abundant metabolite in urine, accounting for on average 38% of the metabolites [84]. The half-life of nicotine is about 2-3 hours [86] and plasma nicotine concentration in smokers usually range between 20-40 ng/ml (0.12-0.25 µM)[84, 86]. The nicotine metabolites have considerable longer half-lives compared to nicotine, on average 16-17 hours [85]. Due to the long elimination time, plasma concentrations of nicotine metabolites in tobacco users tend to accumulate.
throughout the day [85], resulting in high levels of cotinine and trans-3′-hydroxycotinine [85, 87].

**Cardiovascular effects of nicotine**

There is no doubt about the negative effects of smoking on the cardiovascular system [88]. The exact mechanisms by which smoking induces CVD are not entirely known, but are most likely multifactorial. Cigarette smoke is a complex mixture of chemical substances, containing not only nicotine, but also a number of other potentially cardiotoxic substances e.g. carbon monoxide [89]. Nicotine affects cardiovascular biology in many ways, some of the mechanisms being well characterized. By activating the sympathetic nervous system, nicotine induces increased heart rate and myocardial contraction, vasoconstriction in the skin and induces adrenal and neural release of catecholamines [90, 91]. In addition, animal studies have shown that nicotine affects lipid metabolism [92, 93] and accelerates the development of atherosclerosis [93, 94]. Nicotine has also been shown to cause endothelial dysfunction [95-97], induce morphological changes in endothelial cells [98] and increase endothelial cell death [99]. Furthermore, long-term use of oral snuff, where nicotine is absorbed through the oral mucosa, has been shown to induce endothelial dysfunction [100] and increase the risk of fatal myocardial infarction [101, 102]. Taken together, these data suggest a role of nicotine in development and progression of CVD. Although, the concentration of some of the nicotine metabolites in the blood is far higher than nicotine in tobacco users, few earlier studies have examined their effect on the cardiovascular system, and no previous study has examined their effect on ACE.
The abdominal aorta

The abdominal aorta is an area particularly prone to atherosclerosis as well as aneurysm formation [103, 104]. Compared to the more proximal aorta, the abdominal aorta contains fewer lamellar units and has a stiffer wall [105]. Most of the abdominal aortic media lacks vasa vasorum and is instead supplied with oxygen and nutrients by diffusion from the bloodstream [105]. Increased wall thickness, as a result of e.g. ageing [106, 107], may therefore result in impaired nutrition of the abdominal aortic wall. Furthermore, age- and gender-related changes are more pronounced at this site than in other arteries [107, 108]. In addition, the abdominal aorta is exposed to higher systolic and pulse pressures than other central arteries [109].

Abdominal aortic aneurysm (AAA) is defined as a 50% enlargement of the aortic diameter or a localized dilatation [110]. However, in clinical routine a diameter of 3 cm or greater is usually defined as AAA. Risk factors for AAA are e.g. old age, male gender, smoking and hypertension. The prevalence of AAA is low in young populations, but increases with age, reaching 5-8% in men, and 1-2% in women at the age of 65 or above [111]. Rupture of an AAA is associated with a 80% mortality rate [112]. The exact mechanisms involved in aneurysm formation are largely unknown. However, a genetic component has been suggested, as first degree relatives to patients with AAA are more susceptible. Furthermore, previous studies have indicated a possible connection between arterial stiffness and aortic aneurysm [113, 114].
Arterial stiffness

Large artery stiffening is an independent risk factor for cardiovascular morbidity and mortality [115, 116]. Stiffness of large arteries is determined by the content and composition of the arterial wall. Elastin and collagen are the main structural proteins responsible for the elastic properties of large arteries [117]. Elastin is responsible for the mechanical strength at low pressures, while collagen is the main load bearing protein at high pressures [118]. The elasticity varies along the arterial tree; the thoracic aorta being the most elastic area while more distal arteries become gradually stiffer [104, 119], as a result of reduced elastin and increased collagen content [120]. Stiffness of the arterial wall is influenced by age, gender and other recognised cardiovascular risk factors [121-124]. As a consequence of ageing, the structure of the arterial wall is altered, with loss of elastin fibres and increase in collagen content [125]. Men have stiffer arteries than women, at least when measured locally [126-128], although ageing is associated with increased stiffness in both men and women [128, 129]. Ageing is also associated with an increased arterial diameter [113, 130] and thickening of the arterial wall [130]. These age-related changes occur predominantly in elastic arteries, while muscular arteries are less affected [121, 129]. Apart from the classic cardiovascular risk factors, genetic factors have been implicated as important determinants of arterial stiffness, either directly by inducing structural changes in the vessel wall, or indirectly acting through other risk factors [131]. Previously, genetic polymorphisms in e.g. the fibrillin-1 gene [132, 133], MMP genes [134, 135] and a region on chromosome 9p21.3 [136] has been associated with altered arterial stiffness.
Arterial pulse wave and wave reflection

The blood flow in the aorta and other large arteries is pulsatile as a result of the rhythmic ejection of blood from the left ventricle. The left ventricular (LV) ejection generates a pressure wave which travels along the arterial tree with a pulse wave velocity (PWV) of 4-10 m/sec [119]. This pressure wave is reflected at sites of changing impedance e.g. branching points, areas of alterations in arterial elasticity or diameter, and high resistant arterioles, augmenting the forward wave as it moves along the arterial tree [137]. The arterial pressure wave thus represents the sum of the forward and the reflected pulse waves (Figure 5).

![Figure 5. Schematic illustration of the aortic pressure wave in an elderly subject.](image)

The shape of the arterial pressure wave is determined to a large extent by arterial stiffness. In the aorta, with low PWV, the reflecting pulse wave predominantly returns in the diastolic phase of the forward pressure wave, adding to the diastolic pressure resulting in low pulse pressure. With increasing age however, increased stiffness results in premature return of the reflecting wave, adding to the systolic pressure wave [137]. This pressure boost, caused by wave reflection is called augmentation pressure, and generates increased systolic and pulse pressure [137]. Due to increased afterload, a rise in systolic and pulse pressure results in increased cardiac oxygen consumption [119, 137]. In addition, when the reflecting waves arrive earlier in systole, the diastolic pressure decreases, thereby reducing coronary perfusion [119, 137].

