

# C-reactive protein, fibrinogen, soluble thrombomodulin, and vascular diseases

Ph.D. Thesis

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## Summary

Atherosclerosis is a chronic inflammatory disease of worldwide prevalence. No longer regarded as a bland, mechanical process, plaque evolution is now best understood as a pitched battle between proinflammatory and anti-inflammatory cellular and molecular elements. Certain mediators and systemic acute-phase reactants implicated in the pathogenesis of the disease in the future may prove to be (1) useful surrogate markers of disease severity and activity, (2) potential targets for intervention, and (3) end points for titration of therapy and timing of intervention. C-reactive protein is currently the most important of these biomarkers, and may become more so as it appears to identify asymptomatic patients at risk and directly mediate endovascular tissue injury.

We determined the distribution of CRP in a healthy Hungarian population. A representative population distribution of CRP was based on analysis of 207 Hungarians without apparent vascular disease. In our study median CRP level was 1.87 mg/L and ranges of CRP for those with lowest (quintile 1) to highest (quintile 5) vascular risk were 0.05 to 0.73, 0.74 to 1.50, 1.51 to 2.56, 2.57 to 4.77, and >4.78 mg/L. As risk estimates appear to be linear across the spectrum of inflammation, these sequential quintiles can be considered in clinical terms to represent individuals with low, mild, moderate, high, and highest relative risks, respectively, of future cardiovascular disease. According to this supposition, 40% (n=83) of the examined subjects belonged to the high and highest cardiovascular risk group.

Diseases of the blood vessels are among the most frequent cause of serious neurological disorders, ranking third as a cause of death in the adult population in Hungary and probably first as a cause of chronic functional incapacity. Randomized trials have verified the efficacy of carotid endarterectomy for treating and preventing stroke in patients with extracranial cerebrovascular occlusive disease.

To our knowledge, no data on longitudinal measurements of any acute-phase proteins with such long follow-up period after carotid endarterectomy have been published so far, and the effect of TNF- $\alpha$  polymorphism on sTM levels was not tested either. Therefore, we examined 117 patients with severe carotid artery stenosis, who were undergoing eversion endarterectomy at our Department. During the follow-up period (14 months) we observed a sharp, highly significant drop ( $p<0.0001$ ) in the

serum and plasma concentrations of both acute-phase proteins (CRP, fibrinogen). Serum CRP levels decreased from 7.90 (3.20-14.25) mg/L measured preoperatively to 3.00 (1.23-7.93) mg/L at the last follow-up visit. Plasma fibrinogen levels were 410 (346-479) mg/dL and 352 (285-410) mg/dL, respectively. The drop in the CRP levels during the follow-up period was mainly due to the decrease in the highest tertile of the baseline levels. Strong negative correlation ( $R=-0.418$ ,  $p=0.0006$ ) was found between the plasma sTM concentrations and the preoperative duplex scan values. Patients with -308 A TNF- $\alpha$  genotype had significantly lower ( $p=0.0415$ ) preoperative sTM values than their counterparts with no such polymorphism. Fourteen months postsurgery the sTM levels were significantly higher ( $p=0.0002$ ) compared to the preoperative state.

Internal carotid artery restenosis of 50% or greater was detected in 15 patients (13%), but only 4 patients (3%) had severe ( $\geq 70\%$ ) restenosis in the operated region during the follow-up period. Neither CRP nor fibrinogen levels changed significantly compared to the preoperative values till the end of the observation period in the restenosis group. By contrast, in patients with no restenosis CRP and fibrinogen levels significantly decreased as compared to the levels measured before surgery already at the first follow-up visit, and the drop continued till the end of the follow-up. In addition, early postoperative changes in fibrinogen levels predicted restenosis.

Our findings indicate that removal of atherosclerotic plaques from carotid arteries markedly decreases the production of the two acute-phase proteins in patients. This change can be due to the decrease of the inflammatory burden postsurgery or the removed advanced plaques able to produce acute-phase proteins. Then again, sTM may be adsorbed to the atherosclerotic plaques or inflamed endothelium in carotid arteries, but the pathological significance of this adsorption remains to be determined.

## Összefoglalás

Az ateroszklerózis csaknem a Föld teljes népességét érintő idült, gyulladással járó betegség. A plakkok kialakulása nem egyszerűen egy mechanikus folyamat, sokkal inkább a gyulladás irányába ható és a gyulladás ellenes sejtek, valamint molekulák közti egyensúly megbomlásának az eredménye. Az ateroszklerózis létrejöttében résztvevő különböző mediátorok és akut-fázis fehérjék a betegség súlyosságának, aktivitásának megítélésében hasznos kiegészítő markerek, valamint beavatkozási célpontok lehetnek a későbbiekben. Fontos szerepük lehet továbbá a terápia beállításánál és a terápiás idő tervezésénél is. Jelenleg a CRP a legfontosabb vizsgált molekula, mivel úgy tűnik, hogy tünetmentes egyéneknél alkalmas a kardiovaszkuláris rizikóbecslésre és közvetlenül részt vesz az ér belfelszín károsodásának létrejöttében.

Vizsgálataink során meghatároztuk egy egészséges magyar populáció CRP megoszlását. Kétszázhet klinikailag egészséges magyar véradó mintáit felhasználva a CRP szérumszintjének medián értéke 1,87 mg/L-nek bizonyult, az egyes kvintiliseknek megfelelő CRP értékek pedig a legkisebbtől a legnagyobb vaszkuláris rizikót jelentő kvintilisek felé haladva a következők voltak: 0,05-0,73, 0,74-1,50, 1,51-2,56, 2,57-4,77 és >4,78 mg/L. Ha feltételezzük, hogy lineáris összefüggés van a gyulladás mértéke és a kardiovaszkuláris rizikó között, akkor az egyes kvintilisek a kis, a mérsékelt, a közepes, a nagy és a nagyon nagy rizikónak felelnek meg. E feltételezést alapul véve, a vizsgálatunkban résztvevők 40%-a a nagy, illetve a nagyon nagy kardiovaszkuláris rizikót jelentő csoportba tartozott.

A súlyos neurológiai kórképek hátterében, melyek a felnőtt magyar lakosság körében a 3. leggyakoribb halálhoz vezető okok, a tartós magatehetetlenséget tekintve, pedig minden bizonnyal az első helyen állnak, igen sokszor érfali elváltozások szerepelnek. Randomizált tanulmányok igazolták a karotisz endarteriektómia hatékonyságát a stroke prevencióban és közvetve a stroke terápiában olyan betegeknél, akiknek szignifikáns extrakraniális karotisz sztenózisuk van.

Tudomásunk szerint karotisz endarteriektómiát követően az akut-fázis fehérjék szérumszintje és plazmaszintjének hosszú távú változását, illetve a TNF- $\alpha$  polimorfizmus hatását a sTM szintjére eddig nem vizsgálták. Éppen ezért 117 olyan szignifikáns karotisz sztenózissal rendelkező beteget követtünk nyomon, akiknél a

Klinikán everziós endarteriektómia történt. A 14 hónapos követési idő alatt mindkét akut-fázis fehérje (CRP, fibrinogén) szérum, illetve plazma szintje szignifikánsan csökkent ( $p < 0,0001$ ). A CRP szérum szintje a műtét előtti 7,90 (3,20-14,25) mg/L-es értékről 3,00 (1,23-7,93) mg/L-re, míg a fibrinogén plazma szintje 410 (346-479) mg/dL-es preoperatív értékről 352 (285-410) mg/dL-re csökkent az utolsó mérési időpontban. A vizsgálati periódusban elsősorban a legnagyobb preoperatív tercilisbe tartozó CRP koncentrációk csökkentek. Negatív korrelációt ( $R = -0,418$ ,  $p = 0,0006$ ) észleltünk a műtét előtti sTM koncentrációk és a CDS értékek között. A -308 A genotípussal rendelkezők preoperatív sTM szintje szignifikánsan alacsonyabb ( $p = 0,0415$ ) volt, mint a normál genotípusúaké. Tizennégy hónappal a műtétet követően a sTM koncentrációk szignifikánsan magasabbak ( $p = 0,0002$ ) voltak a preoperatív értékekhez képest.

Ötven százalékos vagy azt meghaladó resztenózis 15 betegnél (13%), míg 70%-os vagy annál nagyobb resztenózis 4 betegnél (3%) alakult ki. A resztenózisos csoportban a preoperatív értékekhez képest sem a CRP, sem a fibrinogén szintek nem változtak szignifikánsan a nyomonkövetés során. Ezzel ellentétben a nem resztenózisos betegeknél már 6 héttel a műtétet követően mindkét akut-fázis fehérje koncentrációja szignifikánsan csökkent és ez a csökkenés a későbbiekben is folytatódott. Ráadásul a fibrinogén szintek korai posztoperatív változása a resztenózisra nézve prediktív értékűnek bizonyult.

Vizsgálataink azt mutatják, hogy az ateroszklerotikus karotisz plakkok sebészi eltávolításával az akut-fázis fehérjék képződése jelentősen csökken. Ennek oka vagy az általános gyulladási aktivitás csökkenésében keresendő vagy az ateroszklerotikus plakkok is képesek akut-fázis fehérjék termelésére. Feltételezzük továbbá, hogy a sTM kötődik a plakkokhoz, illetve a gyulladási folyamatokban résztvevő endotéliumhoz. E feltételezés bizonyítása azonban további vizsgálatokat igényel.

## Abbreviations

<b>A:</b>	adenosine	<b>IMT:</b>	intima-media thickness
<b>APC:</b>	activated protein C	<b>iNOS:</b>	inducible nitric oxide synthase
<b>AT<sub>1</sub>-R:</b>	angiotensin type 1 receptor	<b>IQR:</b>	interquartile range
<b>BMI:</b>	body mass index	<b>LDL:</b>	low-density lipoprotein
<b>CAD:</b>	coronary artery disease	<b>MAP:</b>	mitogen-activated protein
<b>cAMP:</b>	cyclic adenosine monophosphate	<b>MCP:</b>	monocyte chemotactic protein
<b>CDS:</b>	carotid duplex scan	<b>M-CSF:</b>	macrophage colony-stimulating factor
<b>CEA:</b>	carotid endarterectomy	<b>MMP:</b>	matrix metalloproteinase
<b>CHD:</b>	coronary heart disease	<b>mRNA:</b>	messenger ribonucleic acid
<b>CI:</b>	confidence interval	<b>NcoI:</b>	restriction enzyme
<b>CRP:</b>	C-reactive protein	<b>NFκB:</b>	nuclear factor κB
<b>CVD:</b>	cardiovascular disease	<b>OR:</b>	odds ratio
<b>DNA:</b>	deoxy-ribonucleic acid	<b>PAD:</b>	peripheral artery disease
<b>EC:</b>	endothelial cell	<b>PAI:</b>	plasminogen activator inhibitor
<b>ECG:</b>	electrocardiogram	<b>PCR:</b>	polymerase chain reaction
<b>EDTA:</b>	ethylene diamine tetra-acetic acid	<b>PDGF:</b>	platelet-derived growth factor
<b>EGF:</b>	epidermal growth factor	<b>PS:</b>	protein S
<b>ELISA:</b>	enzyme-linked immunosorbent assay	<b>PTA:</b>	percutaneous transluminal angioplasty
<b>eNOS:</b>	endothelial nitric oxide synthase	<b>PTX:</b>	pentraxin
<b>ERK:</b>	externally regulated kinase	<b>ROS:</b>	reactive oxygen species
<b>ET:</b>	endothelin	<b>SAA:</b>	serum amyloid A
<b>FGF:</b>	fibroblast growth factor	<b>SMC:</b>	smooth muscle cell
<b>G:</b>	guanine	<b>sTM:</b>	soluble thrombomodulin
<b>GP:</b>	glycoprotein	<b>TAFI:</b>	thrombin-activatable fibrinolysis inhibitor
<b>HDL:</b>	high-density lipoprotein	<b>TC:</b>	total cholesterol
<b>hs-CRP</b>	high-sensitivity C-reactive protein	<b>TGF:</b>	transforming growth factor
<b>ICA:</b>	internal carotid artery	<b>TIA:</b>	transient ischemic attack
<b>ICAM:</b>	intercellular adhesion molecule	<b>TM:</b>	thrombomodulin
<b>IFN:</b>	interferon	<b>TNF:</b>	tumor necrosis factor
<b>IGF:</b>	insulin-like growth factor	<b>VCAM:</b>	vascular cell adhesion molecule
<b>IHD:</b>	ischemic heart disease	<b>VSMC:</b>	vascular smooth muscle cell
<b>IL:</b>	interleukin		

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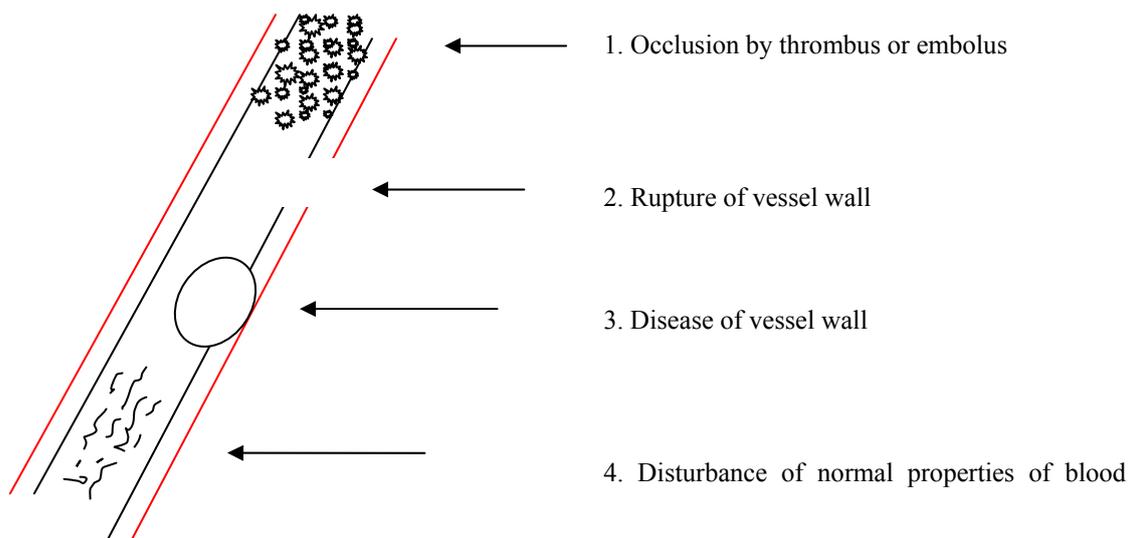
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## 1. Introduction

### 1. 1. Stroke

Stroke is a leading cause of neurological disability and the third most common cause of death, preceded only by coronary artery disease and cancer in Hungary.

Stroke is a generic term, lacking pathological meaning. Cerebrovascular diseases can be defined as those in which brain disease occurs secondary to a pathological disorder of blood vessels (usually arteries) or blood supply. Whatever the mechanism, the resultant effect on the brain is either ischemia/infarction, or hemorrhagic disruption. (Figure 1)



**Figure 1.** Mechanisms in cerebrovascular diseases

Approximately 75% to 80% of all strokes are ischemic. Hemorrhage, including intraparenchymal and subarachnoid, is present in about 15% to 20%. Although most occlusive strokes are due to atherosclerosis and thrombosis and most hemorrhagic strokes are associated with hypertension or aneurysms, strokes of either type may occur at any age from several causes, including cardiac disease, trauma, infection, neoplasm, blood dyscrasia, vascular malformation, immunological disorder, exogenous toxins, etc. (Table 1)

**Table 1.** Causes of cerebrovascular diseases (from Hachinski) [50]

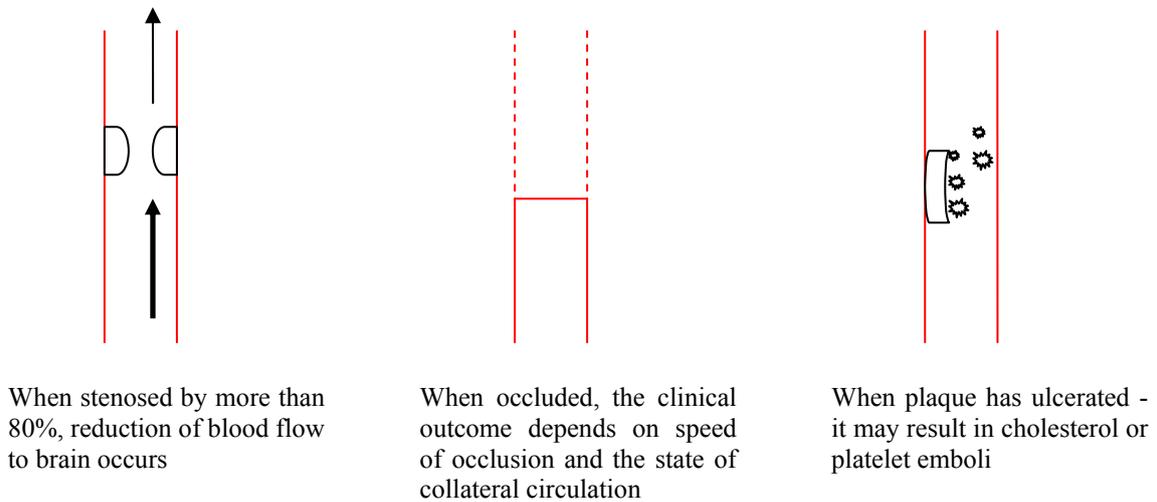
<b>OCCLUSION (50%)</b>	
Atheromatous/thrombotic	Large vessel occlusion or stenosis Branch vessel occlusion or stenosis Perforating vessel occlusion
Nonatheromatous diseases of the vessel wall	Collagen disease Vasculitis Miscellaneous
<b>EMBOLIZATION (25%) from</b>	
Arteries	Intracranial arteries Extracranial arteries Aortic arch
Heart	Valvular heart disease Arrhythmias Ischemic heart disease Bacterial and nonbacterial endocarditis Atrial myxoma Prosthetic valves Patent foramen ovale Cardiomyopathy
<b>HEMORRHAGE (20%)</b>	
Intraparenchymal and/or extraparenchymal	Hypertension Amyloid vasculopathy Aneurysm Arteriovenous malformation Neoplasm Coagulation disorder Anticoagulant therapy Vasculitis Drug abuse Trauma
<b>DISEASES OF BLOOD</b>	
<b>DECREASED CEREBRAL PERFUSION</b>	

## 1. 2. Occlusive and stenotic cerebrovascular diseases

Atherosclerosis is the pathological process most often responsible for cerebrovascular insufficiency. The carotid bifurcation is the predominant location for atherosclerotic disease. (Figure 2)

Originally formulated as an unsophisticated disorder of lipoprotein accumulation, atherosclerosis can now be fundamentally understood as a chronic inflammatory disease of the arterial system. [104, 138] While endothelial injury and dysfunction remain central to the initiation and pathogenesis of the disease,

accumulating evidence suggests that the classically postulated "response to injury" is in fact inflammation itself. [138]



**Figure 2.** Internal carotid artery stenosis and its complications

### 1. 2. 1. The inflammatory nature of plaque progression

The earliest event in atherogenesis appears to be endothelial cell dysfunction. Various noxious insults, including hypertension, diabetes mellitus, smoking, dyslipidemia, and hyperhomocysteinemia, can result in endothelial cell dysfunction which manifests primarily as deficiency of nitric oxide and prostacyclin and an increase in endothelin-1, angiotensin II, plasminogen activator inhibitor-1 (PAI-1), cell adhesion molecules, etc. Following endothelial cell dysfunction, mononuclear cells attach to the endothelium, initially loosely and thereafter adhere firmly to the endothelium and then diapedese into the subendothelial space. The rolling and tethering of leukocytes on the endothelium is orchestrated by adhesion molecules such as selectins (E-selectin, P-selectin), cell adhesion molecules [intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)], and integrins. Chemotaxis and entry of monocytes into the subendothelial space is promoted by monocyte chemoattractant protein-1 (MCP-1), interleukin-8, and fractalkine. Thereafter, macrophage colony-stimulating factor (M-CSF) promotes the differentiation of monocytes into macrophages. Macrophages incorporate lipids from oxidized low-density lipoprotein (LDL) via the

scavenger receptor pathway (CD36, scavenger receptor-A), becoming foam cells, the hallmark of the early fatty streak lesion. Following the fatty streak lesion, smooth muscle cells migrate into the intima, proliferate, and form the fibrous cap. Lipid-laden macrophages, during the process of necrosis and apoptosis, release matrix metalloproteinases (MMPs), which cause a rent in the endothelium. Matrix metalloproteinases mediated areas of fissuring or ulceration that subsequently develop in advanced atherosclerotic plaques are particularly vulnerable to platelet-associated vascular hemorrhage, rupture, thrombosis, embolization, and occlusion. [80, 112, 138]

### **1. 3. Carotid endarterectomy**

During the past 50 years there has been tremendous progress in reducing mortality from stroke. This success in part is related to the evolution of the surgical treatment of extracranial cerebrovascular diseases. Carotid endarterectomy (CEA) is the primary treatment used, and the frequency at which this procedure is performed has steadily increased since the early 1990s. Currently, CEA is one of the most commonly performed vascular operations in Europe.

