Thesis for the degree of Doctor of Philosophy (Ph.D.)

EXAMINATION OF THE SEVERITY AND CLINICOPATHOLOGICAL FEATURES OF ATHEROSCLEROSIS IN STROKE PATIENTS

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1. INTRODUCTION

Cerebrovascular disease is one of the leading causes of death and chronic disability in developed countries. In contrast with coronary patients, 10-20% of stroke survivors demand a lifetime of full nursery care and 30-40% need help in their everyday lives. Specific treatment of completed stroke is feasible only in a portion of patients and success rate in treated patients is relatively low. The goal of primary and secondary vascular prevention is to forestall stroke, myocardial infarct, ischemic events in other vascular territories, and death caused by these events through the recognition and treatment of vascular risk factors. Accordingly, risk assessment is becoming more and more important in vascular prevention.

Most important modifiable risk factors of ischemic stroke are arterial hypertension, hyperlipidaemia, diabetes mellitus, smoking and carotid stenosis, which are all involved in the process of atherosclerosis. Atherosclerosis is a general process affecting the entire arterial system. Co-existence of atherosclerotic changes in different vascular beds is important because patients with multiple lesions have higher incidence of cardiovascular events compared to patients with only one affected vascular region. Close correlation between the atherosclerotic changes of the different arterial regions was proven by multiple studies. Recent works emphasize the role of inflammation in the atherosclerotic process. Based on seroepidemiological studies, *Chlamydia penumoniae, Helicobacter pylori,* and Cytomegalovirus are related to atherosclerosis.

1.1. Importance and examination of intima-media thickness (IMT)

Carotid arteries are easily accessible by ultrasound examinations. Ultrasound is non-invasive, repeatable, no ionizing radiation is used, stenotic and other lesions can be determined quantitatively with high accuracy, but requires an experienced examiner. Modern, high resolution ultrasound systems visualize intima-media layers of the vessel wall as a double line structure. IMT measured in the common carotid artery is considered an early marker of atherosclerosis correlates both with cardiovascular risk factors and the likelihood of cardio- and cerebrovascular events. According to prospective studies, thicker IMT is an independent marker of vascular events even in young age. Higher IMT values are correlated with the risk of recurrent stroke. Repeated, standardized IMT measurement is a suitable method for the follow-up of early atherosclerotic changes and the effect of influencing factors and drugs.

1.2. The role of Chlamydia pneumoniae in atherosclerosis

Beyond the classical risk factors, recent research results emphasize the importance of inflammation in the atherosclerotic process, especially in the formation of initial intima lesion and intimal hyperplasia, later in plaque destabilisation. *Chlamydia pneumoniae* is an air-borne intracellular bacterium disseminated via circulating macrophages and able to infect even the endothelium and the smooth muscle cells in the vicinity of the atherosclerotic plaque. The agent was detected in atherosclerotic plaques by immunohistochemical and polymerase chain reaction (PCR) methods. Detection of *Chlamydia pneumoniae* DNA was possible in 15-55% of endarterectomy specimens and atherosclerotic plaques of autopsied arteries.

1.3. Vessel wall calcification

Calcification is a prominent feature of atherogenesis. Atherosclerotic lesions contain calcium phosphate deposits. Both atherosclerosis and calcification progress with age. The degree of carotid artery lumen reduction proved to be a relevant marker of stroke risk, but comparison of carotid artery calcification and stroke risk brought conflicting results. Calcified carotid plaques are more stable and less prone to be symptomatic than non-calcified soft plaques. Degree of calcification was determined only in atherosclerotic plaques in former studies, so we have limited information about calcification in the early stage of the atherosclerosis. Calcification can be quantified in vivo by electron beam computer tomography (CT), multidetector CT, or magnetic resonance (MR) techniques. Localisation and concentration of calcium deposits can be determined by nuclear microscopy, Raman spectroscopy, inductively coupled plasma atomic emission spectrometry, and particle induced X-ray emission (PIXE) methods. The latter one is suitable for quantitative determination of macro- and microelements with high accuracy elemental localisation.