The pressure wave is progressively amplified as it moves from central (elastic) to more distal (muscular) arteries [138]. This amplification phenomenon is due to increased stiffness along the arterial tree and a decreased distance to reflection sites, resulting in augmentation of the systolic pressure [119]. In healthy young
subjects with elastic arteries, the pressure in peripheral arteries is thus usually higher than in central arteries [139]. This difference decreases to a large extent with age [137, 138], as the stiffness of muscular arteries is less affected by aging [129]. In recent years, there has been a growing interest in studying blood pressure in central arteries as this is the pressure the heart actually meets, and as central pressure has been shown to be a better predictor of cardiovascular outcome than peripheral pressure [139, 140].

Heart failure and RAS
Heart failure is a growing health problem in Western countries. The prevalence of heart failure is low in young populations but increases with age, reaching approximately 10% in those aged ≥65 years [141]. In systolic heart failure, the ventricular contraction is impaired, and in the majority of patients with systolic heart failure, the left ventricle is affected. Major determinants of systolic heart failure are ischemic heart disease and hypertension, but a variety of other cardiac disorders (e.g. dilated cardiomyopathy, heart valve diseases, arrhythmias) may also be the underlying cause [142].

A chronically elevated blood pressure often results in left ventricular hypertrophy (LVH), which represents a strong predictor of cardiovascular morbidity and mortality [143]. Although, hemodynamic factors are considered as main determinants of LVH [144], non-hemodynamic factors may also be of importance [19, 145, 146]. RAS has been recognized as an important system in the pathogenesis of heart failure. Most evidence supporting a role of RAS in heart failure arise from the beneficial effects seen after pharmacological inhibition. ACEi are first line therapy for patients with heart failure, and has been shown to reduce cardiovascular morbidity and mortality in a number of clinical trials [147, 148]. In recent years, accumulating evidence indicates that these beneficial effects are shared by angiotensin II receptor blockers (ARBs) [149, 150].
AIMS

The aims of this thesis were to ...

Study the effect of nicotine and nicotine metabolites on ACE in cultured endothelial cells (HUVECs) and to evaluate the possible influence of the ACE I/D polymorphism (paper I).

Study the association between the ACE I/D polymorphism, circulating ACE level and cardiovascular risk in elderly men and women (paper II).

Study the association of the ACE I/D polymorphism, circulating ACE and the mechanical properties of the abdominal aorta in elderly men and women (paper III).

Study the association of the ACE I/D polymorphism and circulating ACE levels, with central aortic blood pressure, aortic augmentation, pulse pressure amplification and LV function in elderly subjects (paper IV).
MATERIALS AND METHODS

Cell culturing, HUVECs (paper I)

The cultured cells used in paper I, were Human Umbilical Vein Endothelial Cells (HUVECs) isolated from umbilical cords after vaginal deliveries without complications. Umbilical cords are suitable for isolation of endothelial cells as they are easy to obtain and have an unbranched vein of appropriate size. The method for isolation and cultivation of HUVECs was first described by Jaffe et al. in 1973 [151].

Umbilical veins were treated with collagenase for detachment of endothelial cells from the vessel. The collagenase-cell suspension were collected and HUVECs were seeded in cell culture flasks coated with 0.2% gelatin. Endothelial cells grown on uncoated plastic have low spontaneous proliferation and relatively high apoptosis, whereas growth on surfaces of e.g. gelatin is more optimal [152]. Cell culture medium was replaced every 48-72 h and confluent cells were trypsinised and subcultured in new cell culture flasks. As HUVECs are primary culture cells they have limited life span and culturing for more than 2-3 passages results in reduced proliferation rate. In addition, the expression of ACE in HUVECs is reduced by cultivation [153]. HUVECs were thus used at the first or second passage.
Study populations (paper II-IV)

In paper II-IV we studied elderly subjects from a rural municipality in South East of Sweden. All subjects were included in a previous study which started in 1999 [154]. When a follow-up study was performed between 2003 and 2005 we had the opportunity to perform a cross-sectional study in this population. A total of 672 subjects (322 men and 350 women) were included (paper II), of whom 655 subjects had successful examination of their LV function (paper IV). All 672 subjects were also asked to participate in a study regarding mechanical properties of the abdominal aorta as well as central hemodynamics. A total of 452 agreed, resulting in a participation rate of 67%. As the study population consisted of elderly subject, and many of them had quite a long distance to the clinic, the main reason for not participating was transportation problems. Non-participants were slightly older compared to those who chose to participate, however there were no difference in blood pressure or CVD. In addition, there were more men than women who agreed to take part. Determination of abdominal aortic wall mechanics was successful in 406 subjects (paper III), while central aortic hemodynamics were successfully obtained in 422 subjects (paper IV). Figure 6 shows the study populations and the examinations that were performed.

Figure 6. Study populations used in paper II-IV
**Determination of ACE activity**

ACE activity was determined in cultured endothelial cells (paper I) and in serum (paper I-II), using a commercially available radio-enzymatic assay (ACE-direct REA, Bühlmann Laboratories, Schönenbuch, Switzerland). The principle for the assay is based on the cleavage of the synthetic substrate \(^3\)H-hippuryl-glycyl-glycine into \(^3\)H-hippuric acid and glycyl-glycine dipeptide, a reaction catalyzed by ACE. Adding of HCl stops the enzymatic reaction and the scintillation cocktail separates the \(^3\)H-hippuric acid from the glycyl-glycine dipeptide. The amount of \(^3\)H-hippuric acid can be determined using a beta counter, and is proportional to the amount of ACE in the sample. One unit of ACE is defined as the amount of enzyme required to produce 1 µmol \(^3\)H-hippuric acid per minute and litre. Intra-assay variation was 8% for ACE activity determined in cultured cells, and 6% in serum, while inter-assay variation was 7%.