The combined data from the European Carotid Surgical Trial (ECST), North American Symptomatic Carotid Endarterectomy Trial (NASCET) and Veterans' Trials have confirmed that carotid endarterectomy for a 70-99% carotid stenosis (as determined by intra-arterial angiography) reduces stroke risk in symptomatic patients. [139] Similarly, the Asymptomatic Carotid Atherosclerosis Study (ACAS) and the Asymptomatic Carotid Surgical Trial (ACST) have shown a significant, albeit less marked, advantage of surgery for asymptomatic stenoses of the same severity. [52, 101, 183]

Since its introduction by DeBakey in 1953, CEA has undergone a number of technical modifications that have increased its efficacy. Although CEA is a conceptually simple operation, precision and attention to technical details are required to achieve a low rate of stroke.

### **1. 3. 1. Technique of carotid endarterectomy**

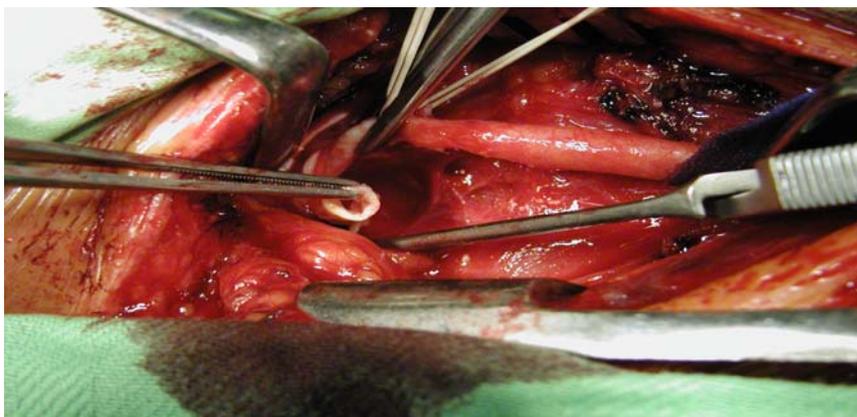
The supine patient is prepared with his head extended and turned away from the side of the operation. The incision must be longitudinal along the anterior border of the sternocleidomastoid muscle. Exposure of the carotid bifurcation is facilitated by dividing the common facial vein, a tributary of the internal jugular vein. The common, external, and internal carotid arteries are identified. Division of the posterior belly of the digastric muscle or mandibular subluxation may be needed if the carotid bifurcation is high or if there is distal plaque that can not be accessed by conventional means. Undiseased segments of the carotid vessels are dissected and controlled so as to decrease the potential for embolization while the arteries are being mobilized. The classical dictum that "the patient should be dissected away from the carotid artery" emphasizes the need for careful technique during exposure. The surgeon must be aware of the mandibular branch of the facial nerve, and the hypoglossal and vagus nerves, although visualization of these nerves is not mandatory. Following systemic heparinization, the distal internal, common, and external carotid arteries are clamped. In recent years the technique of eversion endarterectomy has been popularized. [33] The internal carotid artery is transected obliquely from the common carotid artery. The internal carotid artery wall is everted over the atheromatous core until a distal endpoint is directly visualized. After the internal carotid artery has been completely endarterectomized, significant disease in the common and external carotid arteries is removed. The internal carotid artery is then reanastomosed to the common carotid artery. This technique avoids the need for a suture line in the distal internal carotid artery where the luminal diameter is small. As such, it has been suggested that eversion endarterectomy reduces the incidence of occlusion and restenosis compared to the more conventional endarterectomy techniques. (Figure 3)



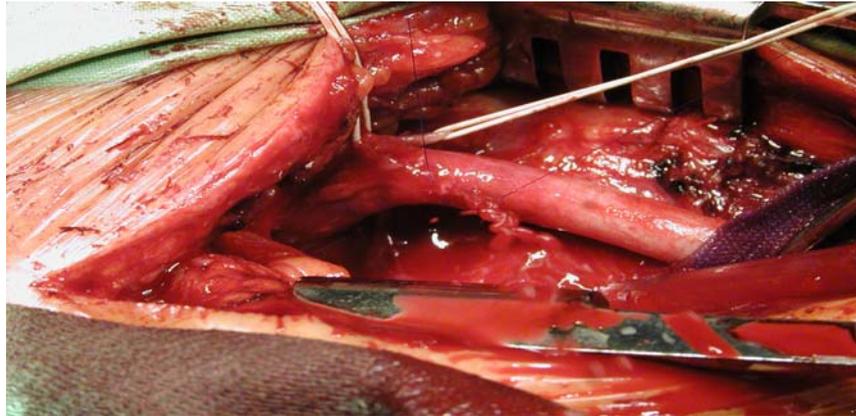
(A) Internal carotid artery is transected obliquely at the carotid bifurcation.



(B) Medial and adventitial layers of the internal carotid artery are everted over the atheromatous core.



(C) Completion of endarterectomy with the distal endpoint directly visualized.



(D) Internal carotid artery is reanastomosed to the common carotid artery.

**Figure 3.** Technique of eversion carotid endarterectomy (from Entz et al.)

### **1. 3. 2. Restenosis after surgery**

The majority of recurrent stenotic lesions are confined to the initial endarterectomy site. The suture line is almost always evident within the region of recurrent disease. Stoney and String were the first to classify recurrent carotid lesions into two distinct types: lesions occurring within 2 years after CEA were attributed to intimal hyperplasia and thereafter, to recurrent atherosclerosis. [155] Although neointimal fibrous plaques do occur more frequently early on, there appears to be a wide spectrum of changes ranging from lesions with only neointimal thickening or atherosclerotic components to complex lesions with both myointimal thickening elements and evidence of recurrent atherosclerosis present at all time periods.

#### **1. 3. 2. 1. Myointimal thickening**

Following completion of the endarterectomy and restoration of flow, the recently denuded surface is covered by a layer of platelets, fibrin, and entrapped red blood cells and leukocytes. The factors that rapidly render the thrombotic layer of blood cells and leukocytes are poorly understood.

Platelets, endothelial and smooth muscle cells at the site of injury produce growth factors, cytokines, and other molecules including platelet-derived growth factor

(PDGF), fibroblast growth factor (FGF), interleukin (IL)-1, insulin-like growth factor (IGF), tumor necrosis factor (TNF)- $\alpha$ , transforming growth factor (TGF)- $\beta$ , angiotensin II, oxygen free radicals, and thrombin, which have been shown to stimulate vascular smooth muscle cell proliferation, migration, and matrix formation. [98] As a potential balance to these mitogens, several factors including IL-1, TGF- $\beta$ , TNF- $\alpha$  (in high concentrations), and interferon (IFN)- $\gamma$  can inhibit smooth muscle cell (SMC) proliferation. These cytokines are usually expressed in low or undetectable concentrations in normal human arteries but are upregulated after injury or in atherosclerotic lesions. [109]

Platelet-derived growth factor BB is a major chemoattractant and mitogen in platelets, and its inhibition with antibodies has been shown to reduce neointimal lesions after endarterectomy by up to 40%. The expression of PDGF may be modulated in an autocrine fashion as well as by cytokines such as IL-1, TNF- $\alpha$  and TGF- $\beta$ , produced by macrophages. Basic FGF released by injured and dying cells is also a potent stimulant of smooth muscle cell proliferation. The inhibition of bFGF by a neutralizing anti-bFGF antibodies reduced medial SMC proliferation by 80%. [44, 98]

In contrast, infusion of IFN- $\gamma$ , the SMC inhibitor produced by T-lymphocytes, reduces the cross-sectional intimal area of experimental lesions by 50%. [44]

The kinetics of intimal thickening have been well documented in a number of animal models including rats, rabbits, and pigs. [44] Of these, the rat carotid artery balloon injury model is the most commonly used. Using thymidine uptake, it has been shown that smooth muscle cell proliferation commences at 24 to 27 hours, peaks at 4 weeks, and returns to normal by approximately 8 weeks. [1] Histologically, these lesions consist almost entirely of SMCs and extracellular matrix. Although human carotid neointimal restenotic lesions have a similar histologic appearance consisting almost entirely of smooth muscle cells and extracellular matrix, unlike the rat, the proliferation rates are low at the time of removal. [1] A few clusters of macrophages are often seen interspersed between the SMCs in the mucopolysaccharide and collagen rich matrix.

### **1. 3. 2. 2. Recurrent atherosclerosis**

Recurrent atherosclerotic lesions may be indistinguishable from primary lesions. Such lesions are characterized by the presence of macrophages, foam cells, cholesterol clefts, collagen, and calcium deposits. Whether intimal thickening is a precursor of atherosclerosis remains unclear. Although both elements may be present within plaque of all ages Glenn et al. have observed predominately fibrous plaques without evidence of atherosclerosis many years after the initial endarterectomy. [44]

### **1. 4. Nonspecific circulating markers of inflammation and atherosclerosis**

Serum and plasma markers of inflammation provide an avenue of insight into the pathophysiology of atherosclerosis and its complications. A number of biomarkers that appear to be linked to inflammation and atherogenesis have been identified, and others are being evaluated.

#### **1. 4. 1. C-reactive protein**

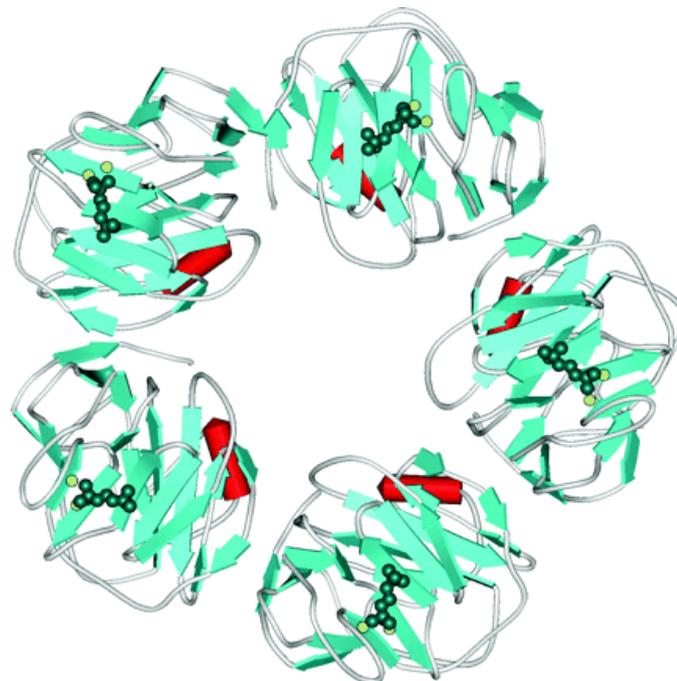
##### **1. 4. 1. 1. Biochemistry and biology**

C-reactive protein (CRP), named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae*, was the first acute-phase protein to be described and is an exquisitely sensitive systemic marker of inflammation and tissue damage.

C-reactive protein is a member of the pentraxin family. It comprises 5 noncovalently associated protomers arranged symmetrically around a central pore and has a molecular weight of 118 000 Da. [150, 159] (Figure 4) In healthy young adult volunteer blood donors, the median concentration of CRP is 0.8 mg/L, the 90th centile is 3.0 mg/L, and the 99th centile is 10 mg/L, but following an acute-phase stimulus, values may increase from less than 50 µg/L to more than 500 mg/L, that is, 10 000-fold. [153] It is a nonglycosylated protein in humans and the gene has been mapped to chromosome 1. The general consensus is that the production of CRP is predominantly

under the control of IL-6. However, IL-1 and tumor necrosis factor may also contribute to hepatic synthesis and secretion of CRP. C-reactive protein has a half-life of ~19 hours and this appears to be constant in health and disease. [174]

Much recent data challenge the dogma that CRP is exclusively produced by the liver. Indeed, cogent data suggest that it is produced in the atherosclerotic lesion (especially by smooth muscle cells and macrophages), the kidney, neurons, and alveolar macrophages. [15, 29, 63, 71, 180, 181] One report found that levels of CRP mRNA within atherosclerotic plaque were 7- and 10-fold higher than levels found in the liver and normal blood vessels, respectively. [180] Also, there is evidence to suggest that the stimulus for the production of CRP might be lipid peroxidation and infection such as cytomegalovirus that triggers a proinflammatory cytokine cascade resulting in CRP release. In this regard, it is interesting that adipose tissue, previously thought to be an inert triglyceride depot, has been shown to produce cytokines such as TNF- $\alpha$  and IL-6, which also could contribute to production of CRP. [184]



The calcium ions are yellow, and phosphocholine is green.

**Figure 4.** Crystal structure of C-reactive protein complexed with phosphocholine (from Thompson et al.) [159]

#### **1. 4. 1. 2. Mechanisms of causal CRP involvement in vascular diseases**

Inflammatory mechanisms play a central role in all phases of atherosclerosis, from initial recruitment of circulating leukocytes to the arterial wall to the rupture of unstable plaques resulting in clinical manifestations of the disease. C-reactive protein may be causally involved in each of these stages by influencing processes such as endothelial dysfunction, lipid-related effects, angiogenesis and apoptosis, thrombosis, complement activation, and monocyte recruitment and activation.

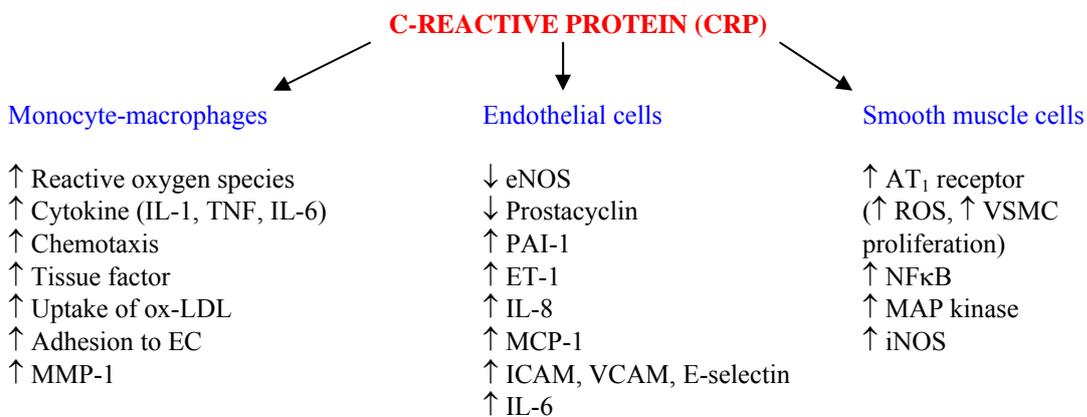
##### **Endothelial dysfunction**

Endothelial dysfunction is one of the early abnormalities in atherosclerosis, characterized by up-regulation of adhesion molecules on the endothelial surface, which allows adhesion and subsequent transmigration of monocytes into the vessel wall. CRP can induce the expression of the adhesion molecules such as ICAM-1, VCAM and E-selectin, in human endothelial cells. [69, 117] The CRP-induced increase in expression of adhesion molecules resulted in elevated adhesion of monocytoid U937 cells to endothelial cells in vitro. [116] These findings were confirmed by others who showed additionally that CRP induced monocyte chemoattractant chemokine-1 (MCP-1) production. [27, 106, 172] The effects are partly mediated via the production of endothelin-1, a potent endothelium-derived vasoactive factor, and by the production of the inflammatory cytokines IL-6 and IL-8. C-reactive protein reduces the expression and bioactivity of endothelial nitric oxide synthase (eNOS) in human aortic endothelial cells. [170, 171] Moreover, CRP reduces prostacyclin activase activity resulting in a decreased prostacyclin release. [170, 171] Less eNOS activity reduces the bioavailability of nitric oxide, which results in inhibition of vasodilatation and stimulation of LDL oxidation, smooth muscle cell proliferation and monocyte adhesion.