1.4. Relations among atheroscleroses of different arterial regions

Atherosclerosis is a general process. Initial diffuse endothelial dysfunction is later associated with local atherosclerotic lesions and thromboembolic complications with or without symptoms. The regional distribution of atherosclerotic lesions shows high personal variability. Co-incidence of atherosclerotic changes in a number of vascular beds is important because patients with multiple lesions have a higher incidence and a worse prognosis of cardiovascular events compared to patients with one affected vascular region only. Indicator vessels that are easily accessible for non-invasive examination techniques have been used in

many trials. Atherosclerotic lesions of the common carotid arteries have been described to represent the total atherosclerotic burden. Before the introduction of the very popular IMT measurements, the degree of carotid atherosclerosis was determined by the severity of stenosis in the carotid arteries, which characterizes only the late phase of the atherosclerotic disease. Anatomical, histological and pathological interpretation and validation of the data raised multiple problems. Common carotid IMT is correlated with the stenosis of the coronary and femoral arteries and the outcome of cardiovascular events. Results of previous studies are controversial. Two authors found only weak correlations between carotid or femoral artery IMT and the severity of coronary artery disease. Others found that femoral artery IMT is a better predictor for coronary disease than IMT measured in the common carotid artery or the carotid bulb. Another work found significant correlations between carotid bulb IMT and coronary artery disease; however, neither common carotid nor femoral artery IMT correlated with the stenosis of the examined coronary segments. In different studies on coronary patients, atherosclerotic changes of the femoral arteries correlated more closely with coronary disease than those of the carotid arteries did. Furthermore, IMT and plaque load of the arteries are different markers of the atherosclerotic burden. While IMT describes the early changes of the vessel wall, number and area of the plaques and the degree of the stenosis characterize the more advanced stage of atherosclerosis.

These controversies and hardly comparable results led us to study the severity of atherosclerosis in the carotid, coronary and femoral arteries searching for clinically relevant correlations. We chose a pathological method, which eliminates the limitations of *in vivo* techniques, describes all stages of the atherosclerosis uniformly, and makes the different sized arteries comparable with each other.

2. OBJECTIVES

1. Old patients with potentially fatal ischemic stroke were screened for cardiovascular risk factors; IMT measurement and *Chlamydia pneumoniae* serology were performed *in vivo.* After death, presence of *Chlamydia pneumoniae* DNA by PCR method and wall thickness of the carotid arteries was examined.

    a. We examined the relation between common carotid artery IMT (as an atherosclerosis marker) and atherosclerosis risk factors – particularly serological markers of *Chlamydia pneumoniae* infection – to see whether *Chlamydia pneumoniae* infection has a role in atherosclerosis.

    b. Comparing *in vivo* measured common carotid IMT and *post mortem* detected atherosclerotic changes of the carotid arteries, we examined how IMT characterizes the severity of carotid atherosclerosis.

    c. Presence of *Chlamydia pneumoniae* DNA in the plaques of the removed carotid arteries was compared with *in vivo* Chlamydia serology, common carotid artery IMT and *post mortem* measured average wall thickness to examine the role of *Chlamydia pneumoniae* in atherosclerosis.

2. IMT was measured *in vivo* in the common carotid arteries and was compared with the calcium content determined *post mortem* by the PIXE method in the same carotid artery wall segments, examining the correlation between IMT and calcification in the early stage of atherosclerosis. We examined the localisation of calcification within the artery wall to identify which layer is involved most intensively.

3. We compared the severity and distribution of atherosclerotic changes in the carotid, coronary and femoral arteries by a *post mortem*, quantitative pathological method in patients who died of acute ischemic stroke. We examined whether atherosclerosis severity of a certain arterial region characterizes the atherosclerotic changes of other regions, searching for an easily accessible, reliable marker vessel of general atherosclerosis.

3. PATIENTS AND METHODS

3.1. Patient group and samples

Patients from the Department of Neurology, University of Debrecen with severe acute ischemic stroke were included in all three studies substantiating this thesis. All patients were older than 50 years to ensure a cohort with probable generalized atherosclerosis. Patients with either hemorrhagic stroke or stroke of special, rare causes (e.g. thrombophilia) were excluded from the studies. Since inflammatory markers and *Chlamydia pneumoniae* infection were also the part of the examinations, patients with pneumonia or other serious infections (sepsis) were excluded as well. Studies were approved by the local ethical committee of the University of Debrecen.