**Determination of ACE level**

ACE level was measured in cell lysate (paper I), in serum (paper I and II) and in plasma (paper II-IV), 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 96-well microplate. Samples are added to the wells and any ACE present in the samples bind to the antibodies. Unbound substances are removed by washing. Biotinylated polyclonal antibodies directed against ACE are added, followed by addition of streptavidine-horseradish peroxidase, attaching to the polyclonal antibody. A substrate is then added, which is converted to a coloured compound by horseradish peroxidase. The intensity of the colour is measured spectrophotometrically and is proportional to the amount of ACE in the sample. Standards with known concentration of ACE were included in each assay and used to calculate the concentration of ACE in samples. Serum and plasma samples were diluted 1:200. Samples were analysed in duplicate and re-analysed if variation from the mean value exceeded 15%. The lower limit of detection was 0.05 ng/ml. Intra-assay variation was 6.5% for analysis of ACE in plasma, and 8% in cell lysate, while inter-assay variation was 12%.
Validation of ACE assays

Although a good correlation between ACE activity and ACE level in plasma has been shown previously [155], we wanted to confirm this using our methods in a population similar to our study population. Serum and plasma were collected from 23 men, 70 years of age, enrolled in a screening program for abdominal aortic aneurysm at Linköping University Hospital. These subjects represent an unselected sample of a normal population of elderly men in Sweden. ACE activity was measured in serum, while ACE level was measured in serum and plasma, according to the methods described above. There was a strong correlation between ACE activity and ACE level in serum (Figure 7A) as well as in serum-plasma (Figure 7B), indicating that ACE level can be used as a surrogate for ACE activity. A similar correlation was also found in a younger healthy population including both men and women (12 men and 9 women, mean age 29 years) (unpublished data).

**Figure 7. Correlation between A) ACE level and ACE activity in serum, and B) between ACE level in plasma and ACE activity in serum, from 23 men, 70 years old.**
Stability of ACE during storage at -70°C

In paper II-IV, ACE level was analysed in plasma stored at -70°C for 3-5 years. As freezing might result in degradation of proteins the stability of ACE in frozen samples was studied. Plasma and serum from 23 men enrolled in a screening program for abdominal aortic aneurysm at Linköping University Hospital was used (see above). The method for preparation of plasma and serum has been described in paper II. Samples were aliquoted and stored in 1.5 ml cryo-tubes at -70°C and analysis of ACE level was planned after 3 weeks, 12, 24 and 48 months. To date, samples stored at -70°C for 3 weeks and 12 months respectively, have been analysed.

As shown in Figure 8, there was no difference in ACE level between the 3 week and the 12 months samples, indicating that there is no degradation of ACE at least during the first year of freezing.

![Figure 8](image.png)

*Figure 8. ACE level (ng/ml) in plasma samples from 23 men after 3 weeks and 12 months of storage at -70°C. Values are mean ± SEM.*
ACE genotyping

A polymerase chain reaction (PCR) method for determination of ACE genotype was described by Rigat and colleagues in 1992 [156]. A few years later, this method was found to result in mistyping of a number of subjects, where carriers of the I/D genotype were incorrectly identified as D/D carriers [157]. More recently, improved methods for ACE genotyping have been described [158-160]. We used a triple primer approach [158], a strategy previously described to be reliable to avoid mistyping of I/D carriers [159]. Three sets of primers were used, allowing detection of a 238 bp fragment for the deletion allele, and two fragments, 155 bp and 525 bp for the insertion allele [158] (Figure 9).

**Figure 9.** Schematic illustration of the method used for ACE genotyping. The forward primer (red) and the reverse primer (blue) allow detection of a 238 bp fragment for the D allele, and 525 bp fragment for the I-allele. By using an I-allele specific primer (green), an additional fragment is amplified for the I-allele, making determination of ACE genotype easier and more accurate.

Amplified DNA was separated by gel electrophoresis using a 1.5% agarose gel stained with ethidium bromide and visualized by UV-light. Determination of ACE genotype was based on length and number of DNA fragments (Figure 10).

**Figure 10.** PCR products separated by gelelectrophoresis and visualised by UV-light. The D/D genotype results in one band, I/I genotype two bands and I/D genotype three bands.
Determination of abdominal aortic wall mechanics

Stiffness of various arteries can be determined non-invasively by a number of different techniques [119]. Carotid-femoral PWV measurements are considered the golden standard for measurement of aortic stiffness [119]. However, as PWV usually is measured between the carotid and femoral arteries, it reflects the mean arterial stiffness of several different territories of the arterial tree. In paper III, we used ultrasound and the Wall Track System, as we wanted to determine arterial stiffness locally in the abdominal aorta. This technique enables high resolution measurements of lumen diameter, pulsatile diameter changes during a cardiac cycle, and intima-media thickness (IMT) [161], which can be used to calculate local aortic stiffness. The aorta was examined approximately 3-4 cm proximal to the aortic bifurcation, a site particularly prone to aneurysm formation [104]. ECG electrodes were connected to the subject. The abdominal aorta was visualized longitudinally and the scanner was then switched to M-mode. The Wall Track System automatically positions two anchors at the posterior and the anterior aortic wall. Manual adjustments of the anchor positions can be made. Pulsatile vessel wall movements were recorded and arterial distension waveforms generated (Figure 11), allowing calculation of lumen diameter and pulsatile diameter changes. IMT of the posterior wall was measured in end-diastole, using the ECG signal for calibration. The Wall Track System uses the radio frequency to automatically determine IMT from the interface between the lumen and the intima, to the interface between the media and adventitia [161]. Each measurement was evaluated and compared with the ultrasonic image. Only measurements which were in agreement with the visual estimation were included. All measurements were carried out by two experienced ultrasonographers on a single occasion, with subjects in the supine position, immediately following brachial blood pressure measurements. Coefficient of variation was 5% for absolute lumen diameter, 21% for pulsatile diameter change and 17% for IMT.

Figure 11. Distention curves from the abdominal aorta determined by the Wall Track System.
Using diastolic lumen diameter, pulsatile diameter change and brachial blood pressure, the compliance coefficient (CC) and distensibility coefficient (DC) were calculated using the formulae [129]:

\[
CC = \pi \left( 2 \times D_{\text{dia}} \times \Delta D + \Delta D^2 \right) / \left( 4 \times \Delta P \right)
\]

\[
DC = \left( 2 \times D_{\text{dia}} \times \Delta D + \Delta D^2 \right) / \left( D_{\text{dia}}^2 \times \Delta P \right)
\]

CC is expressed in \( \text{mm}^2/\text{kPa} \) and DC in \( 10^{-3}/\text{kPa} \). \( D_{\text{dia}} \) is the end diastolic diameter (mm), \( \Delta D \) is the diameter change between systole and diastole (mm), and \( \Delta P \) is the brachial pulse pressure expressed in kPa.