C-reactive protein also affects vascular smooth muscle cells by up-regulating the angiotensin type 1 receptor (AT<sub>1</sub>-R), which mediates the majority of the proinflammatory effects of angiotensin II. [176] C-reactive protein also increases proliferation and migration of vascular smooth muscle cells. [176]

## Monocyte recruitment

C-reactive protein also appears to be involved in recruitment of monocytes, infiltration of monocytes into the vessel wall and subsequent development into foam cells. CRP is deposited in the vessel wall at sites of atherogenesis and has been shown to be chemotactic for freshly isolated human blood monocytes. [162, 163] C-reactive protein promotes MCP-1-mediated chemotaxis through up-regulation of CC chemokine receptor 2 expression in human monocytes. [54] (Figure 5)



**Figure 5.** Potential atherothrombotic effects of CRP on vascular cells

## Complement activation

Another mechanism contributing to cardiovascular disease is complement activation. C-reactive protein is able to activate the classical route of complement activation and it colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions. [119, 163, 179] Griselli et al. demonstrated in an animal model that human CRP and complement activation are major mediators of ischemic myocardial injury. [47] In rats that were injected with CRP infarct size was increased by 40%. Increased levels of complement-CRP complexes are reported in plasma from patients with cardiovascular disease, indicating that CRP induces activation of complement in vivo. [179] Since complement activation leads to the production of a variety of proinflammatory molecules, this is a mechanism by which CRP might aggravate the inflammatory status in the entire body as well as in the atherosclerotic plaque. [161]

## Lipids

The interaction between lipids and CRP is diverse. It has been suggested that CRP could be the factor linking lipoprotein deposition and complement activation in atherosclerotic plaques. Binding of tissue-deposited CRP to enzymatically degraded LDL enhances complement activation, which may be relevant to the development and progression of the atherosclerotic lesion, particularly at early stages of atherosclerosis when low concentrations of enzymatically degraded LDL are present. [5, 6] The reports on interaction between CRP and oxidized LDL are conflicting, but complement activation as a result of this interaction is generally considered unlikely. [17, 157]

The majority of foam cells below the endothelium show positive staining for CRP. [163] Zwaka et al. demonstrated that native LDL coincubated with CRP was taken up by macrophages via macropinocytosis. [186] It was concluded that foam cell formation in human atherogenesis might be caused in part by uptake of CRP-opsonized native LDL.

## Thrombosis

Recently, CRP has also been suggested to directly contribute to cardiovascular disease by inducing a prothrombotic state. It was reported that CRP directly induces tissue factor expression in human monocytes, but this result could not be confirmed, suggesting that other blood cells may be required to mediate its effect. [16, 107, 114]

Danenberg and colleagues studied the prothrombotic effect of CRP in CRP transgenic mice using a model of transluminal wire injury. [22] They observed that in human CRP transgenic mice 28 days after injury 75% of the femoral arteries was occluded compared to 17% in wild-type mice. [22]

C-reactive protein increases the expression and activity of the main inhibitor of fibrinolysis, plasminogen activator inhibitor-1 (PAI-1), in human aortic endothelial cells. [11] Since PAI-1 promotes atherothrombosis and progression of acute coronary syndromes, this effect of CRP may also affect cardiovascular disease. [28] Indeed, in mice transgenic for human PAI-1 it was recently shown that chronically elevated levels of PAI-1 are associated with age-dependent coronary arterial thrombosis. [34]

However, these experiments have generally used commercially sourced CRP of incompletely defined provenance and purity, and there have been few robust specificity controls. The findings must be treated with caution until the purity and structural and functional integrity of the CRP have been rigorously established and the specificity of the observed effects confirmed, for example by comparison with appropriate control proteins and by the use of specific CRP absorbents, ligands, antibodies, and inhibitors of binding.

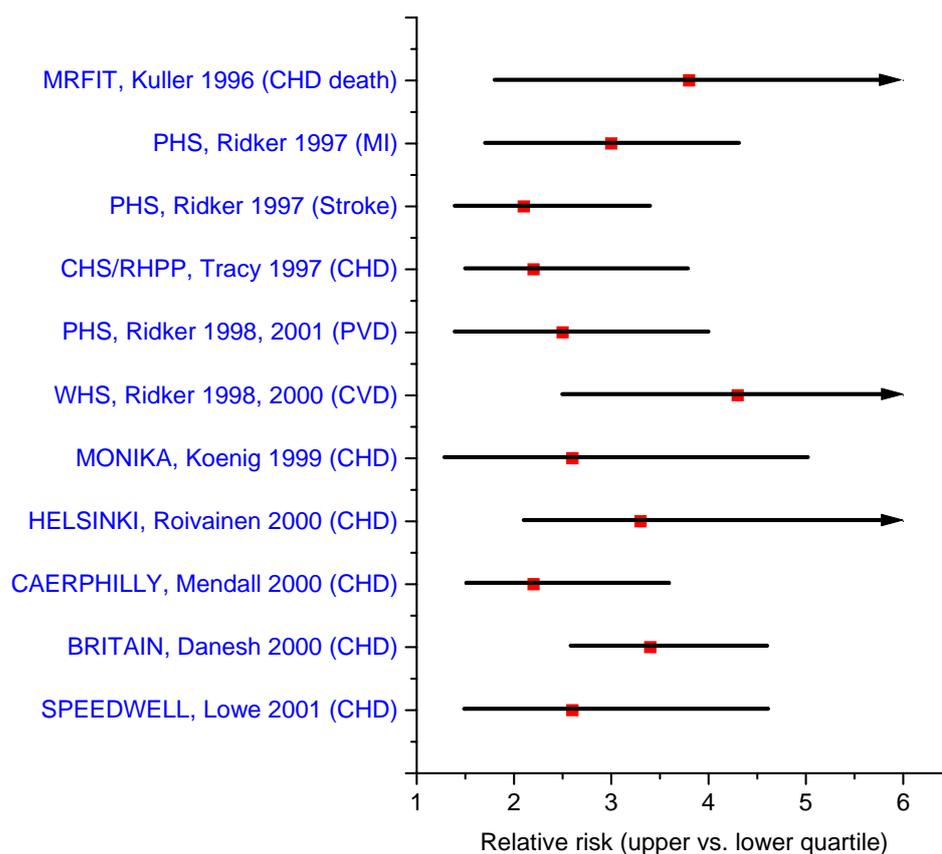
#### **1. 4. 1. 3. Clinical studies**

##### C-reactive protein and cardiovascular risk prediction

Of all the plasma markers of vascular inflammation, CRP has been the most extensively investigated in clinical studies. Baseline levels of CRP are a strong independent predictor of risk of future myocardial infarction, stroke, peripheral vascular disease, and vascular death among healthy individuals without known vascular disease. [25, 55, 73, 75, 86, 130, 131, 132, 133, 135, 165] Furthermore, levels of CRP have been found to predict future risk among patients with stable and unstable angina, in the chronic phase after myocardial infarction, and among patients undergoing revascularization procedures. [7, 13, 18, 57, 58, 82, 84, 96, 103, 134, 164]

Data in support of a role for CRP for cardiovascular risk prediction among apparently healthy individuals are robust and remarkably consistent across several European and US cohorts. [25, 55, 73, 75, 86, 130, 131, 132, 133, 135, 165] (Figure 6) A recent analysis from the WHS thought to compare the risk associated with baseline levels of CRP with other inflammatory and lipid markers of risk. Incident cardiovascular events included death from coronary heart disease, nonfatal myocardial infarction, stroke, and need for coronary revascularization over a mean follow-up of 3 years. [133] Baseline levels of CRP, serum amyloid A (SAA), IL-6, and sICAM-1 were significantly elevated at baseline among the women who subsequently developed cardiovascular events compared to those who did not. Similarly, levels of total cholesterol, LDL-cholesterol, and the ratio of total cholesterol to HDL-cholesterol (TC:HDL ratio) were significantly higher among patients than control subjects. Of all

the inflammatory and lipid markers, CRP was the single most powerful predictor of cardiovascular risk (relative risk for highest compared to lowest quartile =4.4;  $p < 0.001$ ). Multivariate analyses, matched for age and smoking and adjusted for other cardiovascular risk factors, found that only CRP and TC:HDL ratio were independent predictors of future cardiovascular risk.



Risk estimates and 95% confidence intervals are calculated as comparison of top versus bottom quartile within each study group.

**Figure 6.** Prospective studies of CRP as a marker of future cardiovascular risk among individuals without known coronary disease (from Ridker et al.) [129]

Although most studies have shown that CRP is a strong and independent predictor of atherosclerotic risk, the recently reported Reykjavik Study (n=6428) showed a more moderate predictive capability of CRP. [24] Baseline CRP levels were significantly higher in subjects who developed coronary heart disease (CHD) during the

study than in controls, and the odds ratio (OR) for CHD was 1.92 [95% confidence interval (CI) 1.68-2.18] for CHD patients with values in the upper tertile ( $\geq 2.0$  mg/L) as compared with the lower tertile ( $\leq 0.78$  mg/L), after adjustment for age, sex, and year of recruitment. The association between baseline CRP and CHD events remained statistically significant but was attenuated after adjustment for other CHD risk factors (OR 1.45; 95% CI 1.25-1.69). However, the upper tertile cut-off point of 2.0 mg/L used in this study, rather than 3.0 mg/L as recommended by the Centers for Disease Control and Prevention (CDC) and the American Heart Association (AHA), may underestimate the risk associated with CRP level, particularly since the greatest risk appears to be at higher values of CRP. [24]

#### C-reactive protein and carotid atherosclerosis and restenosis

Although CRP predicts progression of atherosclerosis at various sites in the arterial tree, relatively few data are available on the relationship of carotid atherosclerosis and serum concentration of CRP and other acute-phase proteins. As an alternative to the degree of stenosis, intima-media thickness (IMT) has been used in numerous studies to estimate progression of carotid disease. In a family-based cross-sectional study, Folsom et al. found that after adjustment for age and family type, serum CRP was positively but weakly associated with carotid IMT in men and women. [39] A study of middle-aged Dutch women reported a positive association between CRP and common carotid IMT in smokers but not in nonsmokers. [51] By contrast, the Cardiovascular Health Study showed that in older adults, CRP was not associated with internal carotid IMT. [166] Patients with carotid plaques were found to have elevated CRP levels when compared to those without plaques. [9, 10, 48, 126] The implications of an increase in carotid IMT versus an increase in degree of stenosis may differ, particularly in a sample with prevalent atherosclerotic disease. Changes in the degree of stenosis appear to directly reflect progression of atherosclerosis, whereas IMT is considered only as a surrogate marker for carotid disease. However, no data on longitudinal measurements of any acute-phase proteins with long follow-up period (more than a year) have been published after surgical removal of the carotid plaques.

The CRP concentration was noted to be related to the composition of the carotid atherosclerotic plaque, such that higher CRP was found to be associated with a more active or unstable plaque, with greater propensity to stroke. [9, 14]

Schillinger et al. reported a significant increase of serum levels of acute-phase reactants within 48 hours after intervention in response to stent implantation in the carotid artery. The extent of this inflammatory process was associated with recurrent lumen narrowing and in-stent restenosis within 6 months at the treated segment. [144]

## **1. 4. 2. Fibrinogen**

### **1. 4. 2. 1. Pathophysiology**

Fibrinogen is a soluble glycoprotein found in the plasma, with a molecular weight of 340 kDa. [125] It comprises of three pairs of nonidentical polypeptide chains (alpha, beta and gamma chains) linked to each other by disulphide bonds. [125] Fibrinogen has a biological half-life of about 100 hours and is synthesized predominantly in the liver. [125] As a clotting factor, fibrinogen is an essential component of the blood coagulation system, being the precursor of fibrin. However, at the "usual" plasma levels of 200 to 400 mg/dL, its concentration far exceeds the minimum concentration of 50-100 mg/dL necessary for hemostasis.

### **1. 4. 2. 2. Fibrinogen and mechanisms**

Apart from its pivotal role in the coagulation cascade as the substrate for thrombin, there is evidence of multiple mechanisms suggesting that fibrinogen indeed may be involved in both the early and the later stages of the atherothrombotic process. (Figure 7)

#### Atherogenesis

Fibrinogen binding to endothelial cell (EC) receptors (ICAM-1) causes the release of vasoactive mediators. [59] Fibrin(ogen) and fibrin(ogen) degradation products modulate

EC permeability, thus enhancing their deposition in the subendothelial space, and further promote EC migration. Fibrin(ogen) and its degradation products have been shown to promote smooth muscle cell (SMC) chemotaxis and proliferation, and induce monocyte chemotaxis. Fibrin(ogen) provides an adsorptive surface for the extracellular accumulation of LDLs; it further facilitates cholesterol transfer from platelet to monocytes/macrophages and may therefore play a role in foam cell formation. [123, 127] Through all these effects fibrin(ogen) may be involved in the early stages of plaque formation.

#### Platelet aggregation and thrombus formation

Fibrinogen binds to glycoprotein (GP) IIb/IIIa receptors on the platelet membrane and promotes aggregation and formation of platelet-rich thrombi. Elevated plasma fibrinogen levels increase the velocity of platelet aggregation and also increase platelet reactivity. [146]

#### Fibrin thrombus formation

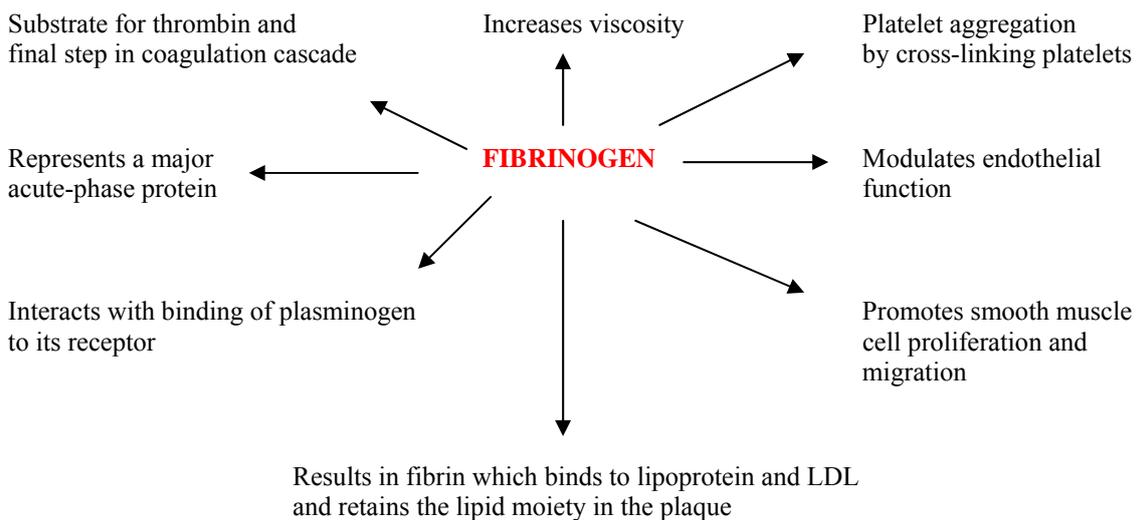
Fibrinogen is the precursor of mural fibrin thrombi and affects thrombus' size, structure, and deformability. Elevated fibrinogen levels lead to larger thrombi and formation of tight and rigid network structures, decrease the deformability of the clot and render it less amenable to endogenous fibrinolysis. [38, 147] Recent data also show that high fibrinogen levels interfere with the binding of plasminogen to its receptor, thus leading to impaired fibrinolysis. [90]

#### Plasma and blood viscosity

Fibrinogen is a major determinant of plasma viscosity (explaining about 50% of its variability), of whole blood viscosity, and of red blood cell aggregation. Elevated blood and plasma viscosity may lead to impaired microcirculatory flow, endothelial shear-stress damage, and predispose to thrombosis. [72]

## Other mechanisms

The fact that fibrinogen is an acute-phase reactant also deserves consideration and atherosclerosis bears similarities to an inflammatory process. Elevated levels of fibrinogen might be an indicator of an underlying low-grade inflammation, the cause of which remains unclear, but may be initiated by various stimuli like oxidized LDL, several cytokines, oxygen free radicals, other factors, and possibly, but not very likely by chronic infections. [8, 23, 138] The "inflammation hypothesis" is further supported by the fact that a variety of other systemic markers of inflammation are also related to vascular diseases. [78] Interestingly, in the ARIC Study, in which a large array of hemostatic parameters was investigated prospectively, only those which were also acute-phase proteins, turned out to be independent predictors of CHD. [40]



**Figure 7.** Plasma fibrinogen, thrombogenesis and atherogenesis

### 1. 4. 2. 3. Clinical observations

#### Epidemiological evidence of an association with vascular disease

Several epidemiological studies have provided prospective data on plasma fibrinogen levels in relation to cardiovascular disease. According to these studies, the risk of

developing a cardiovascular event such as ischemic heart disease (IHD) or stroke is 1.8 to 4.1 times higher in subjects with fibrinogen levels in the top third than in those with levels in the lower third. [35] Preliminary evidence also suggests that reducing fibrinogen levels in patients with high baseline levels and coronary disease may be beneficial. [35]

A meta-analysis of the six prospective epidemiological studies with samples representative of the general population, concluded that plasma fibrinogen was an independent cardiovascular risk factor, the results being uniform despite the diversity of study designs, sample compositions, follow-ups and end-point criteria. [35] In this meta-analysis of 92 147 person-years experience, all prospective studies showed that plasma fibrinogen was associated with subsequent myocardial infarction or stroke. The odds ratio for the events in the upper vs. lower tertile varied between 1.8 (95% CI 1.2-2.5) in the Framingham study and 4.1 (95% CI 2.3-6.9) in the Gottingen risk incidence and prevalence study, with a summary odds ratio of 2.3 (95% CI 1.9-2.8). Furthermore, there was uniform, continuous increase in risk from the lowest to highest tertile. Plasma fibrinogen was associated with "true" risk factors such as diabetes mellitus, hypertension and hypercholesterolemia in the studies included in this meta-analysis. However, even when these factors were included in the multivariate analysis, the association between plasma fibrinogen and cardiovascular disease remained statistically significant, suggesting that fibrinogen is an independent cardiovascular risk factor.

In another meta-analysis, which included 22 studies (13 prospective, 5 cross-sectional, and 4 case-control) trying to determine the role of fibrinogen as a cardiovascular risk factor, the overall estimate of risk of cardiovascular events in subjects with plasma fibrinogen levels in the higher tertile, was twice as high as that of subjects in the lower tertile (OR 1.99; 95% CI 1.85-2.13). [88] High plasma fibrinogen levels were associated with an increased risk of cardiovascular disease in healthy as much as in high-risk individuals.

Thus, there is strong and unequivocal evidence from epidemiological studies that plasma fibrinogen levels are independently related to the presence of, and the subsequent development of vascular disease.

## Fibrinogen and carotid artery disease

Fibrinogen levels correlate with the number of coronary and extracoronary vascular beds involved in atherosclerosis. Whereas the association of fibrinogen with coronary artery disease is thus well established, it is less clear for other vascular diseases.