3.1.1. Studying the connection between IMT, Chlamydia infection and carotid atherosclerosis

Altogether 67 stroke patients were examined (mean age: 77.2 years; male/female: 27/40). Both *in vivo* IMT measurements and Chlamydia antibody titers were determined in 57 cases. In 5 cases only IMT, in 2 cases only antibody titers were measured, in a further 3 patients only *post mortem* examinations were done. At admission, patient history and risk profile were taken and then detailed laboratory examinations were done within 48 hours including CRP and anti-*Chlamydia pneumoniae* IgA and IgG antibody titers. Detailed carotid duplex ultrasound examination with common carotid artery IMT measurement was performed in 62 cases. 40 of the patients deceased due to complications of the stroke, 25 of them had pneumonia. In this study only measurements on the carotid arteries were used. Presence of *Chlamydia pneumoniae* specific DNA was screened by PCR method in the characteristic atherosclerotic plaques of the carotid arteries located in the vicinity of the bifurcation.

3.1.2. Examination of artery wall calcium content

In 3 patients (mean age: 85.6 years; male/female: 1/2) carotid duplex examination and common carotid IMT measurement was performed *in vivo*, artery wall calcium content and localization was determined by PIXE method *post mortem*.

3.1.3. Pathological comparison of the atherosclerosis in different arterial regions

40 deceased patients' (mean age: 75.3 years; male/female: 21/19) carotid, coronary and femoral arteries were examined. At admission, patient history and risk profile were taken,

then detailed laboratory examinations were done. During autopsy, both common carotid arteries were removed *in toto* with the proximal 2-3 cm segments of the internal and external carotid arteries as well as the bilateral femoral arteries. The section of the femoral artery between the inguinal ligament and the bifurcation of the deep femoral artery (DFA) was referred to as the common femoral artery (CFA), and the distal part of the femoral artery below the bifurcation as the superficial femoral artery (SFA). The right coronary artery (RCA) and the anterior descendent branch of the left coronary artery (LAD) were also removed.

3.2. Methods

3.2.1. IMT measurement

Detailed neurosonological examination of the carotid arteries with IMT measurement was performed within 48 hours after admission using a SONOS 4500 ultrasound system equipped with a 3-11 MHz linear transducer. IMT was determined bilaterally in a standardized manner in the common carotid arteries, 10 mm proximally from the flow divider in a plaque-free segment of the distal artery wall in end-diastole. IMT by definition was measured between the leading edge of the first echogenic line (lumen-intima interface) and second echogenic line (upper layer of the adventitia) in the far (deeper) artery wall. In each artery, we performed 3 measurements with positioning the probe laterally and frontally to the carotids as well, this way gained 6 values from each vessel and 12 values per patient.

3.2.2. Examination of *Chlamydia pneumoniae* specific antibodies

Serological examinations were performed in the Department of Medical Microbiology. *Chlamydia pneumoniae* specific IgG and IgA antibody responses were detected in enzyme linked immune sorbent assay (ELISA) tests using chlamydial outer membrane protein (OMP). According to the manufacturer's instructions an antibody index above 1.1 indicated the presence of the specific antibodies.

3.2.3. Detection of *Chlamydia pneumoniae* DNA in carotid plaques by PCR

Polymerase chain reaction (PCR) examinations were also performed in the Department of Medical Microbiology. Specimens derived from the atherosclerotic plaques of the carotid flow divider. After DNA extraction, the *Chlamydia pneumoniae* OMP coding sequence was

amplified with nested PCR technique. PCR products were visualized by agarose gel electrophoresis.

3.2.4. Examination of artery wall calcium content by PIXE method

The examined sectors of the removed carotid bifurcations were labelled with surgical threads at the points where IMT measurements were performed, branches were ligated and then the arteries were filled with a histological embedding material (Cryochrome Blue; Thermo Shandon, Pittsburgh, PA, USA) and frozen to -25°C. The frozen common carotid arteries were cut into 60 μm thick slices in a cryostat. Transverse sections corresponding to the site of the IMT measurements were sandwiched between Pioloform membranes taking care of the reference points. PIXE (particle induced X-ray emission) measurements were carried out at the Debrecen microprobe facility installed on the 5 MV Van de Graaff accelerator at the Institute of Nuclear Research of the Hungarian Academy of Sciences. The proton beam was focused to a spot about 5×5 μm^2 and scanned over the areas of interest (1.25×1.25 mm^2). Two parts of each cryosection were investigated 90° from each other, according to the IMT measurement points. PIXE spectra were obtained using a conventional Be-windowed and an ultrathin windowed Si (Li) detector. Scanning transmission ion microscopy (STIM) was applied to determine energy loss in the samples, which is used for the calculation of the concentrations. True elemental calcium maps and absolute calcium concentration were evaluated with the True PIXE imaging software. Transverse sections of the examined parts were evaluated by light microscopy after hematoxilin-eosin staining by two investigators and compared with video recordings of the ultrasound examinations to decide if PIXE measurements were performed at the proper location.