CC is the absolute change in cross-sectional area during a cardiac cycle for a given increase in aortic pressure, assuming that the length of the vessel is constant during the pulse wave. A low CC indicates reduced vessel buffering capacity. DC is the relative change in aortic diameter during a cardiac cycle for a given increase in pressure. A decreased DC indicates reduced elasticity of the vessel. There is a non-linear relationship between pressure and diameter change in the abdominal aorta [162, 163]. The aorta is very distensible at low pressures and small diameters, but becomes gradually stiffer with increasing pressure and diameter [162, 163]. Stiffness \( \beta \) is an index which seems to be less dependent on pressure changes [162], and may be used as a complement to CC and DC. Stiffness \( \beta \) was calculated according to [162, 164]:

\[
\text{Stiffness } \beta = \ln \left( P_{\text{sys}} / P_{\text{dia}} \right) / \left( \Delta D / D_{\text{dia}} \right)
\]

\( P_{\text{sys}} \) and \( P_{\text{dia}} \) represent the systolic and diastolic brachial blood pressures in mmHg. Stiffness \( \beta \) varies inversely with DC and CC.

Brachial blood pressure, determined using an oscillometric technique (Dinamap model PRO 200 Monitor, Critikon, Tampa, FL, USA) was used as a surrogate for abdominal aortic pressure, in order to calculate CC, DC and stiffness \( \beta \). Simultaneous measurement of aortic pressure would be ideal, but is difficult to perform and unethical in large populations of elderly subjects. The systolic pressure in the brachial artery and in the abdominal aorta are in good agreement [163]. Diastolic pressure on the other hand, is slightly higher in the brachial artery, leading to a systematic underestimation of aortic stiffness [163]. However, as no age- or gender-related differences have been observed [163], this systematic bias should not affect comparative studies between groups.
Central aortic hemodynamics

There is a growing interest in studying blood pressure in central arteries, as this is the pressure the heart actually meets. Invasive measurement is the most accurate assessment of central aortic pressure, however this is not very ethical in large populations of elderly subjects. Instead, two non-invasive techniques are commonly used; direct estimations of central aortic pressure from the carotid pressure waveform, or calculation of central aortic pressure using tonometric recordings of the radial pressure waveform and a generalized transfer function. In contrast to the carotid artery, the radial artery is supported by bone structures, making it easier to obtain high quality pressure curves [119]. In Paper IV, central aortic pressure was determined from the radial artery. A Millar pressure tonometer was placed on the radial artery and pressure waveforms were recorded. Using brachial blood pressure for calibration, the SphygmoCor system was used to synthesize aortic pressure waveforms (Figure 12).

This method has been validated and showed good agreement with invasively determined pressure, when using intra-radial pressure for calibration [165, 166]. Using brachial blood pressure for calibration, underestimation of central aortic pulse pressure and systolic pressure has been reported [167, 168]. However, this bias is probably less pronounced in elderly populations like ours, as the pressure

Figure 12. Radial and aortic pressure waveforms obtained by applanation tonometry and the SphygmoCor system. \( P_{sys} \): systolic pressure, \( P_{dia} \): diastolic pressure, Aug: augmentation pressure, PP: Pulse pressure.
differences in the arterial tree decrease with age [125]. Moreover, a systematic underestimation of central blood pressure should not affect comparative studies between groups.

From the central aortic pressure waveform a number of hemodynamic parameters can be obtained. Pulse pressure is the difference between the systolic and diastolic pressure. Augmentation pressure is the difference between the first and the second systolic peak and represents the pressure boost caused by wave reflection. Augmentation index (Alx) is the augmentation pressure expressed as percentage of the aortic pulse pressure, and is considered as an indirect measure of aortic stiffness. Alx is dependent on heart rate and Alx is therefore adjusted to a heart rate of 75 beats/minute (Alx HR75). Time to reflection represents the time to return of the reflected wave.

**Echocardiographic evaluation of left ventricular function**

In paper IV, LV function was determined semi-quantitatively by visual estimation using doppler echocardiography (Acuson XP 128c system, Mountain View, CA, USA), a method regularly used in both clinical routine and research at our department. The method has been shown to be in good agreement with Simpsons biplane method as well as radionuclide imaging [169-171]. A good correlation between visual estimation and Simpsons biplane method has also been confirmed in our lab [172]. Subjects were classified into four groups based on LV function; normal function, mild dysfunction, moderate dysfunction or severe dysfunction, corresponding to LV ejection fraction of >50%, 40-49%, 30-39%, <30 % respectively. Examinations were performed by three experienced physicians-echocardiographers with the subjects in the left supine position.
RESULTS AND DISCUSSION

Effect of nicotine and nicotine metabolites on ACE in vitro

Cardiovascular effects of nicotine have been extensively studied, and it seems as nicotine can promote development of atherosclerosis [93-96]. The impact of nicotine metabolites on the cardiovascular system is however rather unknown, despite high plasma concentrations of some of the metabolites in daily smokers. In paper I, the effect of nicotine as well as nicotine metabolites on ACE in human endothelial cells and in human serum was studied. Nicotine, and the five most abundant metabolites found in plasma from tobacco users were used; cotinine, cotinine-N-oxide, nicotine-1´-N-oxide, norcotinine, and trans-3´-hydroxycotinine in concentrations similar to those observed in plasma from daily smokers [84-87].