In a population sample of adults free of clinically overt atherosclerotic disease, Paramo et al. found that an elevated plasma fibrinogen concentration was related to carotid IMT, a surrogate marker of atherosclerosis, independently of a wide range of important confounding variables, confirming previous data in a smaller population. [115] A significant relationship was also detected when fibrinogen was compared to the presence of carotid plaques. [48, 89, 115, 125] Carotid plaques of hyperfibrinogenemic patients were shown to have a different morphological aspect with higher numbers of inflammatory cells and macrophage foam cells, a thinner fibrous cap, and a higher incidence of a rupture and thrombosis. [89]

Fibrinogen levels predict restenosis after angioplasty. [100, 145] Preinterventional fibrinogen levels and other acute-phase reactants, as signs of a chronic inflammatory process, are more predictive than postinterventional levels. [145]

### **1. 4. 3. Thrombomodulin**

#### **1. 4. 3. 1. Structure and function**

Thrombomodulin (TM) is composed of five structural domains. Extending from a short cytoplasmic tail and transmembrane domain is a serine/threonine-rich region to which a chondroitin sulfate moiety that optimizes anticoagulant function is attached. [108] Next is a domain that consists of six epidermal growth factor (EGF)-like repeats, four of which are responsible for the protein's anticoagulant and antifibrinolytic functions. [74, 156] The NH<sub>2</sub>-terminal domain has two modules. The first, adjacent to the EGF-like domain, is an ~70-amino acid residue hydrophobic region. The second, which is ~155-amino acid residues long, has homology to C-type lectins, which in many proteins participate in immune and inflammatory processes. [30, 121]

Thrombomodulin is mostly located on endothelial cells of arteries, veins, and capillaries attached at the carboxy-terminal end of the molecule. [140] There is, however, a small amount of circulating, soluble thrombomodulin. [61] Soluble thrombomodulin (sTM) is not only a parameter of endothelial cell destruction itself but also in particular an early marker of initial endothelial cell membrane changes induced by neutrophil-derived proteases and oxygen radicals. [85] In vitro data provide evidence for sTM as an early indirect parameter of disease activity in vascular diseases with endothelial cell injury. [62]

The complex regulation of TM underlines its importance in a wide variety of pathophysiologic conditions and biological systems. [37] Thrombomodulin is transcriptionally up-regulated by thrombin, vascular endothelial growth factor, histamine, dibutyryl cAMP, retinoic acid, theophylline, heat shock, and statins, whereas shear stress, hemodynamic forces, hypoxia, oxidized LDL, and TGF- $\beta$  will suppress TM gene expression. [169] Although TNF- $\alpha$  and IL-1 $\beta$  up-regulate macrophage expression of TM, these cytokines suppress TM in endothelial cells at transcriptional and posttranscriptional levels. [169] Recently, a polymorphism affecting TNF- $\alpha$  transcription has been identified in the promoter region of the gene, at nucleotide position -308. [178] The "TNF2" allele is a more powerful transcriptional activator than the common allele with a 6- to 7-fold increase in the inducible level of TNF- $\alpha$  gene transcription. [178] To our knowledge, the effect of TNF- $\alpha$  polymorphisms on sTM levels was not tested as yet.

#### **1. 4. 3. 2. Biological role of thrombomodulin**

Activation of protein C by thrombin-thrombomodulin

Activated protein C (APC) is a natural anticoagulant in that it suppresses further thrombin formation by proteolytically destroying coagulation factors Va and VIIIa, facilitated by the cofactor for APC, protein S (PS). Activated protein C also may increase fibrinolytic activity by neutralizing plasminogen activator inhibitor 1 (PAI-1).

The role of APC extends beyond hemostasis. [67] Activated protein C has potent anti-inflammatory properties. This molecule directly dampens inflammation by

inhibiting monocyte/macrophage expression of tissue factor and tumor necrosis factor (TNF)- $\alpha$ , nuclear factor (NF)- $\kappa$ B translocation, cytokine signaling, TNF- $\alpha$ -induced up-regulation of cell surface leukocyte adhesion molecules, and leukocyte-endothelial cell interactions. [136] Many of these protective effects of APC are mediated by proteolytic cleavage of protease-activated receptor 1. [136]

Protein C is transformed to its active form by thrombin-mediated cleavage of protein C at the N-terminus. Effective activation of protein C by thrombin requires TM, as a cofactor for thrombin, amplifying this event >1000-fold. When complexed with TM, thrombin has reduced procoagulant activity as exhibited by its reduced ability to cleave fibrinogen, activate factor V, and trigger platelet activation. Thus, thrombin's substrate specificity is entirely switched by TM.

#### Activation of thrombin-activatable fibrinolysis inhibitor by thrombin-thrombomodulin

Thrombomodulin is also a cofactor for thrombin-mediated activation of TAFI. [4] TAFI (thrombin-activatable fibrinolysis inhibitor) is a plasma procarboxypeptidase B that, when activated to TAFIa, catalyzes the removal of the C-terminal basic amino acid residues Lys and Arg. Inhibition of fibrinolysis is accomplished by removal of Lys residues from modified fibrinogen, which impedes the conversion of plasminogen to plasmin. [4] Although the *in vivo* significance of TAFIa as a regulator of fibrinolysis has not been clearly established, its potential role as a natural anti-inflammatory molecule is currently being explored, with recognition of its ability to inactivate the potent anaphylatoxins C3a and C5a and the proinflammatory mediators bradykinin and osteopontin. [105]

#### Thrombomodulin and inflammation

The lectin-like domain of TM was demonstrated to have direct anti-inflammatory properties, conferring protection by interfering with neutrophil adhesion to endothelial cells. [20] Recent studies further suggest that the lectin-like domain of TM may be important to maintain the integrity of cell-cell interactions, and thus might also prevent leukocyte transmigration. [20]

## Thrombomodulin and cell proliferation

Thrombomodulin may also modulate pathological alterations of the vessel wall that occur in restenosis or vein graft atherosclerosis. [70, 160, 177] Retroviral TM delivery to mechanically dilated rabbit femoral arteries reduces thrombus burden, neointima formation, inflammatory cell infiltration, and matrix degradation. [177] In part, these effects appear to be due to a TM-dependent inhibition of smooth muscle cell proliferation, possibly by modifying thrombin-receptor-dependent intracellular signaling processes. [46, 77] The antiproliferative effect of TM is also observed in endothelial cells, where TM modifies thrombin-receptor-dependent intracellular signal transduction by the MAP-kinase/ERK (externally regulated kinases) pathway. [113] In contrast, recombinant soluble TM consisting only of the six EGF domains (thus lacking the lectin-like domain) enhances proliferation of fibroblasts and smooth muscle cells through an unknown mechanism. [53, 160]

### **1. 4. 3. 3. Studies of use of thrombomodulin in clinical practice**

#### Prediction of vascular diseases using soluble thrombomodulin values in plasma

Clinical cross-sectional studies analysing the association of plasma TM levels and arteriosclerotic disease have remained ambivalent at best, yet plasma TM levels at baseline, which probably reflect the expression level of endothelial TM, are inversely correlated with the incidence of coronary artery disease (CAD). [10, 120, 142, 148, 149]

In the Atherosclerosis Risk In Communities study population, the relationship between sTM and coronary heart disease (CHD) was evaluated prospectively in a nested case-cohort study. The initial Atherosclerosis Risk In Communities study population was examined on a 3-year cycle four times. In addition, the development of CHD since the initial examination was monitored. In this report, 449 cases of CHD and 753 random cohort samples were analysed. The results show an inverse association between sTM and CHD events. After adjustment for age, sex, race and conventional CHD risk factors, the relative risk of CHD events in participants with sTM at the highest quintile was 0.31 (95% CI 0.14-0.69) of those at the lowest quartile. [142]

It is not clear from this and other studies whether the protective effect on arteriosclerosis is mediated by augmented surface-associated TM, or is related to the anti-inflammatory effects conferred by soluble TM. [2, 20, 99, 168]

#### Association between soluble thrombomodulin and carotid artery disease

Only a few clinical studies exist on sTM and atherosclerosis. Most of them have examined peripheral artery disease (PAD), but the results have been conflicting. Some studies have reported a positive association, while others have failed to find any association. [12, 120, 149, 151]

The relation between sTM and carotid IMT has been studied cross-sectionally by Salomaa et al., who reported a positive association between these two variables among 803 white subjects. [142] Fujiwara et al. found cross-sectional positive correlations between sTM and plaque score in diabetic patients compared to 72 control subjects. [41] Ramsis et al. found an independent positive association of sTM with carotid atherosclerosis, defined as a mean carotid IMT >1.1 mm in 36 healthy males. [124] Other cross-sectional studies concerning the relation between sTM and carotid stenosis showed either no, or a positive relation. [10, 64, 120, 121, 149]

## 2. Objectives

### 1.

Although, results of epidemiological studies demonstrated an association between low-grade inflammation and vascular risk, application of CRP testing in clinical practice requires estimates of risk across a spectrum of CRP levels. However, distribution of CRP is rightward skewed such that clinical application will likely require recasting measured CRP levels into an ordinal system. A useful approach to this problem is to rank CRP values into population based quintiles.

*Application of this quintile approach to CRP testing requires knowledge of the population distribution of CRP. Therefore we aimed to determine the distribution of CRP in a healthy Hungarian population.*

### 2.

At present, carotid endarterectomy is considered a proven treatment modality for the prevention of stroke in both asymptomatic and symptomatic patients with hemodynamically significant stenoses. The intermediate and long-term durability of the procedure may be affected by the incidence of restenosis due to either myointimal hyperplasia or recurrent atherosclerosis. The incidence of recurrence has been quite variable, ranging from <2% to as much as 36% in the literature. [43, 52, 68, 101, 139, 183]

*Our study had as one of its primary objectives to define the incidence of restenosis after eversion carotid endarterectomy in a prospective study at our Department.*

### 3.

Endarterectomy is followed by a long-lasting healing and remodeling process. Vascular repair and remodeling is a very complex phenomenon that involves a local intense inflammatory response, smooth muscle cell proliferation and migration, extracellular matrix production and contraction. Thus it may be considered the vascular manifestation of a general biological response to tissue injury, reflecting the systemic wound-healing process. In this light it has recently been suggested that systemic and local inflammatory

states may have an important role in the functional and organic changes that characterize vessel remodeling after vascular surgery.

*To investigate whether changes in systemic inflammatory status may be associated with vascular healing and remodeling after elective carotid endarterectomy we followed-up for 14 months not only the clinical status of the patients after CEA, but we have measured serum CRP and plasma fibrinogen levels before operation, and 4 days, 6 weeks and 14 months postsurgery as well.*

#### 4.

Soluble thrombomodulin is a circulating marker of endothelial damage. [85] Plasma levels of sTM are related to carotid atherosclerosis; [41, 142] however, to our knowledge, their predictive value on restenosis after carotid endarterectomy has not been studied to date.

*Therefore, we hypothesized that postsurgical plasma levels of sTM would be associated with recurrent lumen narrowing after carotid eversion endarterectomy.*

#### 5.

Increasing evidence shows that inflammatory mediators play a major role in determining the degree of plaque inflammation and contributing to its evolution from uncomplicated to complex atheroma. [80, 112, 138] In this complex inflammatory network of mediators, TNF- $\alpha$ , a proinflammatory cytokine with pleiotropic biological effects, appears to be a critical candidate. Recently, TNF- $\alpha$  expression and production have been demonstrated to be directly affected by a polymorphism at nucleotide position -308 in the promoter region of the human gene. [178] TNF- $\alpha$  has been shown to have a suppressive effect on TM expression of endothelial cells. [169]

*For these reasons, we thought to investigate the effect of TNF- $\alpha$  polymorphism on sTM levels in patients with severe carotid artery stenosis and restenosis.*

### **3. Patients and methods**

#### **3. 1. Study I. (Distribution of C-reactive protein levels determined by ultrasensitive method in a healthy Hungarian population)**

The study group consisted of 207 apparently healthy volunteer blood donors. The median age was 46 years, and the interquartile range (IQR) was 40-52 years. Seventy-nine (38%) of 207 patients were men and 128 (62%) were women.

#### **Clinical and laboratory data of the subjects**

All participants underwent baseline clinical examinations, which included medical history, physical examination, ECG, chest X-ray and laboratory tests. From each patient, the following clinical data were obtained: age, gender, body mass index (BMI), smoking habits, diabetes mellitus and blood pressure. Serum and blood samples were assayed for CRP, total cholesterol, triglycerides, erythrocyte sedimentation rate, hemoglobin and for white blood cell count. (Definitions, laboratory and statistical methods see later on.)

#### **3. 2. Study II. (Determination of the serum and plasma levels of two inflammatory markers, CRP and fibrinogen before and after eversion carotid endarterectomy; Changes in the plasma concentration of soluble thrombomodulin after eversion endarterectomy in patients with severe carotid artery stenosis)**

In this prospective study (depending on the inclusion and exclusion criteria), we enrolled 117 patients admitted to our Department with severe internal carotid artery stenosis between 2002 and 2003. The study was performed in accordance with the Declaration of Helsinki and was approved by the local Ethics Committee. All the participants provided informed consent.

## **Inclusion and exclusion criteria**

Patients with symptomatic stenosis in the ICA (internal carotid artery) greater than 70% according to the Carotid Endarterectomy Trialists' criteria and quantified angiographically and asymptomatic patients with stenosis greater than 80% were eligible for inclusion.

We excluded patients who were severely disabled as a result of stroke or dementia, patients with acute metabolic dysfunction or renal insufficiency who were not undergoing dialysis. None of the participants had liver or hematologic diseases. Patients with intercurrent inflammatory, infectious, or neoplastic conditions associated with an acute-phase response were also excluded. No patients with acute (Fontaine stage III) or chronic (Fontaine stage IV) critical limb ischemia, with severe cardiac disease, or with acute stroke were eligible for eversion carotid endarterectomy during the study period.

## **Follow-up**

Patients had medical check-up (questionnaire, physical and ultrasound examination) at 5.7 (4.6-8.0) weeks [median (interquartile range)], 6.8 (6.2-7.9) months, and finally 13.8 (12.3-19.0) months after the operation. Blood samples were drawn preoperatively, 4 (1-6) days, 5.7 (4.6-8.0) weeks after the surgery, and finally 13.8 (12.3-19.0) months postsurgery.

## **Patients' data**

At admission, the patient's medical history and data from the physical examination were recorded by using a standard questionnaire. Routine laboratory test values, urinalysis results, and chest radiographic findings were used to exclude coexistent inflammatory diseases. Clinical history and physical examination were evaluated with special attention to cardiovascular risk factors and comorbidities as follows: age; gender; current history of smoking; presence of dyslipidemia, arterial hypertension, diabetes mellitus, peripheral and coronary artery disease; history of cerebrovascular events;

current use of medications; and prior ipsilateral or contralateral eversion endarterectomy or stent implantation in the carotid artery.

## **Definitions**

Dyslipidemia was defined as an elevation of LDL-cholesterol above 3.0 mmol/L or total cholesterol greater than 5.0 mmol/L, and it was considered to be present in all patients receiving lipid-lowering medication.

Arterial hypertension was diagnosed in patients with resting blood pressure values above 140/90 mmHg measured repetitively (at least twice) and was assumed to be present in patients taking antihypertensive drugs.

A subject was considered diabetic if he or she reported medical history of diabetes mellitus, or use of antidiabetic drugs, or who had a fasting blood glucose level greater than 5.83 mmol/L, in patients with oral glucose tolerance test results suggestive of disease, and in patients with a glycated hemoglobin A<sub>1C</sub> level greater than 6.2%.

Peripheral artery disease was evaluated with clinical history, ankle-brachial index measurements, and angiographic findings and was classified as present (Fontaine stage I or II) or absent.

Coronary artery disease was categorized according to the Canadian Cardiovascular Society classification, and routine evaluation included stress exercise testing, myocardial scintigraphy, and coronary angiography in selected cases.

Neurologic events were categorized as transient ischemic attacks, minor stroke, and major stroke. Stroke was defined as a neurological deficit that persisted longer than 24 hours evaluated by a neurologist according to the modified Rankin stroke scale.

## **Duplex ultrasound examination**

All carotid duplex scans were performed by a single radiologist in a Vascular Laboratory using the colour scanner and a 7.5 MHz probe (Toshiba, Aplio, Model SSA-700A). The common, internal and external carotid arteries on both sides were examined in the standard fashion. [31] The carotid waveforms, peak systolic and end diastolic velocities were recorded for the internal carotid artery and spectral measurements were

taken with a Doppler angle of 50° to 60°. The diagnostic criteria for internal carotid artery stenosis was based on peak systolic and end diastolic velocities as well as internal carotid artery:common carotid artery velocity ratios and modified to detect carotid stenosis at the 70% level, in accordance with the recommended intervention threshold from the Carotid Endarterectomy Trialists. [31, 139] The stenoses and restenoses were further categorized as mild (30-49%), moderate (50-69%), severe (70-99%) and total occlusion. An internal carotid artery stenosis of 70% or greater was regarded as hemodynamically significant.

### **Angiographic evaluation**

The preoperative internal carotid artery stenosis on the angiograms was determined by NASCET (North American Symptomatic Carotid Endarterectomy Trial) criteria. [139] % ICA stenosis =  $[1 - (\text{narrowest ICA diameter} / \text{normal distal cervical ICA diameter})] \times 100\%$ .

### **Carotid eversion endarterectomy**

The procedures were performed with the patient under deep general anesthesia by experienced vascular surgeons. All CEAs were carried out using intravenous heparin (10 000 U) before carotid cross-clamping; blood pressure was maintained at average preoperative levels or slightly higher. The action of heparin was suspended with protamine. Completion imaging was never performed. Only one patient had postoperative complication (recurrent laryngeal nerve injury). Patients were discharged 72 to 96 hours after CEA.

### **Laboratory tests**

Venous blood was sampled using the standard venepuncture method, from an antecubital vein between 8.00 a.m. and 10.00 a.m. after an overnight fast. Venous blood was taken into vacutainer tubes containing no anticoagulant or with EDTA, or sodium citrate (Becton Dickinson, Vacutainer Systems, Plymouth, UK). All samples were

separated by centrifugation within 15 minutes at 2500 g for 20 minutes (4°C). The processed plasma and serum samples were aliquoted and stored at -70°C until analysis. Plasma fibrinogen concentrations were promptly determined. Simultaneously, serum was prepared and sent to our Laboratory for analysis of C-reactive protein, total cholesterol, triglycerides, HDL- and LDL-cholesterol.

## Lipids

Serum concentrations of total cholesterol and triglycerides (Roche/Hitachi), HDL- and LDL-cholesterol (Human, Wiesbaden, Germany) were measured in a Cobas Mira Plus (Diagnostics Basel, Switzerland) clinical chemistry analyser. The intra-assay coefficients of variation were 1.2%, 1.4%, 4.8% and 4.5%, respectively.

## C-reactive protein

C-reactive protein concentrations were measured in serum samples with the ultrasensitive particle-enhanced immunoturbidimetric assay in Cobas Mira Plus (Diagnostics Basel, Switzerland) analyser. The working range of the assay was 0.07 to 1100 mg/L, with intra-assay variation coefficients ranging from 0.6% to 1.3% and interassay variation from 1.3% to 6% at different levels of CRP.

## Fibrinogen

Plasma fibrinogen levels were promptly determined in citrate anticoagulated samples by using the method of Clauss (ST4 BIO, STAGO, Asnières sur Seine, France). The detection level of fibrinogen was 20 mg/dL. The intra- and interassay coefficients of variation for the fibrinogen assay were 4.0% and 5.5%, respectively.