3.2.5. Post mortem examination of the atherosclerosis in the removed arteries

Removed native arteries were filled with stained histological embedding medium (Cryochrome Blue), frozen to -25°C and cut into 3-mm-thick slices. Five to eight slices per vessel were evaluated. Consecutive slices were photographed with a high-resolution digital camera equipped with a macro lens. A ruler was used on the images for off-line calibration. Digital images were analyzed with scientific image-analyzer software. The following parameters were measured on each cross-section: lumen area (mm^2), vessel cross sectional area (mm^2), lumen circumference (mm), vessel circumference (mm), maximum wall thickness (mm), minimum wall thickness (mm) and vessel wall thickness at two different locations (equidistant from the two previous measurements). The native, frozen cross sections did not

allow a reliable identification of the plaque borders, so plaque area was not measured. Occluded arteries were excluded from analysis.

Using the primary data the following parameters were calculated:

1. *Average wall thickness (mm):* average of the 4 wall thickness measurements

2. *Relative wall area:* (vessel cross sectional area - lumen area) / vessel cross sectional area

3. *Longitudinal vessel wall irregularity:* the maximal value of wall thickness within one vessel / the minimal value of wall thickness within one vessel

4. *Cross sectional wall irregularity:* $\sum_{i=1}^{4} |\text{average wall thickness - wall thickness}_i| / 4$

5. *Calculated average wall thickness (mm):* vessel circumference / 2π - lumen circumference / 2π

6. *Wall index:* average wall thickness / (vessel circumference / 2π)

7. *Severity score:* relative wall area × cross sectional wall irregularity × longitudinal wall irregularity

The parameters describe different aspects of atherosclerotic changes of the arteries. Parameters 1-6 describe the local wall abnormalities in one slice and the longitudinal changes in one vessel. The *Severity score* consists of basic parameters (2, 3, 4), each regarding quantitative wall changes expressed only in relative values in order to eliminate the effect of size difference between arteries, ensuring the comparability of arteries of different size and localization.

3.3. Statistical analysis

In the study comparing IMT, Chlamydia infection and carotid atherosclerosis, testing of agreement between two categorical measurements was performed by calculating the kappa statistic. The effect of categorical variables on continuous dependent variables was investigated by non-parametric Mann-Whitney U test. Spearman rank order correlation was used for correlating continuous variables.

In the study comparing IMT and calcium content, normality of continuous variables was checked by the Saphiro-Wilk test. When appropriate due to non-normality of the distribution, Spearman correlation and Friedman ANOVA were used.

During pathological analysis of the atherosclerosis in different arterial regions, the distribution of the calculated parameters was not normal, so Spearman rank order correlation was used for correlating the parameters of different vascular territories. For comparing the parameters of the different vessel groups Friedman ANOVA was used. The effect of categorical variables (gender, left ventricular hypertrophy) was investigated by Mann-Whitney U test. To investigate and control for the potential confounding effect of risk factors and associated clinical data on the correlation estimates, we applied multiple linear regression analysis with these factors as covariates. $P<0.05$ was considered significant. Statistica for Windows 6.1® software was used for data analysis.

4. RESULTS

4.1. Studying the connection between IMT, Chlamydia infection and carotid atherosclerosis

27 males and 40 females were examined. Males were significantly younger (74 vs. 79 years, p=0.009). Frequency of stroke risk factors was: hypertension 79%, diabetes mellitus 42%, smoking 10%, coronary atherosclerosis 69%, and symptomatic carotid artery stenosis 24%. Mean common carotid artery IMT values correlated with hypertension (0.9 vs. 0.83 mm, p=0.024) and age (Spearman r=0.25, p=0.05). The inpatient standard deviation of IMT used to assess the IMT irregularity was higher in the hypertensive patients than in normotensives (0.13 mm vs. 0.09 mm, p=0.024). *In vivo* diagnosis of *Chlamydia pneumoniae* infection was based on the detection of highly specific IgG and IgA antibodies against chlamydial OMP. IgG antibodies indicating lifetime exposure were detected in 73% of patients, while IgA indicating recent or ongoing infection was found in 49%. In patients with IgA seropositivity, CRP was non-significantly higher than in IgA negatives (57.0 vs. 37.5 mg/l, p=0.43). There was no difference between the IMT values of IgG positive and negative (0.91 vs. 0.9 mm, p=0.86) or IgA positive and negative patients (0.88 vs. 0.9 mm, p=0.53). Similarly, common carotid IMT irregularity did not correlate with the presence of either IgG or IgA antibodies. In summary, the systemic markers of *Chlamydia pneumoniae* infection were not associated with the examined *in vivo* markers of common carotid atherosclerosis.