Nicotine, as well as some of the nicotine metabolites induced an increase in ACE activity in HUVECs (Figure 13). The effect was dose-dependent, except for nicotine 10 µM. The effect of nicotine and nicotine metabolites on ACE activity was studied after 10 min-24h incubation. A slight increase in ACE activity was seen after 10 min, which in some cases sustained up to 24 h (data not shown). The most pronounced effect was however seen after 30-60 min, and a 60 min incubation time was therefore chosen for subsequent experiments.
Figure 13. Effect of nicotine and nicotine metabolites on ACE activity in HUVECs. ACE activity was measured in HUVECs following incubation with A) nicotine, B) cotinine-N-oxide, C) norcotinine, D) trans-3-hydroxycotinine, E) nicotine-1´-N-oxide and F) cotinine for 1 h. Values are mean ± SEM. n = 11-16
The expression of ACE was slightly increased by nicotine, cotinine-N-oxide and nicotine-1'N-oxide, although no dose-dependent effects were seen (Figure 3, paper I). Pre-treatment of HUVECs with the protein synthesis inhibitor cycloheximide, followed by incubation with nicotine showed that nicotine was still able to induce an elevation in ACE activity, although the effect was slightly reduced at the highest concentrations of nicotine (Figure 14). If the increase in ACE activity was entirely due to increased expression of the enzyme, incubation with a protein synthesis inhibitor would abolish the effect. This suggests that the increase in ACE activity after treatment with nicotine and nicotine metabolites is mainly due to altered enzymatic activity, but also due to a slight increase in the expression, at least at high concentrations.

![Figure 14](image-url)  
*Figure 14. Effect of nicotine on ACE activity after pre-incubation with cycloheximide (10µM). Despite pre-treatment with a protein synthesis inhibitor, nicotine induced an increased ACE activity, although the effect was slightly reduced at 1 and 10 µM.*

To further evaluate the mechanism of ACE regulation, ACE activity was analyzed in serum from 3 healthy non-tobacco using volunteers after incubation with nicotine (Figure 5, paper I). There was no effect of nicotine on serum ACE, indicating that the regulation of ACE by nicotine only takes place within cells/tissues.

Previous studies investigating the effect of nicotine on ACE have produced conflicting results. Saijonmaa and colleagues [69] found no effects of nicotine alone after 4, 18 or 24 hours incubation, but together with vascular endothelial growth factor (VEGF), nicotine potentiated the VEGF-induced up-regulation of ACE in HUVECs. The difference between our study and the study by Saijonmaa et al. may be related to methodological differences as well as the time of incubation. Zhang and co-workers [173], on the other hand, showed an up-regulation of ACE
mRNA in cultured human coronary artery endothelial cells after 24 hours incubation with nicotine, and Sugiyama et al. [174] showed increased serum ACE activity in dogs 30-60 min after intravenous administration of nicotine. Although the effect of nicotine was studied at different time points, and despite differences in methods as well as cells/animal models, the data by Zhang et al. and Sugiyama et al. are in agreement with our findings, and supports a nicotine-induced increase in ACE activity. Interestingly, a protective effect of the ACEi captopril on nicotine-induced endothelial dysfunction has been shown in vitro and in vivo [175], suggesting that nicotine may promote atherosclerosis by inducing an up-regulation of ACE. Our data also suggests a similar effect of some of the nicotine metabolites (i.e. norcotinine, trans-3’-hydroxycotinine and cotinine-N-oxide). Due to high concentrations of nicotine metabolites in plasma, it is of great importance that the effect of the metabolites is considered.

**Individual differences in the response to nicotine and nicotine metabolites**

Although, mean values showed an increased ACE activity following treatment with nicotine or nicotine metabolites, there were individual differences in the response; some experiments showed a 50% increase, whereas in others no or a moderate effect was seen (Figure 15).

![Figure 15](image-url). Illustration of individual differences in ACE activity in HUVECs from three umbilical cords, after treatment with trans-3’-hydroxycotinine.
This difference was not related to either basal ACE activity or time of culture. In each separate experiment, HUVECs obtained from different umbilical cords with individual genetic background, were used. As the ACE I/D polymorphism is known to influence ACE level [51] and as previous studies have reported that the effects of e.g. nitric oxide and ACEi, are dependent on ACE genotype [80, 176], we hypothesized that the variation in response to nicotine and nicotine metabolites seen in our study might be related to the ACE genotype. However, our data could not confirm such interaction, and the explanation for this variation remains unknown. One might speculate that lifestyle factors (e.g. tobacco or diet) would explain differences in response to nicotine and nicotine metabolites. However, as the donors of the umbilical cords were anonymous, no such information was available leaving this relationship to be further elucidated. If this individual difference also applies in vivo, it may be of importance regarding risk for CVD in tobacco users, where some subjects might be more affected.
Association between ACE and cardiovascular risk

Numerous studies have investigated the link between the ACE I/D polymorphism and CVD. Although a relationship between the ACE I/D polymorphism and plasma ACE level has been repeatedly confirmed [51, 52], other factors have been shown to influence circulating ACE levels [70, 73, 177-180]. Despite this, few studies have investigated the role of circulating ACE in CVD. In addition to elucidate the relationship between ACE genotype and CVD, this study also focuses on the link between ACE level, cardiovascular risk factors and CVD.

ACE level – influence of ACE genotype, age and gender

A study by Rigat et al. [51] including 80 healthy middle aged men and women showed that 47% of the variation in circulating ACE was due to the ACE I/D polymorphism. Subsequent studies in larger populations have confirmed the impact of the ACE I/D polymorphism on circulating ACE, but have showed a less pronounced effect, accounting for approximately 20% of the variation [52]. Our study showed a significant association between the ACE I/D polymorphism and circulating ACE, although there were large variations within each group (Figure 16). Only 10% of the variation in men and 17% in women were due to the ACE I/D polymorphism.

![Figure 16. Impact of the ACE I/D polymorphism on circulating ACE level in elderly men and women.](image-url)
There were no gender differences in ACE level in the overall population or between genotypes. In previous studies, gender differences have been reported among children, boys having higher circulating levels of ACE than girls of same age [181, 182]. However, this gender-related difference seems to diminish with age, as most investigations in adults failed to identify such association [51, 183]. The frequency of the D allele in our population was 0.51, which is in accordance with previous studies in Caucasian populations [61, 184]. However, ethnic differences in allele frequency [185-187], as well as the impact of ACE I/D polymorphism on circulating ACE have been shown [181, 188]. Furthermore, there was a positive association between age and ACE level in our study. A negative association between age and ACE level has been shown in children [181, 189], however, studies in adults showed no influence of age on ACE level [68, 190, 191]. Whether the age related increase in ACE seen in our population is due to higher prevalence of diseases in older subjects, or to age per se remains unknown. However, age obviously needs to be taken into consideration when studying circulating ACE levels, at least in elderly subjects.