## Thrombomodulin

Soluble TM concentrations were measured in citrated plasma stored at -70°C until assay. A prototype two-site ELISA (enzyme-linked immunosorbent assay) was used for the

determination of sTM. The test was performed according to the manufacturer's instructions [sCD141 (Thrombomodulin) ELISA kit (Diacalone, France)]. The intra-assay and the interassay coefficients of variation were 6.0% and 8.2%, respectively. The precoated 96-well plates were washed and incubated for one hour at room temperature with the diluted plasma samples in duplicates (50  $\mu$ L supernatant and 75  $\mu$ L sample buffer) or the provided standards. After washing the plates were further incubated with the peroxidase-conjugated secondary anti-TM antibody (100  $\mu$ L/well) for the next hour and finally with the substrate solution (tetramethylbenzidine; TMB) at room temperature in the dark for 10 minutes. With exception of the last step plates were washed between each incubation step. Finally, the reaction was stopped with 0.5M H<sub>2</sub>SO<sub>4</sub> and the optical density was measured after 30 minutes of colour stabilisation by an automated ELISA plate reader at 450 nm (Titerek Multiscan Plus MKII; ICN/Flow, Meckenheim, Germany).

#### TNF- $\alpha$ gene promoter polymorphism

Genomic DNA was extracted from peripheral blood leukocytes collected in EDTA using the method of Miller and co-workers. [97] TNF- $\alpha$  polymorphism was detected using primers containing a single base-pair mismatch adjacent to the polymorphic site in order to introduce a restriction site into the wild-type nucleotide sequences after amplification. Two primers were prepared: A1 = 5'-ATCTGGAGGAAGCGGTAGTG and M1 = 5'-AATAGGTTTTGAGGGCCCATG [contains a mismatch (underlined) corresponding to G at position -313]. Primers A1 and M1 were used to amplify fragments containing the -308 polymorphism. DNA samples were amplified in 50  $\mu$ L of KCl reaction buffer (Bioline, London, UK) containing 200  $\mu$ mol/L dNTP, 0.25  $\mu$ mol/L primer(s), 1  $\mu$ g of DNA sample and 2 units of Taq polymerase (Bioline) for 35 cycles at 94°C for 1 minute, 59°C for 1 minute and 70°C for 45 seconds followed by 1 cycle at 70°C for 10 minutes. The PCR products were digested at 37°C with NcoI (restriction enzyme) to detect the -308 polymorphism and were examined by 10% acrylamide gel electrophoresis.

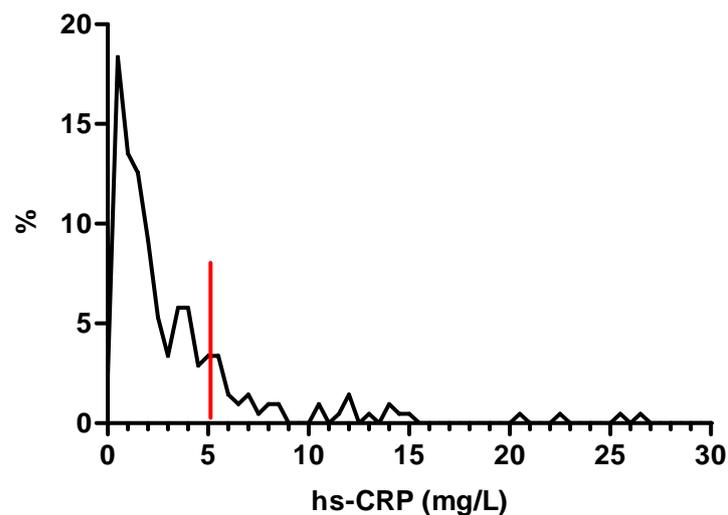
## **Statistical analysis**

Data were collected in MS Excel 2002 and were mostly analysed with the Statistica 6.0 version for Windows statistical package (StatSoft Inc., Tulsa, OK, USA, [www.statsoft.com](http://www.statsoft.com)) and with GraphPad Prism V 3.0 software package (GraphPad Software Inc., San Diego, California, USA, [www.graphpad.com](http://www.graphpad.com)). Two-group comparisons were done with the Mann-Whitney U test, difference in the values within a group over a period of time was measured by nonparametric ANOVA (Kruskal-Wallis multiple comparison test); comparison of categorical variables was calculated with Fisher exact test. Multiple regression analysis was done by the SPSS 10.0 software (SPSS Inc., Chicago, IL, USA, [www.spss.com](http://www.spss.com)). When evaluating a possible relationship between the preoperative duplex scan values and sTM levels, Spearman rank correlation coefficient was calculated. Data are presented as mean±S.D. or median (interquartile range) for skewed variables. A value of  $p < 0.05$  was considered statistically significant.

## 4. Results

### 4. 1. Distribution of C-reactive protein levels determined by ultrasensitive method in a healthy Hungarian population

Elevated C-reactive protein concentration is a suggested risk marker for cardio- and cerebrovascular diseases. We aimed at investigating the distribution and determinants of CRP levels in a Hungarian population. Using blood samples from 207 apparently healthy Hungarian blood donors the mean CRP concentration was  $3.57 \pm 5.33$  mg/L (mean $\pm$ S.D.). Table 2 shows the median value and the Figure 8 represents the distribution of CRP in a healthy Hungarian population. It appears, that in 81% (n=168) of the subjects the serum CRP levels were less than 5 mg/L.



**Figure 8.** CRP distribution profile in a healthy Hungarian population

### Comparison of CRP distribution profiles in a Hungarian and an American population

In the literature there is no data about the CRP distribution profiles in the Central or East European countries. We compared our results to the data of a North American study published by Ridker et al., which is one of the most carefully documented paper

in this field. [129] A Hungarian CRP distribution was similar to an American one, but we noted slightly higher CRP values in the third, fourth and fifth quintiles. An American representative population distribution profile of CRP was based on analysis of more than 5000 people (nonwhite subjects were excluded) without apparent cardiovascular disease. In this survey, median CRP level was 1.6 mg/L and ranges of CRP for those with lowest (quintile 1) to highest (quintile 5) vascular risk were 0.1 to 0.6, 0.7 to 1.1, 1.2 to 1.9, 2.0 to 3.7, and >3.8 mg/L. [129] In a Hungarian population the median CRP level was 1.87 mg/L, and the quintiles were the following: 0.05 to 0.73, 0.74 to 1.50, 1.51 to 2.56, 2.57 to 4.77, and >4.78 mg/L, respectively. (Table 2)

**Table 2.** Ranges of CRP in a healthy Hungarian and an American population

Quintile (mg/L)	Hungarian population (n=207)	American population (n>5000) [129]
1: 1%-20%	0.05-0.73	0.1-0.6
2: 21%-40%	0.74-1.50	0.7-1.1
3: 41%-60%	1.51-2.56	1.2-1.9
4: 61%-80%	2.57-4.77	2.0-3.7
5: 81%-100%	>4.78	>3.8
Median (mg/L)	1.87	1.6

### **Differences in clinical data and laboratory parameters in patients with low or high CRP levels**

We compared the apparently healthy blood donors with equal or less (n=168) and with higher than 5 mg/L CRP levels (n=39) in respect of age, gender, body mass index (BMI), smoking habits, diabetes mellitus, blood pressure, serum total cholesterol and triglycerides concentrations, erythrocyte sedimentation rate, hemoglobin values, white blood cell and neutrophil count. There was a significant difference between the two groups in BMI (p=0.0015), total cholesterol (p=0.0136), triglycerides (p<0.0001), erythrocyte sedimentation rate (p<0.0001), white blood cell count (p<0.001) and absolute neutrophil count (p=0.001). The number of smokers were higher among those with high C-reactive protein levels (the difference was not statistically significant). When we compared the smokers (median: 1.81 mg/L) and the nonsmokers C-reactive protein concentrations (median: 2.12 mg/L) the difference was also not significant

(p=0.839). Gender distribution did not differ between groups of subjects with low or high CRP values (p=0.169; p=0.153). (Table 3)

There was a marginally significant difference (p=0.075) between age of individuals with low or high CRP concentrations, therefore further evaluations were done: based on their age the volunteer blood donors were divided into four groups. It turned out that the CRP concentrations were significantly higher with the advancement of age. (Table 4) A significant correlation was noted between age and CRP levels (R=0.22, p=0.001). There was also a significant positive correlation between C-reactive protein concentrations and BMI (R=0.373, p<0.0001), total cholesterol (R=0.198, p=0.005), triglycerides (R=0.361, p<0.0001), white blood cell (R=0.296, p<0.0001) and absolute neutrophil count (R=0.269, p<0.0001).

**Table 3.** Clinical data and laboratory parameters of subjects with low or high serum CRP levels

Parameters	CRP ≤5 mg/L (n=168)	CRP >5 mg/L (n=39)	p value*
Age (years)	48 (38.5-54)	52 (47-55)	0.075
Gender (male/female)	68/100	11/28	0.200
BMI (kg/m <sup>2</sup> )	24.6 (22.3-28.1)	27.7 (25.6-30.0)	<0.001
Smokers (%)	25	29	0.660
Smoking (cigarette/day)	12 (9-20)	20 (10-20)	0.240
Diabetes mellitus (%)	0	0	-
Systolic blood pressure (mmHg)	131 (120-141.5)	127 (120-147)	0.920
Diastolic blood pressure (mmHg)	76 (57-84)	80 (75.5-86)	0.051
Total cholesterol (mmol/L)	5.49 (4.76-6.18)	5.86 (5.32-6.55)	0.014
Triglycerides (mmol/L)	1.15 (0.81-1.70)	1.77 (1.27-2.19)	<0.0001
Erythrocyte sedimentation rate (mm/h)	12 (6-20)	22 (15-37)	<0.0001
Hemoglobin (g/L)	139 (130-148)	137 (132-148)	0.650
White blood cell count (G/L)	6.47 (5.40-7.43)	7.36 (6.31-9.12)	<0.001
Neutrophil count (%)	59 (54-64.5)	61 (56-63.5)	0.207
Absolute neutrophil count	3.7 (3.04-4.52)	4.38 (3.71-5.45)	<0.001

\*P values were calculated by the Mann-Whitney U test and Fisher exact test (in case of categorical variables).

**Table 4.** Age distribution of CRP concentrations

	Age (years)			
	20-39	40-49	50-59	60-79
n	46	66	74	21
CRP (mg/L) median (25th-75th percentile)	1.41 (0.46-3.38)	1.69 (0.89-4.56)	2.26 (1.24-4.67)	2.50 (1.16-4.78)

## **4. 2. Determination of the serum and plasma levels of two inflammatory markers, CRP and fibrinogen before and after eversion carotid endarterectomy**

### **Comparison of the baseline CRP and fibrinogen levels measured in patients and healthy subjects**

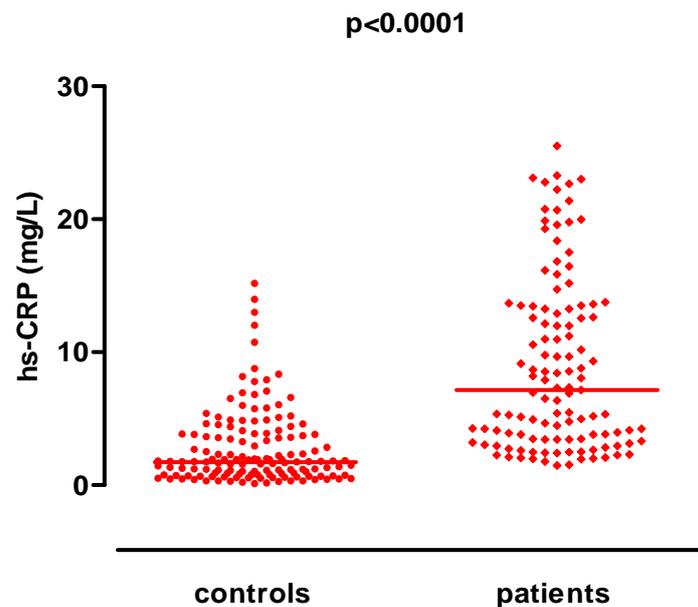
It has recently been suggested that systemic and local inflammatory molecules may have an important role in the functional and organic changes that characterize vessel healing and remodeling after invasive therapeutic procedures. To investigate whether changes in systemic inflammatory status may be associated with remodeling after vascular surgery, we chose the model of the internal carotid artery subjected to elective endarterectomy.

Within the study period, 117 patients were scheduled for eversion carotid endarterectomy. The median age was 64 years, and the IQR, from the 25th to the 75th percentile, was 56-76 years. Seventy-eight (67%) of 117 patients were men (median age, 69 years; IQR, 63-76 years) and 39 (33%) were women (median age, 71 years; IQR, 64-75 years). One hundred and forty-five healthy subjects (81 males, 64 females; median age, 53 years; IQR, 45-60 years) who volunteered for a regular medical check-up and gave informed consent served as control.

Serum levels of CRP were determined in 145 healthy controls and in the preoperative blood samples from 117 patients. Preoperative samples were taken immediately after the admission of the subjects, before the preoperative medication was started. The patients had significantly higher ( $p < 0.0001$ ) median preoperative CRP levels [7.90 (3.45-13.65) mg/L] than the healthy controls [1.93 (1.09-4.07) mg/L]. (Figure 9) Since there were significant differences in the gender distribution and mean age of the patients and controls we calculated by multiple logistic regression if the difference in the CRP level between the two groups remained significant after adjustment for age and gender. A significant difference ( $p < 0.0001$ ) in the CRP concentration was found between patients and controls even after these adjustments.

Since plasma fibrinogen values were not available for the group of healthy subjects, we used laboratory reference values for the evaluation. Fibrinogen levels exceeding the upper limit of the reference values (400 mg/dL) were found in 68 patients, that is in 58% of the patients tested.

Thirty-seven patients had no symptoms, 61 patients had transient ischemic attack (TIA), 16 and 3 patients had minor and major stroke, respectively, before operation. There were no significant differences between asymptomatic patients and those with preoperative symptoms (TIA or stroke) in the CRP ( $p=0.6025$ ) or fibrinogen levels ( $p=0.7302$ ) measured before operation or CRP and fibrinogen levels measured at the end of the observation period ( $p=0.8768$  and  $p=0.4419$ , respectively).



P value was calculated by the Mann-Whitney U test.

**Figure 9.** Levels of C-reactive protein in serum samples from 117 patients taken before operation and from 145 healthy controls

### **Mortality and complications during the follow-up period**

Seven patients died before the end of the follow-up period. The cause of death was acute myocardial infarction in 4 patients. One patient committed suicide, one patient died due to pulmonary embolism and one in cancer. There was no significant difference ( $p=0.2525$ ) in the preoperative CRP levels of the 4 patients who subsequently died in myocardial infarction [4.19 (2.53-6.29) mg/L] and the whole group of patients [7.06 (3.08-13.65) mg/L].

During the 14 months follow-up none of the patients developed stroke, but one patient underwent coronary artery bypass surgery and 2 patients had revascularization on the lower limb. Most carotid restenoses were asymptomatic and hemodynamically nonsignificant. Internal carotid artery stenosis of 50% or greater was detected in 15 patients (13%), but only 4 patients (3%) had severe ( $\geq 70\%$ ) restenosis in the operated region. Two patients who had early recurrent stenosis were assigned for carotid stenting. (Carotid stent placement for recurrent carotid artery stenosis was indicated for patients with restenosis if diameter reduction was  $\geq 80\%$  in the ICA.)

### Early changes in the fibrinogen and CRP levels after the surgery

Levels of the two acute-phase proteins were determined before operation, 4 (1-6) days and 5.7 (4.6-8.0) weeks postsurgery in 90 patients. (Table 5)

There was a sharp, highly significant rise in the serum and plasma concentrations of both proteins during the acute postsurgery period which returned to the baseline values at the first follow-up visit.

**Table 5.** Changes in the serum and plasma concentrations of CRP and fibrinogen during the early phase of the postsurgery period in 90 patients

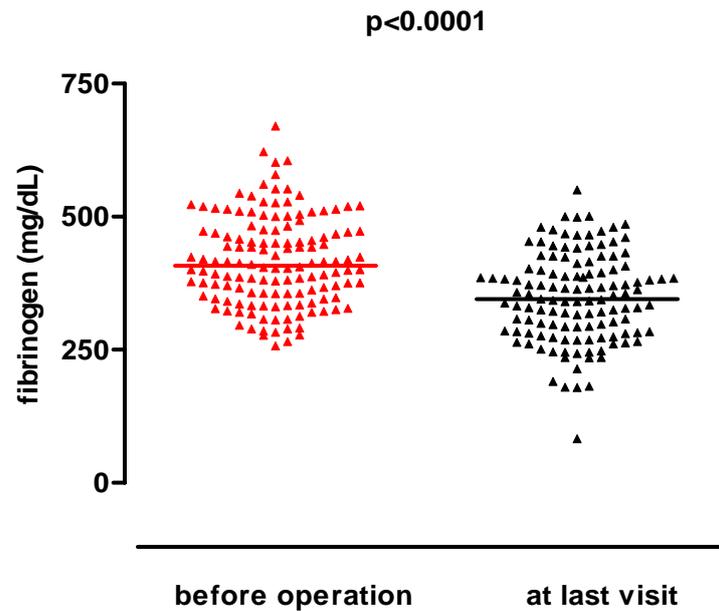
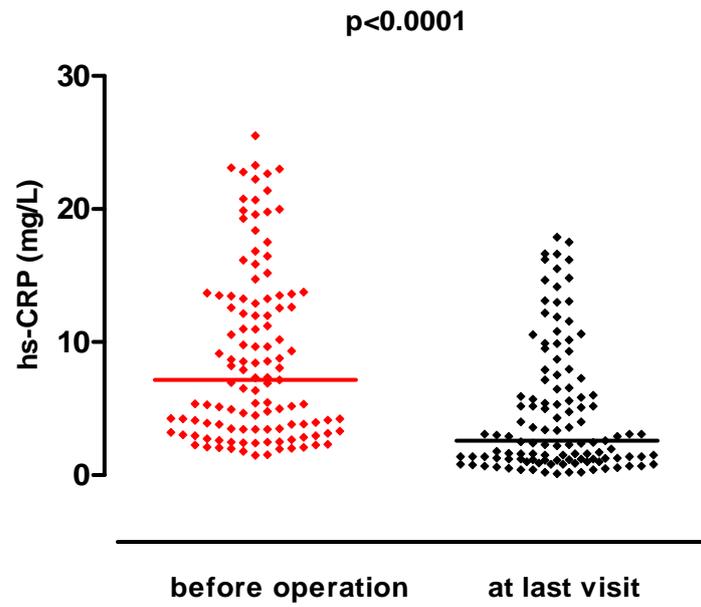
	Before operation (BO)	4 days postsurgery	p values* (compared to the BO values)	5.7 (4.6-8.0) weeks postsurgery	p values* (compared to the 4 days values)
CRP (mg/L) median (interquartile range)	6.44 (2.91-13.38)	17.44 (10.79-29.58)	<0.001	4.31 (2.05-8.56)	<0.001
Fibrinogen (mg/dL) median (interquartile range)	416 (356-474)	511 (452-581)	<0.001	387 (333-466)	<0.001

\*Dunn post hoc test was used after repeated measures ANOVA (Friedman test).

### **Changes in the CRP and fibrinogen levels during the follow-up period**

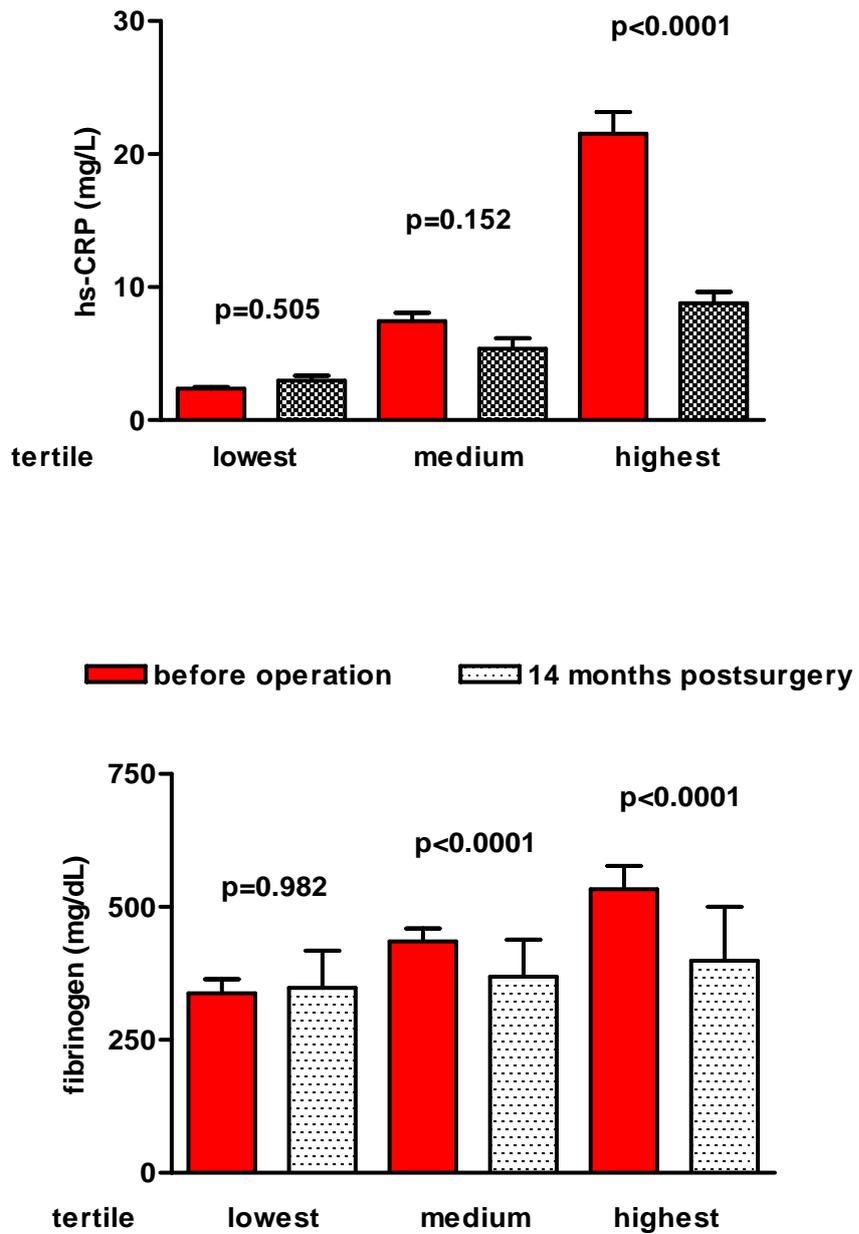
Concentrations of the acute-phase proteins were measured before the operation and at the end of the follow-up period, that is 13.8 (12.3-19.0) months after endarterectomy (in the followings: at final visit) in all patients (n=117). (Figure 10) Both CRP and fibrinogen levels markedly decreased ( $p<0.0001$ ) during the follow-up period. Serum CRP levels decreased from 7.90 (3.20-14.25) mg/L measured preoperatively to 3.00 (1.23-7.93) mg/L at the last follow-up visit. Plasma fibrinogen levels were 410 (346-479) mg/dL and 352 (285-410) mg/dL, respectively.

When the patients were divided according to the tertiles of the baseline CRP and fibrinogen levels and the concentrations of the two acute-phase proteins measured before the operation and at the final visit were compared, it turned out that the drop of the CRP levels during the follow-up period was mainly due to the decrease in the highest tertile of the baseline levels. In case of fibrinogen highly significant drop occurred in both the medium and the highest tertile, while no changes were found in patients with plasma fibrinogen concentration in the lowest tertile. (Figure 11) We compared the extent of carotid stenosis in patients with CRP levels in the highest tertile and the rest of patients. Not only the operated side but the mean of the operated and the contralateral side were considered. There were, however, no difference between patients with CRP levels in the highest tertile and the rest of the patients either in the extent of carotid stenosis in the operated side ( $p=0.7157$ ) or the mean value of the two sides ( $p=0.4061$ ). Similarly, no difference was found in case of high fibrinogen levels.



P values for the Wilcoxon signed-ranks test were indicated.

**Figure 10.** Serum concentration of CRP and plasma concentration of fibrinogen in 117 patients before carotid endarterectomy and 13.8 (12.3-19.0) months postsurgery



P values for the Wilcoxon signed-ranks test were indicated.

**Figure 11.** Changes in the serum CRP and the plasma fibrinogen levels during the follow-up period as compared to the respective tertiles of the baseline levels of the two acute-phase proteins

## Dynamics of the changes in acute-phase protein levels

In 37 patients acute-phase protein levels were not determined at the first visit since they failed to appear. Nonetheless, all these patients attended the last follow-up visit. In 80 patients, however, CRP and fibrinogen measurements were made before operation, and at both 5.7 weeks and at the final visit which allowed to study the dynamics of changes in the levels of the two acute-phase proteins. (Table 6) C-reactive protein levels significantly decreased as compared to the levels measured before surgery as soon as at the first follow-up visit, and the values continued to drop till the final visit. Plasma fibrinogen concentrations did not change during 6 weeks after eversion endarterectomy but thereafter a significant decrease in this marker was noted, too.

**Table 6.** Serum and plasma concentration of CRP and fibrinogen before and after carotid endarterectomy

	Median (25th-75th percentile)			Friedman test, p value
	Before operation	5.7 weeks after the operation	13.8 months after the operation	
CRP (mg/L)	6.95 (3.00-13.55)	4.31 (2.19-9.26)*	3.01 (1.23-8.31)**	<0.0001
Fibrinogen (mg/dL)	416 (348-478)	380 (330-460)	371 (307-420)**	<0.0001

Dunn post hoc test was used after repeated measures ANOVA (Friedman test). Values were compared to the preoperative concentrations, \*p<0.01, \*\*p<0.001.

## Incidence of restenosis at the last follow-up visit

Based on literature data the patients were divided into two subgroups: 15 patients who developed restenosis of  $\geq 50\%$  during the follow-up formed the restenosis group, while the other 102 patients belonged to the no restenosis group. No difference was found in the restenosis rate between the symptomatic and asymptomatic patients.

Demographic characteristics, some laboratory test values and data from case history of the two groups are summarized in Table 7. There was no significant difference between the two groups in either parameter tested except serum total cholesterol values, which were significantly higher in the restenosis group.

Baseline CRP and fibrinogen levels were about the same in the two groups. (Table 7) As it was shown above, median levels of both acute-phase proteins significantly increased at day 4 postsurgery. C-reactive protein and fibrinogen concentrations measured 4 days after the surgery were not found to be related to restenosis. Comparison of fibrinogen concentrations, however, revealed a significant increase to baseline values in the restenosis group at day 4 postsurgery. No such increment was seen with CRP. In the no restenosis group neither fibrinogen, nor CRP increased significantly at day 4. Early postoperative increase in the plasma fibrinogen levels was associated with a higher risk of restenosis: we found a significant positive correlation ( $R=0.212$ ,  $p=0.0223$ ) between the early increase in the fibrinogen concentrations and the restenosis rate measured 14 months after the operation.

Since the two groups differed in the baseline total cholesterol concentrations, too, we calculated by multiple linear regression adjusted to the serum cholesterol levels, as well as gender and age of the patients the 14 months restenosis rate in patients with high and low early fibrinogen changes for restenosis in the follow-up period. A highly significant regression coefficient ( $B=12.66$ , S.E. of  $B=4.11$ ,  $t=3.08$ ,  $p=0.003$ ) was seen for the early fibrinogen changes even after these adjustments.

**Table 7.** Comparison of the baseline data of the two groups of patients who underwent carotid endarterectomy and did or did not develop restenosis over the follow-up period of 14 months duration

	Median (25th-75th percentile) for the continuous data		p value*
	Restenosis group (n=15)	No restenosis group (n=102)	
Age (years)	60 (55-74)	66.5 (58-72)	0.516
Gender (male/female)	8/7	70/32	0.685
Preoperative BMI (kg/m <sup>2</sup> )	25.39 (24.77-26.84)	25.60 (23.14-28.69)	0.748
Hypertension (yes/no)	11/4	91/11	0.541
Diabetes mellitus (yes/no)	7/8	30/72	0.677
Smoking (yes/no)	5/10	42/60	0.697
Total cholesterol (mmol/L)	7.13 (6.15-7.97)	6.08 (5.41-7.02)	0.047
Triglycerides (mmol/L)	2.49 (1.32-4.09)	1.97 (1.42-2.55)	0.370
HDL-cholesterol (mmol/L)	1.40 (1.00-1.80)	1.30 (1.00-1.43)	0.441
LDL-cholesterol (mmol/L)	3.20 (2.90-4.80)	3.10 (2.70-4.43)	0.476
CRP (mg/L)	3.84 (2.44-12.15)	8.00 (3.40-14.86)	0.304
Fibrinogen (mg/dL)	348 (317-416)	413 (350-482)	0.137
(CRP levels 4 days postsurgery) - (levels before operation) (mg/L)	5.29 (3.79-12.83)	9.90 (2.01-21.11)	0.290
(Fibrinogen levels 4 days postsurgery) - (levels before operation) (mg/dL)	122 (115-206)	76 (19-144)	0.035

\*P values were calculated by the Mann-Whitney U test and Fisher exact test (in case of categorical variables).

### **Long-term changes in the CRP and fibrinogen concentrations after carotid endarterectomy in patients with and without restenosis**

Changes in the serum CRP and plasma fibrinogen levels taken from the patients of the two groups are shown in Table 8 and 9, respectively.

There were sharp differences between the restenosis and the no restenosis groups in the changes of both inflammatory markers. Neither CRP nor fibrinogen levels changed significantly compared to the preoperative values till the end of the observation period in the restenosis group. By contrast, in patients with no restenosis CRP levels significantly decreased as compared to the levels measured before surgery already at the first follow-up visit, and the drop continued till the end of the follow-up. (Table 8) In

the same group, plasma fibrinogen concentrations did not change during 6 weeks after endarterectomy but thereafter a significant decrease in this marker was noted, too. (Table 9)

**Table 8.** Serum concentration of CRP (mg/L) in patients with or without restenosis before and after carotid endarterectomy

	Median (25th-75th percentile)			Friedman test, p value
	Before operation	5.7 weeks after the operation	13.8 months after the operation	
No restenosis (n=65)	7.31 (3.17-13.74)	4.36 (2.02-9.26)*	3.10 (1.33-9.01)**	<0.0001
Restenosis (n=15)	3.16 (2.15-11.26)	3.55 (3.09-13.92)	2.43 (1.06-10.98)	0.1561
All patients (n=80)	6.95 (3.00-13.55)	4.31 (2.19-9.26)*	3.01 (1.23-8.31)**	<0.0001

Dunn post hoc test was used after repeated measures ANOVA (Friedman test). Values were compared to the preoperative concentrations, \*p<0.01, \*\*p<0.001.

**Table 9.** Plasma concentration of fibrinogen (mg/dL) in patients with or without restenosis before and after carotid endarterectomy

	Median (25th-75th percentile)			Friedman test, p value
	Before operation	5.7 weeks after the operation	13.8 months after the operation	
No restenosis (n=65)	415 (346-506)	376 (324-460)	363 (291-397)*	<0.0001
Restenosis (n=15)	422 (359-478)	393 (322-486)	405 (314-457)	0.1860
All patients (n=80)	416 (348-478)	380 (330-460)	371 (307-420)*	<0.0001

Dunn post hoc test was used after repeated measures ANOVA (Friedman test). Values were compared to the preoperative concentrations, \*p<0.001.

### **Lack of medical treatment to influence changes in the fibrinogen level**

As it was expected for a group of patients with severe atherosclerosis, most patients received drugs which may affect fibrinogen levels, such as beta receptor blocking drugs (62 patients), ACE-inhibitors (86 patients), other antihypertensive drugs (18 patients),

statins (23 patients) or platelet aggregation inhibitors (94 patients). In most patients (99/117), however, no changes occurred postsurgery in the medical treatment compared to the preoperative period. When the two groups were compared no significant difference ( $p=0.6771$ ) was detected in the extent of the decrease in fibrinogen levels during the follow-up period.

In the whole study group 34 patients were under statin or fibrate therapy. In these patients the preoperative serum CRP concentrations were slightly lower ( $p=0.045$ ) than in those without such medications.

#### **4. 3. Changes in the plasma concentration of soluble thrombomodulin after eversion endarterectomy in patients with severe carotid artery stenosis**

##### **Soluble thrombomodulin levels measured in patients before operation and in healthy controls**

The objectives of the study were as follows: to examine the effects of thromboendarterectomy on sTM levels as a parameter of endothelial cell injury leading to abnormal hemostasis as well as to examine the clinical significance of sTM as a marker of inflammation.

In the framework of a prospective study (described in the foregoing) sTM levels were determined in 64 patients. The median age was 66 years, and the IQR, from the 25th to the 75th percentile, was 57-73 years. Forty-five of 64 patients were men and 19 were women.

In citrated plasma samples from 64 healthy volunteers the sTM levels ranged from 2.39 to 7.90 ng/mL, while in the patients it ranged from 0.48 to 15.32 ng/mL. In the study group the sTM levels in 11% of the patients, but only in 1% of the control individuals were equal or less than 2.39 ng/mL. There was no significant difference ( $p=0.545$ ) between the plasma sTM levels measured in patients and healthy subjects.

Preoperative serum total cholesterol and triglycerides levels were significantly higher in our patients, than in the control group ( $p=0.005$ ;  $p<0.0001$ ). The incidence of diabetes mellitus was 31% in the study group, whereas in the control group no diabetic patient was noted. (Table 10)

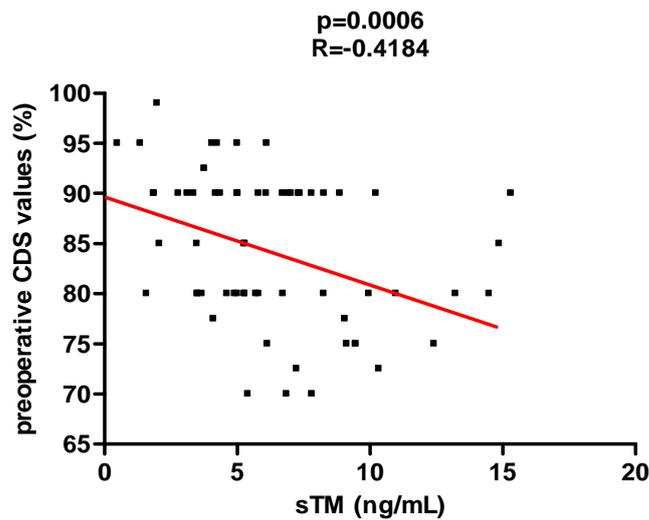
**Table 10.** Clinical and laboratory characteristics of the study and the control population

	Median (25th-75th percentile) for the continuous data		p value*
	Study group (n=64)	Control group (n=64)	
Age (years)	66 (57-73)	57 (52-67)	<0.0001
Gender (male/female)	45/19	39/25	0.176
Preoperative BMI (kg/m <sup>2</sup> )	26.22 (24.73-28.72)	25.99 (23.75-29.06)	0.434
Diabetes mellitus (yes/no)	20/44	0/64	<0.0001
Smoking (yes/no)	24/40	16/48	0.908
Total cholesterol (mmol/L)	6.11 (5.54-7.07)	5.54 (5.12-6.34)	0.005
Triglycerides (mmol/L)	2.02 (1.35-2.78)	1.23 (0.99-1.93)	<0.0001
Preoperative plasma sTM (ng/mL)	5.56 (3.91-8.04)	5.59 (4.60-7.81)	0.545

\*P values were calculated by the Mann-Whitney U test and Fisher exact test (in case of categorical variables).

**Negative correlation between baseline plasma sTM levels and the preoperative duplex scan values**

Negative correlation ( $R=-0.418$ ,  $p=0.0006$ ) was found between the plasma sTM concentrations measured in 64 patients before operation and the preoperative carotid duplex scan (CDS) values. (Figure 12)



Spearman correlation coefficient and its significance were indicated.

**Figure 12.** Correlation between the preoperative duplex scan values and the plasma sTM concentrations

### **Preoperative sTM levels in the carriers and in the noncarriers of the TNF2 allele**

Endothelial cell expression of thrombomodulin is potently inhibited by TNF- $\alpha$ . Recently, a polymorphism affecting TNF- $\alpha$  transcription has been identified in the promoter region of the gene, at nucleotide position -308. [178] The "TNF2" allele is a more powerful transcriptional activator than the common allele with a 6- to 7-fold increase in the inducible level of TNF- $\alpha$  gene transcription. For these reasons, we thought to investigate the effect of TNF- $\alpha$  polymorphism on sTM levels in patients with severe carotid artery stenosis.

Fifteen/sixty-four (23%) of the patients and 22/64 (34%) of the controls carried the G→A substitution at position -308 in the TNF- $\alpha$  gene promoter region (designated the TNF2 allele) ( $p=0.4297$ ). [164] Distribution of the TNF- $\alpha$  alleles corresponded to the Hardy-Weinberg equilibrium in both populations.

The preoperative sTM concentration was significantly lower ( $p=0.0415$ ) in patients with TNF2 allele [4.19 (1.98-6.73) ng/mL], than in the noncarriers [5.81 (4.31-8.27) ng/mL]. In the control group the sTM levels were almost the same independently of the TNF- $\alpha$  genotype [5.61 (4.41-9.01) and 5.35 (3.05-8.61) ng/mL;  $p=0.691$ ]. It was also found, that the presurgical duplex scan values were significantly higher in patients with than in those without TNF2 allele ( $p=0.037$ ).