**ACE level and cardiovascular risk factors**

To examine the relationship between ACE level and cardiovascular risk, subjects were divided into 4 groups based on the number of cardiovascular risk factors. Risk factors were diabetes, heredity for CVD, hyperlipidemia and hypertension. In men, there was a positive association between number of risk factor and ACE, while no association was seen in women (Figure 17).

![Figure 17](image_url)

*Figure 17. Circulating ACE levels in men and women according to number of cardiovascular risk factors. P-values are ONE-way ANOVA adjusted for age and ACE genotype.*
Elevated ACE levels may represent one of the cellular mechanisms involved in the vascular damage associated with cardiovascular risk factors, possibly mediated by increased production of ang II, increased degradation of bradykinin and altered levels of other angiotensin peptides e.g. angiotensin 1-7. To further investigate which particular risk factors that are associated with higher ACE levels in men a multivariable analysis including risk factors and CVD was performed.

Higher ACE levels were seen in men who smoked compared to non-smoking men, while no difference was found in women. However, no interaction between gender and smoking was seen. The lack of association in women may be due to the low proportion of female smokers. An increased release of ACE from cultured endothelial cells after exposure to cigarette smoke has been reported previously [192]. In addition, in vivo studies have indicated that the acute effects of smoking is an increased serum ACE activity [174, 193], whereas results regarding the long-term effects are inconsistent [194-196]. A decreased ACE activity has been reported in daily smokers [194, 195], however, a relatively small number of subjects were studied and no confounders were considered. A larger study, on the other hand, which included correction for a number of potentially confounding factors (e.g. ACE-genotype, age, and gender) showed higher ACE level among smokers, which is in line with our findings [196]. Taken together, our data, as well as previous studies suggests that smoking induces an up-regulation of circulating ACE [192, 196], possibly mediated by nicotine and nicotine metabolites [69, 173, 197].

Furthermore, a gender-dependent association between ACE and diabetes was found, where diabetic men had higher ACE levels compared to men without diabetes, while no such difference was seen among women. Higher ACE levels have previously been reported in diabetic patients compared to healthy controls [198-200], however, no difference between genders was reported. At present, the rationale for this gender difference is unknown and needs to be further elucidated and confirmed in future studies. Diabetic nephropathy is a common complication in diabetics, and previous studies have shown up-regulation of ACE in diabetic nephropathy [198, 199, 201]. In our study, adjustment for glomerular filtration rate did not affect the association between ACE and diabetes, suggesting that this association is not due to impaired renal function. An up-regulation of ACE may contribute to increased risk for vascular complications in diabetics. This is supported by the findings that ACEi and ARBs reduces the risk for
cardiovascular complications among diabetic subjects, more effectively than placebo or other anti-hypertensive medications [202, 203].

ACE inhibitor treatment

Among the 672 studied subjects, 21% (n=141) were treated with ACEi. ACEi bind to the active sites of ACE, inhibiting the conversion of ang I to ang II, as well as the degradation of bradykinin. Our data showed that treated subjects had higher levels of circulating ACE, compared to non-treated subjects. In treated men, ACE level was 300 ng/ml compared to 195 ng/ml in non-treated men (p<0.001). The corresponding levels for women were 343 ng/ml and 208 ng/ml (p<0.001), respectively. An up-regulation of ACE after treatment with ACEi has previously been reported in cultured endothelial cells [76, 77, 204], in different tissues in animal models [77, 205], as well as in human plasma [52, 78, 206]. Furthermore, we found no effect of ARBs on ACE levels, which is in accordance with previous findings [204, 206]. Recently, Kohlstedt et al. [207] showed that binding of ACEi to ACE induce an intracellular signal in endothelial cells leading to induction of ACE mRNA expression. This molecular mechanism is probably responsible for the up-regulation of ACE during ACEi treatment, and provides an explanation for the lack of effect of ARBs. Due to interference of ACEi treatment on circulating ACE levels, subjects who were on this treatment were excluded from statistical analyses of ACE levels.
Association between ACE and abdominal aortic wall mechanics

The abdominal aorta is a site particularly prone to aneurysm formation [103]. Age- and gender-related changes are more pronounced at this site compared to other vascular territories. The mechanical properties of the abdominal aorta are measures of aortic wall integrity. Impaired wall integrity, along with other local hemodynamic and/or inflammatory factors may be of importance for aneurysm formation. Previous studies have indicated that RAS might have a role in regulation of vessel wall mechanics [208, 209] as well as in AAA [210, 211]. In our study we investigated the link between ACE and abdominal aortic wall mechanics.

ACE I/D polymorphism and abdominal aortic wall mechanics

Differences in abdominal aortic wall properties between ACE genotypes were analysed in men and women. Men carrying the D allele had significantly lower pulsatile diameter change (p=0.014) and DC (p=0.017) than men carrying the I/I genotype (Figure 18). Carriers of the I/D and D/D genotype had similar stiffness values, while carriers of the I/I genotype were the ones that differed. Furthermore, in men, correction for a large number of potential confounders (age, MAP, BMI, heart rate, LDL, C-reactive protein, diabetes mellitus, smoking, antihypertensive treatment and ACE level) resulted in additional associations between the ACE D allele and both reduced CC (p=0.045) and increased stiffness β (p=0.048), and strengthening of the association between the D allele and DC (p=0.003). In women, however, there were no differences in abdominal aortic wall mechanics between genotypes.

Figure 18. Distensibility coefficient in men carrying the ACE D allele compared to men carrying the I/I genotype. Data are mean values ± SEM.
No previous study has investigated the impact of the ACE I/D polymorphism on mechanical properties of the abdominal aorta. However, the ACE D allele has been associated with increased stiffness of the carotid artery in a general population [212] and in older subjects [213]. A number of studies have investigated the connection between ACE I/D polymorphism and carotid-femoral PWV, some showing no impact of the ACE I/D polymorphism on PWV [213-216], while the I allele was associated with higher PWV in diabetics [215], in hypertensives [217] and in healthy middle-aged subjects [218]. PWV is usually measured between the carotid and femoral artery and reflects the mean arterial stiffness of several arterial territories, including both elastic and muscular arteries. PWV may thus reflect other aspects of vascular function than those measured in our study. In addition, the elastic behaviour of various types of arteries may be different, and genetic determinants may have different outcome depending on the type of artery studied [213, 219].

Circulating ACE and arterial stiffness

The mechanism by which the ACE D allele modulates arterial stiffness is unknown. A plausible mechanism could be an increased generation of ang II, due to higher levels of ACE in carriers of the D allele. Ang II influences vascular homeostasis, vascular tone, vascular smooth muscle cell growth [12, 13] and affects the production of collagen and elastin in the vessel wall [15, 16], thereby promoting stiffening of the artery. In addition, a role of ACE in arterial stiffening is supported by the finding that ACEi and ARBs reduce arterial stiffness beyond that expected from the reduction in blood pressure alone [208, 209, 220]. Although an association between the ACE I/D polymorphism and circulating ACE levels has been confirmed repeatedly, we and others have shown large variations between carriers of the same genotype [52]. Thus, we hypothesized that ACE level would show a stronger association to arterial stiffness than the ACE I/D polymorphism. Surprisingly, although the D allele was associated with abdominal aortic stiffness, there was no link between circulating ACE and aortic stiffness. To the best of our knowledge, no previous study has investigated the association between ACE level and arterial stiffness. We do not know whether levels of circulating ACE correlate with the levels in the arterial wall. Thus, there might be a link between ACE in the vessel wall and abdominal aortic stiffness. The beneficial effect of ACEi and ARBs on arterial stiffness may thus result from local effects within the vessels wall. On the other hand, the ACE D allele may be in linkage disequilibrium with genetic variations in other genes, which in turn may influence aortic stiffness. The ACE I/D polymorphism may thus represent a
marker for another genetic polymorphism involved in regulation of vessel wall mechanics. The identity of such polymorphism is however unknown.

**ACE, abdominal aortic stiffness and aneurysm**

The abdominal aorta is the most common site for aneurysm formation [103]. The mechanisms involved in aneurysm formation are largely unknown, however it has been argued that a combination of local hemodynamic factors, as well as factors affecting wall strength (genetic factors, proteolytic activity, inflammation etc.) are of importance [103].

The prevalence of AAA in our population was 8% in men and 1% in women, which is similar to previous reports [111]. There was a tendency towards higher stiffness values in men with AAA compared to those without, however, this failed to reach statistical significance, possibly due to the low number of subjects with AAA. Increased abdominal aortic stiffness in subjects with AAA has on the other hand been shown previously [113, 114].

A link between RAS and abdominal aortic aneurysm has been shown in experimental studies [210, 211]. Accumulation of ACE has been reported in the abdominal aortic wall in patients with aneurysm [210], and ang II infusion induces AAA in mice [211]. Furthermore, previous studies have indicated a possible association between the ACE I/D polymorphism and AAA [221-224]. Interestingly, Jones et al. reported that the increased risk for AAA was equal for I/D and D/D carriers, when compared to I/I carriers [223]. The same pattern was shown in our study; increased stiffness in carriers of the D allele independent of one or two copies of the allele.

Our study showed an association between the ACE D allele and aortic stiffness in men, whereas no association was found in women. This gender difference is particularly interesting in the context of aneurysm formation, as AAA is more common in men [103]. Previous studies investigating the link between the ACE I/D polymorphism and arterial stiffness of other vascular territories have analyzed mixed populations [212, 213, 216, 217]. The lack of association between the ACE D allele and aortic stiffness in women may therefore conceal the association in men, if mixed populations are studied.

Taken together, this study provided evidence of an association between the ACE D allele and aortic stiffness in men. An increased abdominal aortic stiffness indicates impaired vessel wall integrity, which may be of importance in aneurysm
formation. However, as the ACE D allele is a common allele, it cannot be the sole factor responsible, but together with other local factors, the ACE D allele may predispose to aneurysm formation. An illustration of this hypothesis is shown in Figure 19.

Figure 19. Schematic illustration of a suggested link between the ACE D allele, abdominal aortic wall integrity and abdominal aortic aneurysm.
Association between ACE, left ventricular function and central hemodynamics

LV dysfunction is a common final outcome for a number of cardiac disorders. Ischemic heart disease and hypertension are important determinants of LV dysfunction, but a variety of other cardiac disorders (e.g. dilated cardiomyopathy, heart valve diseases, arrhythmias) may also be the underlying cause [142]. Pharmacological inhibition of ACE is the standard treatment for patients with heart failure and has been shown to reduce morbidity and mortality [147]. A large meta-analysis of previously published trials demonstrated a significant improvement in LV function in subjects treated with ACEi compared to placebo [225]. In our study, the relation between ACE, LV function and central hemodynamics was investigated in a cohort of elderly men and women. Subjects with LV dysfunction had higher levels of circulating ACE than subjects with normal LV function, and there seem to be a relationship between ACE level and degree of LV dysfunction (Figure 20).

![Figure 20](image)

**Figure 20.** Association between circulating ACE level and LV dysfunction in elderly subjects. Values are mean ± SEM. P-value was calculated using ONE-way ANOVA.
The underlying cause for LV dysfunction is not known for all subjects included, and the rationale for higher ACE levels among those with LV dysfunction is unknown. Previous data have indicated a role of ACE in both cardiac remodeling and ischemic heart disease. A role of ACE in LV remodeling is supported by studies in rats, showing increased conversion of ang I to ang II as well as increased expression of ACE in hypertrophic myocardium [226-228]. In humans, a positive association between circulating ACE and LV wall thickness [229, 230] and LV mass index [229, 231] has been reported. However, in contrast to our findings, no correlation between ACE and LV function was found [230]. An increased expression of ACE may be involved in cardiac remodelling through an increased production of ang II and degradation of bradykinin. In the heart, ang II has hypertrophic effects [19] and promotes fibrosis [20]. An increased degradation of bradykinin may also promote cardiac hypertrophy, as a result of a reduced production of nitric oxide and prostacyclin. On the other hand, no difference in circulating ACE between normotensive and hypertensive subjects with or without LV hypertrophy was found [145] and no relation between circulating ACE and LV mass was detected in healthy normotensive men [183]. Furthermore, accumulation of ACE has been reported in coronary atherosclerotic lesions [42, 232], and enhanced cardiac ACE [233], as well as circulating ACE levels [52, 234] has been shown in patients who have suffered a myocardial infarction. In addition, in subjects with ischemic heart disease a higher cardiac ACE was associated with reduced LV function [235], and up-regulation of cardiac ACE has been shown in patients with heart failure [228, 236].