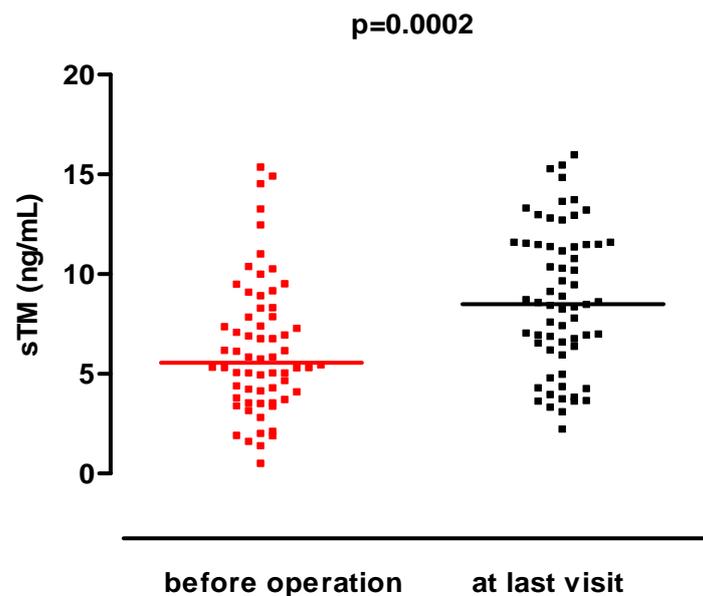
### **Postoperative changes in the sTM levels**

Fourteen months postsurgery the sTM levels [8.49 (6.25-11.46) ng/mL] were significantly higher ( $p=0.0002$ ) compared to the preoperative state [5.56 (3.91-8.04) ng/mL]. (Figure 13) By contrast, other laboratory or clinical variables (total cholesterol, triglycerides, HDL- and LDL-cholesterol, BMI) did not change during the follow-up period. The 14 months sTM levels did not differ significantly in the carriers and noncarriers of the TNF2 allele [7.00 (4.23-11.45) and 8.67 (6.33-11.48) ng/mL;  $p=0.105$ ).

According to the duplex scan values we divided the study group into two subgroups: restenosis subgroup ( $\geq 50\%$  stenosis at 14 months postsurgery,  $n=15$ ) and no restenosis subgroup (no or  $< 50\%$  stenosis at 14 months postsurgery,  $n=49$ ). In the

restenosis subgroup the serum total cholesterol, triglycerides and LDL-cholesterol were significantly higher ( $p=0.027$ ;  $p=0.033$ ;  $p=0.022$ ) compared to the no restenosis subgroup. The median sTM concentration in the restenosis subgroup was 5.28 ng/mL and in the no restenosis subgroup was 5.80 ng/mL before surgery, while at 14 months follow-up the sTM level among the restenotic patients was 9.09 ng/mL and among the nonrestenotic patients was 8.33 ng/mL. Neither the preoperative, nor the postoperative sTM levels were significantly different ( $p=0.943$ ;  $p=0.674$ ) in the two subgroups. The plasma sTM concentrations significantly increased in both subgroups during the follow-up period. (Table 11)

The incidence of subjects with -308 A TNF- $\alpha$  polymorphism was similar in the restenosis (29%) and in the no restenosis group (26%).



P value for the Wilcoxon signed-ranks test was indicated.

**Figure 13.** Comparison of the plasma thrombomodulin levels measured in patients with carotid atherosclerosis before and 14 months after carotid endarterectomy

**Table 11.** Plasma concentration of sTM (ng/mL) in patients with and without restenosis before and after carotid endarterectomy

Groups	Median (25th-75th percentile)		p value*
	Before surgery	14 months postsurgery	
No restenosis (n=49)	5.80 (3.72-8.27)	8.33 (5.43-11.56)	<0.0001
Restenosis (n=15)	5.28 (4.19-7.82)	9.09 (7.00-11.45)	0.005
p value**	0.943	0.674	

\* P values for the Wilcoxon signed-ranks test were indicated.

\*\* P values were calculated by the Mann-Whitney U test.

## 5. Discussion

### 5. 1. Distribution of C-reactive protein levels determined by ultrasensitive method in a healthy Hungarian population

Basic science and epidemiological studies have developed an impressive case that atherogenesis is essentially an inflammatory response to a variety of risk factors and the consequences of this response lead to the development of acute coronary and cerebrovascular syndromes. Although several cytokines, acute-phase reactants, and cellular responses to inflammatory stimuli potentially might be predictive of clinical disease, the laboratory tests to assess inflammation are limited to those that are employable in clinical settings, can be standardized, and have adequate precision. Laboratory and clinical evidence favors CRP as the marker that is ready for clinical application; it is the only one of the analytes with acceptable laboratory standards, considering assay availability, stability, World Health Organization standardization, and precision. [118, 182]

C-reactive protein measured by ultrasensitive method has been studied in nested case-control and prospective studies, which have shown graded, dose-response relationships to clinical cardiovascular disease (CVD) that remain after adjustment for other risk factors, with moderately strong associations between the lower and upper tertiles (RR  $\approx$ 2.0). C-reactive protein seems to add predictive value above that of currently established risk factors. The evidence, however, is not entirely consistent across published studies, and in particular, additional prospective studies are needed to more precisely define at various strata and to assure consistency in other age, sex and ethnic groups.

Our achievements showed that the CRP measurement - independently of gender and smoking - could be a good marker for cardio- and cerebrovascular risk estimation in healthy adults. The results corresponding to the others found in the literature. [75, 130, 133, 165] In respect of age we reported an important difference compared to the international data: it turned out that the CRP concentrations were significantly higher with the advancement of age. Similarly to us, in a Brazilian study aging was demonstrated to be associated with increase of CRP concentration in men and in elderly

men and women. [3] In an Australian aboriginal community CRP concentrations varied with age and were greatest in the 45-54-year age group. [91] We suggest that the increase in CRP with the advancement of age is related to the increased production of interleukin-6 associated with aging, which stimulates CRP production in the liver. On the other hand, these findings may be related to differences in the characteristics of studied populations, sample sizes and deserve further evaluation of the modulation of CRP level by other demographic and clinical variables.

Although epidemiological studies demonstrated association between low-grade inflammation and vascular risk, application of CRP testing in clinical practice requires estimates of risk across a spectrum of CRP levels. However, distribution of CRP is rightward skewed such that clinical application will likely require recasting measured CRP levels into an ordinal system. A useful approach to this problem is to divide CRP values into population based quintiles. Application of this quintile approach to CRP testing requires knowledge of the population distribution of CRP. Therefore we determined the distribution of CRP in a healthy Hungarian population. A representative population distribution of CRP was based on analysis of 207 Hungarians without apparent vascular disease. As risk estimates appear to be linear across the spectrum of inflammation, the sequential quintiles can be considered in clinical terms to represent individuals with low, mild, moderate, high, and highest relative risks, respectively, of future cardiovascular disease. According to this supposition, 40% (n=83) of the examined subjects belonged to the high and highest cardiovascular risk group.

On the basis of the available evidence, the screening of the entire adult population for CRP as a public health measure is not recommended. It is reasonable to measure CRP as an adjunct to the major risk factors to further assess absolute risk for coronary artery and cerebrovascular disease primary prevention. The finding of a high relative risk level of CRP ( $>3.0$  mg/L) may allow for intensification of medical therapy to further reduce risk and to motivate some patients to improve their lifestyle or comply with medications prescribed to reduce their risk. Individuals at low risk will be unlikely to have a high risk identified through CRP testing. Individuals at high risk or with established atherosclerotic disease generally should be treated intensively regardless of their CRP levels, so the utility of CRP in secondary prevention appears to be more limited.

C-reactive protein levels increase with acute infection and trauma. [129] Thus, testing should be avoided within a 2 to 3 weeks window in patients who have had an upper respiratory infection or other acute illness. Individuals with clinically apparent inflammatory conditions such as rheumatoid arthritis or lupus are likely to have elevations of CRP well into the clinical range; CRP evaluation for the purpose of vascular risk prediction may be of limited value in such patients. However, for most individuals, CRP levels appear to be stable over long periods of time. [118] These latter data support the possibility that enhanced inflammatory response and, hence, increased propensity to plaque rupture may involve important genetic determinants.

In contrast to results for cytokines such as IL-6, no circadian variation appears to exist for CRP. [93] Thus, clinical testing for CRP can be accomplished without regard for the time of day. Two separate measurements of CRP are adequate to classify a person's risk level and to account for the increased within-individual variability. [111, 141] Several factors have been identified as being associated with increased or decreased levels of CRP. For example, body weight and the metabolic syndrome are consistently associated with elevated CRP, and weight loss is associated with reduction in CRP levels, with some authors suggesting that CRP is merely a marker for obesity and insulin resistance. [92, 158]

Recent data describing CRP within atheromatous plaque, as a correlate of endothelial dysfunction, and as having a direct role in cell adhesion molecular expression raise the possibility that CRP may also be a potential target for therapy. No specific therapy has been evaluated for its ability to reduce CRP, nor does any direct evidence indicate that reduction of CRP necessarily will result in reduced risk of cardio- and cerebrovascular events. Prevention of cardiovascular events in persons with high-normal or elevated plasma CRP levels may be achieved by anti-inflammatory agents like aspirin, reducing the synthesis of CRP (cytokine-antagonists?), preventing the binding of CRP to membranes (phosphorylcholine-like drugs?) or inhibiting CRP-induced activation of the classical complement pathway (C1-esterase-inhibitor?). [131, 134] Future studies should reveal whether these approaches are indeed efficacious.

Several limitations of CRP evaluation require consideration. Inflammatory markers are nonspecific, increase with acute infection or trauma, and have been shown to predict total mortality as well as cardio- and cerebrovascular events. The need to

avoid CRP evaluation during times of infection or trauma and among individuals with known systemic inflammatory conditions thus may limit clinical utility. On the other hand, although cost effectiveness of CRP testing has not been formally evaluated, testing for CRP is inexpensive and likely to prove cost effective, particularly when compared to techniques such as electron beam calcium scanning or magnetic resonance imaging.

Although limitations inherent to inflammatory screening remain, available data suggest that CRP has the potential to play an important role as an adjunct for global risk assessment in primary prevention of cardio- and cerebrovascular diseases.

### **5. 2. 1. Determination of the serum and plasma levels of two inflammatory markers, CRP and fibrinogen before and after eversion carotid endarterectomy**

We have reported here on novel observations obtained in 117 patients with severe carotid atherosclerosis who underwent eversion endarterectomy and followed-up for a median time of 13.8 months.

We found a sharp and highly significant decrease in the concentrations of two acute-phase proteins, serum CRP and plasma fibrinogen after surgical removal of the atherosclerotic plaques. As far as we know, no data on longitudinal measurements of either acute-phase proteins with such long follow-up period after endarterectomy have been published till now.

Apparently it is surprising that removal of atherosclerotic plaques by surgical intervention from one side of the arterial tree resulted in such dramatic changes in the production of CRP and fibrinogen. No definite explanation for this observation can be given at the present time. Since most patients were given the same medical treatment postsurgery as in the preoperative period, it seems probable, that the operation itself resulted in a marked attenuation of the inflammatory burden of the patients. The overall decrease of serum CRP is due to an almost three-fold drop that occurred in patients who had the baseline CRP levels in the highest tertile that is produced the highest amounts of CRP. A similar but less sharp difference was noted in case of fibrinogen, overall drop of the plasma fibrinogen levels during the follow-up period was due to decrease in patients with baseline fibrinogen levels in the medium and the highest tertiles.

Parallel to our findings, Mezzetti et al. reported a rapid and significant increase in the serum concentration of ceruloplasmin in the first 24 hours, no change until the third day, and subsequent lowering to initial values after one month in 45 patients undergoing elective carotid endarterectomy. [94] Hashimoto et al. who concluded that CRP concentration was a marker of carotid atherosclerotic activity rather than the extent of atherosclerosis also seems to be relevant for understanding the mechanism of our present observations. [56] In addition to this indirect mechanism, however, it is possible that the plaques also significantly contributed to the production of the acute-phase proteins prior to removal. Various cytokines, growth factors, and inflammatory cells are abundant in atheromatous plaques. [65, 138] Patients with more extensive plaques have

higher expression of adhesion molecules. [26] C-reactive protein belongs to the pentraxin protein family. According to the studies of Rolph et al. another member of the family PTX3 is produced in advanced carotid atherosclerotic plaques. [137] More importantly, Yasojima et al. detected 10.2-fold higher CRP mRNA and higher CRP levels in postmortem samples from atherosclerotic plaques compared to the normal arterial tissue. [180]

Other findings of our prospective study such as the significantly higher CRP and fibrinogen levels in patients with severe carotid atherosclerosis are in agreement with the results of previous studies. [48, 87, 126] On the other hand, in contrast to the observations of Rerkasem et al. we did not find significantly elevated CRP concentrations in symptomatic compared to asymptomatic patients with carotid artery disease. [126]

It remains questionable whether elevated levels of inflammatory biomarkers uncover the presence of a single vulnerable lesion or identify a "vulnerable patient" exhibiting several coexisting high-risk lesions in different arterial segments. Actually, it appears more likely that elevation of CRP and fibrinogen indicates the systemic nature of progressive atherosclerotic disease, which suggests that patients with enhanced inflammation are generally at high risk for progression of atherosclerotic disease and may exhibit multiple vulnerable lesions. The concept of early identification of vulnerable patients who are susceptible to cardio- and cerebrovascular adverse events seems appealing, and measurement of inflammatory biomarkers may be a potent adjunctive tool for this purpose.

### **5. 2. 2. Restenosis rate**

After results of large-scale trials demonstrated that carotid endarterectomy is superior to medical treatment of extracranial carotid artery atherosclerosis in selected symptomatic patients with 70% or greater stenosis of the internal carotid artery (NASCET), in symptomatic patients with 80% or greater stenosis (ECST), and in asymptomatic patients with 60% or greater stenosis (ACAS), there was a sharp increase in the number of patients who underwent plaque removal with surgical intervention. [52, 101, 139, 183] Carotid eversion endarterectomy is a durable procedure; however, restenosis and

occasionally recurrent symptoms may develop. Restenosis is the result of either myointimal hyperplasia, which tends to develop within the first 2 years following CEA, or recurrent atherosclerosis, which is most predominant after 2 years. Thus, depending on the time interval after operation, recurrent plaque may have differing morphological characteristics. Symptoms are relatively rare in patients who develop intimal hyperplasia because these lesions are smooth, nonulcerated, and do not act as a nidus for cholesterol or platelets. Beyond 2 years, symptoms are more frequent and are related to embolization from atherosclerotic plaque. Recurrent stenosis and symptoms are more frequent in women, in patients with atherosclerotic risk factors such as cigarette smoking and hypercholesterolemia, or if residual disease remains following the initial endarterectomy. [78]

Postoperative surveillance protocols using duplex ultrasound are frequently employed in patients following CEA. Recurrent stenoses of greater than 50% are identified in 12% to 36% of endarterectomized vessels. However, the incidence of stenosis greater than 80% is only around 2% per year and the incidence of recurrent symptoms is even less. [43, 68] At our Department internal carotid artery stenosis of 50% or greater was detected in 15 patients (13%), but only 4 patients (3%) had severe ( $\geq 70\%$ ) restenosis in the operated region during the follow-up period. The frequency of 3% of patients with restenosis of 70% or greater is in harmony well with previously published data. [43, 45, 68]

Inflammatory processes are now known to be active in all stages of atherosclerosis, including plaque initiation, growth, and late complications. [81] Less certain is the degree to which restenosis, another vascular "response to injury" process, shares similarities with the inflammatory pathophysiology of spontaneous atherosclerosis. A number of circulating indicators of vascular inflammatory activity have been proposed, such as soluble adhesion molecules, cytokines, acute-phase reactants (e.g. CRP, fibrinogen, and serum amyloid A), and leukocyte count. [118, 128] Among them, fibrinogen is one of the most interesting, as it presents a possible marker for both inflammation and thrombosis and may hint a link between the two. The clinical utility of these indicators will depend on the ability to measure their levels in serum or plasma accurately and reliably, and on their predictive value as demonstrated in clinical studies.

Studies from Europe have suggested a relation between C-reactive protein and restenosis or the need for repeat revascularization after angioplasty, but other reports, including some from the United States, have not. [13, 42, 58, 60, 173, 175, 185] In a relatively small (n=75) but carefully documented, quantitative study, Zhou et al. found no association between CRP and angiographic restenosis after angioplasty and endarterectomy. [185] Similarly, Horne et al. noted in their assessment of 415 patients undergoing percutaneous coronary intervention, that CRP was not associated with increased restenosis. [60] Data published on the association of the acute-phase response with restenosis after carotid endarterectomy are scarce and it is well known that carotid arteries present different problems than do those of the coronary arterial system, on which most data have been published. Lippi et al. studied the possible usefulness of fibrinogen measurements in identifying subjects at risk for occlusive complications following vascular surgery (including carotid endarterectomy) and endovascular procedures but did not find differences in the plasma fibrinogen levels between patients with and without restenosis. [83] By contrast, recently Schillinger et al. observed an association between the intensity of the acute-phase response, i.e. CRP levels measured 48 hours postsurgery and the 6 months in-stent restenosis rate after stent implantation in the carotid arteries. [144] The process leading to restenosis is different after eversion endarterectomy and stent implantation, nevertheless these findings are in line with the association we noted between early fibrinogen response and subsequent restenosis after carotid endarterectomy.

The lack of the decrease in the CRP and fibrinogen levels in patients with restenosis also indicates that inflammatory burden was markedly attenuated after surgery in patients with no restenosis. Myointimal hyperplastic lesions are most probable a consequence of complement activation via the lectin pathway and production of different inflammatory cytokines due to reperfusion injury. Several studies are needed yet to elucidate the mechanism of the attenuation of the overall inflammation after carotid endarterectomy.

The second interesting information which came out of our study is the predictive value of the measurement of early changes (from baseline to 4 days postsurgery) of the plasma fibrinogen levels: restenosis rate was significantly higher in patients with high (above median) early increase in the plasma fibrinogen levels compared to those with

no change or decrease in the plasma fibrinogen concentrations. By contrast we did not find any correlation between early changes of the CRP levels and subsequent restenosis. On the other hand, the present observations are in line with the earlier findings of Montalescot et al. obtained in patients after coronary angioplasty: patients with high fibrinogen concentration at follow-up within 6 months had higher restenosis rates than patients with low fibrinogen level. [100]

Nowadays, the potential uses for inflammatory markers fall into three categories: prevention techniques, new types of treatment, and response to conventional therapies. Why should predicting restenosis matter? We know from the results of coronary stent placement that there are other interventional techniques already available that may further reduce restenosis in the carotid artery. Results with intravascular irradiation following coronary angioplasty and stent placement have been excellent. [110] Brachytherapy is currently recommended and approved for treatment of restenosis following coronary stent placement rather than for de novo lesions. Recent studies in the coronary circulation have described remarkable results with negligible rates of restenosis by using stents coated with paclitaxel (Taxol; Bristol-Myers Squibb) or sirolimus (rapamycin). [49, 102, 152] (Coated grafts and threads are under development in vascular surgery.) All these approaches have succeeded by using a locally active method to control the proliferation of smooth muscle cells, which occurs following vessel angioplasty or stent placement. However, our locally effective methods of controlling stent restenosis are not without limitations. Brachytherapy is technically difficult. Drug-eluting stents are much more expensive than their noneluting cousins.

If we already have a solution, should we care about the mechanisms of restenosis? The answer should be yes. Therefore knowledge of predictive factors of restenosis after surgical or interventional procedures aiming to re-establish circulation in the carotid arteries narrowed by atherosclerotic plaques may lead to selective use of these procedures and/or to again evaluate the potential for systemic drug therapy in patients at an increased risk for restenosis.

Atherosclerosis and problems with its therapy, including restenosis, are baffling and complex processes that remain largely enigmatic. Fortunately, this subject continues to be one of the most frequently studied, and new theories and possible

treatments are surfacing at a rapid rate. Clearly, inflammation plays a role, but further research must be supported at all levels.

### **5. 3. Changes in the plasma concentration of soluble thrombomodulin after eversion endarterectomy in patients with severe carotid artery stenosis**

Only a few studies were published on the relationship between plasma thrombomodulin concentrations and atherosclerotic vascular diseases. No data on longitudinal measurements of sTM are, however, available and the effect of TNF- $\alpha$  polymorphisms on sTM levels was not tested either. Our present work that was aimed to address these questions resulted in several novel observations.

We found an inverse correlation between the preoperative duplex scan values and sTM concentrations. Patients whose plasma sTM levels were higher had less severe carotid artery stenosis, than those with lower sTM concentrations. Parallel to this finding, Salomaa et al. found that the risk of coronary heart disease gradually decreased with increasing quintile of sTM. [142] On the other hand, in contrast to our present results, the same author showed a positive association between sTM levels and carotid intima-media thickness (IMT) among 803 Caucasian subjects. [142] Petit et al. reported a positive trend between these two variables, but no association with plaques was found. [122] Some other studies have reported a positive association between sTM concentrations and carotid artery disease, while others have failed to find any association. [10, 64, 120, 121, 124, 149] Thrombomodulin has been shown to have not just anticoagulant, but anti-inflammatory effect, as well. [36] It is not clear yet, if there is a relationship between the endothelial TM activity and the plasma sTM concentration, but such connection has been suggested by Salomaa and coworkers and by other studies, too. [142, 149] These findings and the inflammatory theory of atherosclerosis can provide explanation why the presurgical duplex scan values were higher in patients with lower sTM levels.

Until now there is no data about the changes in plasma concentration of sTM after carotid surgery. Our second measurement was 13.8 (12.3-19.0) months after the operation. It was interesting that the sTM level of the whole study population increased highly significantly at the end of the follow-up period. The observed negative correlation between sTM plasma levels and the preoperative duplex scan values as well as the marked increase in the sTM plasma concentrations after surgical removal of the plaques indicate that sTM may be adsorbed to the atherosclerotic plaques in carotid

arteries. The pathological significance of this adsorption remains to be determined. On the other hand, atherosclerotic plaques are known to be active inflammatory loci, which can produce different cytokines, including TNF- $\alpha$ , which can suppress the functional TM. Therefore, it can not be excluded that removal (together with the plaques) of the activated endothelium that covers the plaques leads to the decrease of this suppression and consequently to increase in the sTM levels measured in patients with more severe carotid atherosclerosis. Not all endothelial cell proteins, however, are down-regulated at sites of atherosclerosis. In line with this assumption we noted a markedly elevated P-selectin levels in the same patients before operation (unpublished). These changes are likely to work in concert to favour atherothrombosis and the adhesion of leukocytes and platelets.

Up to the present, few studies have examined the role of thrombomodulin in the pathophysiology of restenosis after vascular procedures. Tsakiris et al. found in 71 patients with peripheral arterial occlusive disease 6 months after transluminal angioplasty a trend for higher TM in those who developed restenosis. [167] Mihara et al. gave an account of no significant difference in the TM level in patients with and without restenosis after percutaneous transluminal coronary angioplasty. [95] In our study in both subgroups (restenosis, no restenosis) the 14 months postoperative sTM concentrations were significantly higher, but neither the preoperative nor the postoperative sTM levels were different in the two subgroups. Thus our present findings indicate that soluble TM is not a contributory marker for recurrent carotid artery stenosis.

Increased plasma concentrations of TNF- $\alpha$  have been found in patients with premature coronary artery disease. [66] However, it remains unclear whether elevated serum TNF- $\alpha$  in patients with manifest atherosclerosis derives from atherosclerotic plaques or from nonvascular sources. Be that as it may, the primary proinflammatory cytokine TNF- $\alpha$ , in turn, elicits the expression of the messenger cytokine IL-6, which induces expression of hepatic genes encoding acute-phase reactants, as well as the production of other effector molecules in the inflammatory response, such as cellular adhesion molecules for leukocytes. [81] Adhesion of circulating leukocytes to endothelial cells with ensuing transendothelial migration is considered an important early step in atherogenesis, and increased expression of cellular adhesion molecules

may accordingly be one mechanism by which TNF- $\alpha$  is implicated in atherothrombotic disease. [21] As the TNF2 allele is a more powerful transcriptional activator than the common allele with a 6- to 7-fold increase in the inducible level of TNF- $\alpha$  gene transcription and TNF- $\alpha$  is known to be able to suppress the expression of TM on endothelial cells it seemed to be interesting to determine the association between TNF- $\alpha$  polymorphism and plasma sTM levels in patients with and without carotid atherosclerosis.

As it was expected, the preoperative sTM concentration was significantly lower among the carriers of the TNF2 allele, while we could not find such difference 14 months after the operations, in the restenosis and in the control group. Moreover, the presurgical carotid duplex scan values were significantly higher in patients with TNF2 allele. It seems probable that the lower sTM levels measured in the TNF2 allele carriers are secondary to the higher extent of carotid atherosclerosis in this group of patients. This premise is in harmony with the findings of Skoog et al. and Elkind et al. who observed that the plasma TNF- $\alpha$  concentration was associated with the degree of early atherosclerosis and correlated with metabolic and cellular perturbations that were considered to be important for the vascular processes. [32, 154]

From the rabbit experiments in which TM was overexpressed in injured common femoral arteries, it can be inferred that TM plays an important role in preventing leukocyte migration into the vessel wall. [177] Therefore, down-regulation of TM would be likely to facilitate the leukocyte influx into the plaques. Activated inflammatory cells have been shown to increase the decay of the plaque cap leading to plaque rupture. [143] Inflammatory mediators generated locally, such as TNF- $\alpha$ , can down-regulate TM by blocking gene transcription. [19, 76] Preliminary results from our laboratory support the hypothesis that thrombomodulin is down-regulated in carotid arteries with atherosclerosis. These changes would be expected to result in reduced inhibition of thrombogenic and anti-inflammatory activity on the endothelium overlying atherosclerotic regions.

#### **5. 4. Novel findings**

**1.** *We characterized the distribution profile of serum C-reactive protein measured by ultrasensitive method in an apparently healthy Hungarian population (207 blood donors).*

**2.** *In patients with severe carotid artery stenosis the concentration of two acute-phase proteins, serum C-reactive protein and plasma fibrinogen significantly decreased after surgical removal of the atherosclerotic plaques (6 weeks and 14 months postsurgery).*

**3.** *Neither C-reactive protein nor fibrinogen levels changed significantly till the end of the observation period in restenotic patients as compared to the preoperative values. By contrast, in patients with no restenosis C-reactive protein and fibrinogen levels significantly decreased as compared to the levels measured before surgery already at the first follow-up visit, and continued to drop till the end of the follow-up.*

**4.** *Early postoperative changes in fibrinogen levels predicted restenosis.*

**5.** *An inverse correlation was observed between the preoperative duplex scan values and soluble thrombomodulin levels. The soluble thrombomodulin concentration of the whole study population increased highly significantly at the end of the follow-up period.*

**6.** *The preoperative soluble thrombomodulin level was significantly lower among the carriers of the tumor necrosis factor 2 allele, while we could not find such difference 14 months after the operations, in the restenosis and in the control group. Moreover, the presurgical carotid duplex scan values were significantly higher in patients with tumor necrosis factor 2 allele.*

**7.** *Soluble thrombomodulin is not a marker for recurrent carotid artery stenosis.*

## 6. Future perspectives

### 1.

*Our studies were limited by their small sample size. An increase in the number of restenotic patients is required, therefore the studies should be continued.*

*In the CRP measurements two sequential samples have been found to be appropriate for clinical use. Although, in most of the published studies the serum CRP concentrations have been determined only once, in the future we are planning to measure it twice to classify a person's risk level and to account for the increased within-individual variability.*

*It would be useful to investigate the long-term changes in the serum and plasma concentrations of acute-phase proteins and sTM after endarterectomy at different arterial segments to confirm our present results.*

### 2.

The early phase of atherosclerosis involves the recruitment of inflammatory cells from the circulation and their transendothelial migration. This process is predominantly mediated by cellular adhesion molecules, which are expressed on the vascular endothelium and on circulating leukocytes in response to several inflammatory stimuli.

*We would like to firmly establish the potential clinical and therapeutic utilities of (soluble) adhesion molecules [P, E and L-selectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, platelet-endothelial cell adhesion molecule (PECAM-1)] since the results in both fields hold the promise that in future, adhesion molecules might add information for clinical risk prediction and serve as therapeutic targets.*

### 3.

During carotid eversion endarterectomy, a short period (15 to 20 minutes) of cross-clamping occurs, which in turn may result in ischemic-reperfusion injury. Thus, we suggested that mannose-binding lectin (MBL), the activator of the lectin pathway of the complement system is deposited after carotid endarterectomy and that complement activation products may activate endothelial cells. (Rugonfalvi et al., *Stroke* 2005)

Activated endothelial cells produce cytokines, growth factors and other molecules, which have been shown to be essential for smooth muscle cell proliferation, migration, matrix formation, and for triggering neointimal hyperplasia, giving rise to a vicious circle resulting in restenosis.

*Therefore, we have started to study the possible predictive value of different components of the complement system [C5b-9, C3a, C4d, Bb, total hemolytic complement activity (CH50), alternative pathway complement activity (AH50), MBL-associated serine protease (MASP-2), C3, C4, C9, C1 inhibitor (C1-INH),  $\alpha$ 2-HS-glycoprotein] for restenosis after carotid endarterectomy.*

#### 4.

Negative arterial remodeling plays an important role in the pathogenesis of carotid artery restenosis. Smooth muscle cell proliferation in restenosis is regulated through the interaction of growth factors and their receptors. We have found an initial postoperative increase in the serum vascular endothelial growth factor (VEGF) concentrations which predicted restenosis ( $\geq 50\%$ ) after carotid endarterectomy. Since restenosis occurred mainly in patients who were homozygous carriers of the normal MBL genotype a prediction comprising MBL genotyping and VEGF measurement should be considered. (Szabó et al., *Brain* - submitted for publication) This combined determination may have a very strong predictive value. This finding - if it is found reproducible in other cohorts - may have important clinical and theoretical implications.

*We aim to investigate the predictive value of other growth factors like platelet-derived growth factor (PDGF)-BB, insulin-like growth factor (IGF)-1 and fibroblast growth factor (FGF) for recurrent carotid artery stenosis, too.*

#### 5.

Percutaneous transluminal angioplasty and stenting seems to be, at present, the treatment of choice for atherosclerotic stenoses of internal carotid arteries. During carotid artery stenting, the atherosclerotic plaques are only compressed compared to endarterectomy techniques, when the plaques are removed from the artery. Using self-expanding stents poststenting dilation of the distal part of the stent with a balloon is

usually required. The ischemic interval during carotid cross-clamping (15 to 20 minutes) is quite long compared to the poststenting dilation (2 to 3 seconds).

*Therefore, it seems to be interesting to study the possible differences between the two techniques in respect of restenosis rate, acute-phase response, complement activation, expression of adhesion molecules, growth factors and their genetic background. To get to the bottom of this problem, we have started another investigation containing patients who were undergoing internal carotid artery stenting.*

## **6.**

The use of animal models in the study of atherosclerosis is essential to answer many questions. For instance, evaluation of a risk factor as a single independent variable, with almost complete exclusion of other factors, can best be performed in animals free of intercurrent diseases or abnormalities and with well known genetic characteristics.

*In the near future, parallel to the clinical studies we are going to focus on animal models in order to understand the possible molecular, cellular, and genetic background of our clinical findings.*

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## 9. List of publications

### 1. Peer reviewed papers with relevance to the current work

**I. Dósa E**, Szabó A, Entz L, Selmecei L, Walentin Sz, Kleiber M, Rugonfalvi-Kiss Sz, Hosszúfalusi N, Füst Gy, Prohászka Z. Distribution of C-reactive protein levels determined by ultrasensitive method in a healthy Hungarian population. (in Hungarian) *Orvosi Hetilap* 2004; **145**:567-572.

**II. Dósa E**, Rugonfalvi-Kiss Sz, Prohászka Z, Szabó A, Karádi I, Selmecei L, Romics L, Füst Gy, Acsády Gy, Entz L. Marked decrease in the levels of two inflammatory markers, hs-C-reactive protein and fibrinogen in patients with severe carotid atherosclerosis after eversion carotid endarterectomy. *Inflamm Res* 2004; **53**:631-635.

**III. Dósa E**, Szabó A, Prohászka Z, Karádi I, Rugonfalvi-Kiss Sz, Apor A, Járányi Zs, Hüttl K, Selmecei L, Acsády Gy, Füst Gy, Entz L. Changes in the plasma concentration of soluble thrombomodulin in patients with severe carotid artery stenosis after eversion endarterectomy. *Inflamm Res* 2005; **54**:289-294.

### 2. Abstracts

**IV. Dósa E**, Entz L, Szabó A, Rugonfalvi-Kiss Sz, Veres A, Bíró A, Prohászka Z, Selmecei L, Füst Gy. Recurrent stenosis rate following carotid endarterectomy. *Cardiovasc Surg* 2002(10) Suppl. 1, p92.

**V. Dósa E**, Rugonfalvi-Kiss Sz, Prohászka Z, Szabó A, Tamás L, Selmecei L, Füst Gy, Entz L. Role of the levels of two inflammatory markers and the genotype of mannose-binding lectin in the pathophysiology of restenosis after carotid endarterectomy. *Eur Surg Res* 2004(36) Suppl. 1, p15.

**VI. Dósa E**, Rugonfalvi-Kiss Sz, Prohászka Z, Szabó A, Tamás L, Selmecei L, Füst Gy, Entz L. Changes in the serum and plasma concentrations of CRP and fibrinogen after carotid endarterectomy. (in Hungarian) *Magy Seb* 2004(57)3: p150.

**VII. Dósa E**, Szabó A, Selmecei L, Prohászka Z, Füst Gy, Tamás L, Entz L. Acute-phase proteins and their association with atherosclerosis and restenosis. (in Hungarian) *Érbetegségek* 2004. Suppl. p13.

**VIII. Dósa E**, Prohászka Z, Füst Gy, Entz L. Soluble thrombomodulin and TNF-alfa genotype in patients with severe carotid artery stenosis and restenosis. *Eur Surg Res* 2005(37) Suppl. 1, p79.

**IX. Dósa E**, Szabó A, Selmecei L, Füst Gy, Entz L. Role of the inflammatory markers in the pathophysiology of internal carotid artery stenosis and restenosis. (in Hungarian) *Magy Seb* 2005(58)4: p280.

**X. Dósa E**, Szabó A, Rugonfalvi-Kiss Sz, Selmecei L, Füst Gy, Entz L. Markers of inflammation and vascular disease. *J Vasc Res* 2005(42) Suppl. 2, p67.

**XI. Dósa E**, Szabó A, Selmecei L, Füst Gy, Entz L. Role of soluble thrombomodulin and TNF-alfa genotype in the pathogenesis of severe carotid artery stenosis and restenosis. (in Hungarian) *Érbetegségek* 2005. Suppl. 2, p37.

### **3. Other publications**

#### **3. 1. Peer reviewed papers**

1. Szabó A, Brázda E, **Dósa E**, Apor A, Szabolcs Z, Entz L. Long-term restenosis rate of eversion endarterectomy on the internal carotid artery. *Eur J Vasc Endovasc Surg* 2004; **27**:537-539.

2. Rugonfalvi-Kiss Sz, **Dósa E**, Madsen HO, Endrész V, Prohászka Z, Laki J, Karádi I, Gönczöl É, Selmecei L, Romics L, Füst Gy, Entz L, Garred P. High rate of early

restenosis after eversion endarterectomy in homozygous carriers of the normal mannose-binding lectin genotype. *Stroke* 2005; **36**:944-948.

**3.** Bíró A, **Dósa E**, Horváth A, Prohászka Z, Rugonfalvi-Kiss Sz, Szabó A, Karádi I, Acsády Gy, Selmei L, Entz L, Füst Gy, Romics L. Dramatic changes in the serum levels of anti-cholesterol antibodies after eversion endarterectomy in patients with severe carotid atherosclerosis. *Immunol Lett* 2005; **99**:51-56.

**4.** Szabó A, Laki J, Madsen HO, **Dósa E**, Prohászka Z, Rugonfalvi-Kiss Sz, Kókai M, Acsády Gy, Karádi I, Entz L, Selmei L, Romics L, Füst Gy, Garred P, Cervenák L. Early rise in serum vascular endothelial growth factor and platelet derived growth factor predisposes for restenosis after eversion endarterectomy in patients with a normal mannose-binding lectin genotype. *Brain* (submitted for publication)

**5.** Széplaki G, Varga L, Laki J, **Dósa E**, Madsen HO, Prohászka Z, Rugonfalvi-Kiss Sz, Szabó A, Acsády Gy, Karádi I, Selmei L, Romics L, Garred P, Füst Gy, Entz L. The third component of complement (C3) is associated with early restenosis after carotid endarterectomy. *Atherosclerosis* (submitted for publication)

### **3. 2. Published abstracts**

**6.** **Dósa E**, Szabó A, Apor A, Rugonfalvi-Kiss Sz, Selmei L, Füst Gy, Entz L. Predictive factors of carotid artery restenosis. (in Hungarian) *Magy Seb* 2003(56)3-4: p144.

**7.** **Dósa E**, Szabó A, Apor A, Rugonfalvi-Kiss Sz, Selmei L, Füst Gy, Entz L. Role of MBL (mannan-binding lectin) genotype and serum LDL-cholesterol concentrations in the pathogenesis of internal carotid artery restenosis. (in Hungarian) *Érbetegségek* 2003. Suppl. p35.