In recent years, there has been a growing interest in studying blood pressure in central arteries, as this is the pressure the heart actually meets. In addition central pressure is more strongly related to cardiovascular outcome than the peripheral pressure [139, 140]. Previous studies have indicated that ACEi lower central blood pressure more effectively than other hypertensive drugs [237, 238], suggesting a role of ACE in regulation of central hemodynamics. Ang II, the main effector peptide of ACE, is a powerful vasoconstrictor with well-described effects on blood pressure. As ang II raises blood pressure, one might expect to find a connection between ACE and blood pressure. Previous data have indicated a weak but significant association between ACE level and peripheral systolic pressure [155, 239]. Although some data indicate a possible connection between ACE and peripheral pressure, this may not hold true for central pressure. Our study was the first to investigate the association between circulating ACE and central hemodynamics. There was no association between circulating ACE and
central aortic blood pressure, aortic augmentation or pulse pressure amplification.

As a result of an increased afterload, chronically elevated blood pressure may result in LVH. However, LVH has been observed in normotensive subjects, and the degree of hypertension cannot explain the variance in LV mass [240, 241]. Although lowering of blood pressure has beneficial effects on LVH, clinical trials have indicated that ACEi decrease LV mass more effectively than other hypertensive drugs [242]. This finding has rendered the hypothesis that the hemodynamic burden cannot be the sole determinant of LV structure and function [243], and non-hemodynamic factors may also be of importance [19, 145, 146]. However, the possibility that this effect is due to a more pronounced effect of ACEi on central pressure compared to peripheral pressure cannot be excluded [237]. Our data showing an association between circulating ACE and LV dysfunction, while there was no correlation between ACE and central blood pressure supports the hypothesis of a pressure-independent role of ACE in cardiac remodeling.

The ACE I/D polymorphism is probably one of the most frequently studied candidate genes in CVD. Our study showed no difference in D allele frequency between subjects with normal LV function and those with LV dysfunction. However, a larger population with higher prevalence of LV dysfunction might have been necessary to detect such an effect. The ACE D/D genotype has been associated with increased risk of a number of different cardiac complications, including myocardial infarction [58, 59], LVH [244] and heart failure [67], although data are inconsistent [61, 183]. Today, there is no consensus regarding the importance of the ACE genotype for CVD. The D/D genotype alone might not be a risk factor for CVD, but together with other factors it may increase the risk for cardiovascular complications and may be of particular importance in specific subgroups.

The impact of the ACE I/D polymorphism on peripheral blood pressure has been extensively studied, but the results are contradictory [61, 65, 66, 245]. However, to our knowledge, only one previous study has investigated the impact of the ACE I/D polymorphism on central pressure [218]. In agreement with Dima et al. [218], we found no difference in central aortic blood pressure, aortic augmentation or pulse pressure amplification between genotypes. Thus, at present, there is no evidence for direct impact of the ACE I/D polymorphism or circulating ACE on
central blood pressure. However, the relation between tissue ACE and central hemodynamics is still unknown.
LIMITATIONS

There are some limitations in our studies that should be pointed out. A weakness in paper II-IV is the relatively small sample size (n= 406-672), leading to a low number of subjects in subgroups (with respect to e.g. gender, genotype, LV function). Also, as in all population based studies in elderly, there might be a survival bias in our study, as some subjects obviously died before initiation of the study. A selection against a specific ACE genotype is however contradicted by the fact that the population was in accordance with the Hardy Weinberg equilibrium.

We reported a positive association between number of cardiovascular risk factors and circulating ACE (paper II), as well as an association between ACE and LV dysfunction (paper IV). An up-regulation of ACE might be involved in the pathogenesis of CVD. However, we cannot exclude the possibility that an increased ACE level is a non-pathological phenomenon, resulting from e.g. increased shedding of ACE from ACE containing cells.

Furthermore, the abdominal aortic wall properties were calculated using the brachial blood pressure for calibration. This may introduce a systematic bias as the diastolic pressure is slightly higher in the brachial artery compared to the abdominal aorta. However, as no age- or gender-related differences have been observed, this systematic bias should not affect comparative studies between groups.

The SphygmoCor system uses a generalized transfer function in order to determine central hemodynamics. Although this transfer function has been validated in a number of different populations, it is not individualized and may thus not be accurate for all subjects. Furthermore, a second limitation is the calibration of the radial artery pulse wave with brachial pressure. This may introduce an error as it neglect the brachial-to-radial pressure amplification. However, as the pressure differences in the arterial tree decrease with age, this bias is probably less pronounced in elderly subjects.
CONCLUSIONS

Based on results from paper I-IV, the following conclusions can be drawn:

Nicotine and nicotine metabolites induce an increased ACE activity in cultured human endothelial cells (HUVECs), and act primarily by affecting the enzymatic activity, but may also induce an increased expression. The regulation of ACE by nicotine seems to be restricted to ACE located in cells/tissues. There is an individual variation in nicotine/nicotine metabolite induced up-regulation of ACE, that appears to be independent of the ACE I/D polymorphism.

In elderly men, cardiovascular risk factors (such as smoking and diabetes) are associated with higher levels of circulating ACE in men. The previously reported association between the D/D genotype and CVD was not confirmed. Increased ACE level may represent one of the cellular mechanisms involved in producing the vascular damage associated with cardiovascular risk factors.

Men carrying the ACE D allele have higher abdominal aortic stiffness compared to men carrying the I/I genotype. However, there is no association between circulating ACE level and aortic stiffness, suggesting that the effect of the D allele is due to factors other than elevated levels of circulating ACE. Increased abdominal aortic stiffness suggests impaired vessel wall integrity, which combined with local hemodynamic and/or inflammatory factors may have a role in aneurysm formation.

Subjects with LV dysfunction have higher levels of circulating ACE compared to those with normal LV function, and there is a relationship between circulating ACE and degree of LV dysfunction. ACE might play a role in the pathogenesis of left ventricular dysfunction and our findings suggest a direct effect on the heart rather than affecting central blood pressure.
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