Post mortem analysis of carotid atherosclerosis and *Chlamydia pneumoniae* infection were performed in 40 patients. Comparing *post mortem* measured *Average wall thickness* and *in vivo* measured CCA-IMT, Spearman correlation showed significant correlation with the

common carotid and external carotid arteries (CCA: r=0.51, p=0.002; ECA: r=0.58, p<0.001; ICA: r=0.34, p=0.052). Irregularity data of IMT and wall thickness correlated significantly only in the common carotid arteries (CCA: r=0.34, p=0.045; ECA: r=0.2, p=0.24; ICA: r=0.3, p=0.078).

Chlamydia pneumoniae specific DNA sequences could be detected by PCR method from the characteristic plaques of the internal or common carotid arteries in 54% of the examined patients. Presence of DNA was only in partial agreement with IgA seropositivity (kappa value: 0.357, p=0.058) and showed no significant correlation either with IMT (0.93 vs. 0.95 mm, p=0.46) or *post mortem* measured *Average wall thickness* (CCA: 1.36 vs. 1.37 mm, p=0.95; ICA: 1.00 vs. 0.97 mm, p=0.74; ECA: 0.93 vs. 0.83 mm, p=0.2). We could not find significant correlation between DNA presence and irregularity of wall thickness.

4.2. Examination of artery wall calcium content

In this study, bilateral common carotid arteries of 3 stroke victims were examined. IMT was measured *in vivo*, then calcium content of the artery wall was measured *post mortem* by the PIXE method. PIXE measurements were performed in two wall segments in every artery, so 12 samples were created. Mean (SD) elemental calcium content of the samples was 6578 (3806) ppm, mean (SD) IMT was 1.0 (0.37) mm. Comparing the calcium content of the samples and the identical IMT values, no significant correlation was found (Spearman r=0.545, p= 0.066).

Each high resolution PIXE calcium distribution map was divided into 16×16 equal portions and the calcium content of each pixel was calculated. Quartiles of the calcium content of all pixels were determined in each calcium distribution map and the *Lower limit of the upper quartile$_i$* of each specimen was used for further analysis. The *Lower limit of the upper quartile$_i$* and IMT correlated significantly (Spearman r= 0.69, p= 0.01).

After pooling all pixels of the 12 specimens (12×16×16), the quartiles of the pooled data were calculated. To indicate calcium accumulation within the different specimens, number of the pixels in the *Pooled Upper Quartile$_{a\ 12}$* was counted in each sample. Significant correlation was found between IMT values and the number of pixels in the *Pooled Upper Quartile$_{a\ 12}$* (Spearman r= 0.66, p= 0.02).

The size of the elemental calcium distribution maps was 1.25×1.25 mm^2. The side of these maps was approximately parallel with the lumen-intima border in 8 of the original 12 specimens. In these 8 specimens median calcium content of the 16 layers from the intimal to

the adventitial side was calculated using the data of the 16×16 pixels. Median calcium content of the different layers showed an uneven distribution (Friedman ANOVA, p<0.0001). In light of the mean [SD] IMT of the 8 specimens (0.90 [0.14] mm), the thickness of the layers (1.25/16 = 0.078125 mm), and the localisation of the calcium-richest eleventh layer (at 0.859 mm), we determined that calcium accumulated mainly in the tunica media layer of the artery wall. To confirm this finding we also calculated the number of pixels in the *Pooled Upper Quartile$_{a8}$* of the 8 specimens. These data also supported the finding that calcium content was highest in the media layer within the artery wall.

4.3. *Pathological comparison of the atherosclerosis in different arterial regions*

489 arteries of 40 stroke victims were analyzed *post mortem*. Significant difference was found between *Average wall thickness* and *Calculated average wall thickness*, so we used them as separate entities during further analysis. We used Spearman rank order correlation for pair-wise comparison of the *Calculated average wall thickness, Wall index,* and *Severity score* (parameters 5, 6, 7). Significant and consistent correlations were found between the parameters of CCA and LAD, ICA and ECA, ICA and DFA, ICA and CFA. There was no correlation between the parameters of the CCA and the ICA. The strongest correlations were found between the femoral arteries (CFA-DFA-SFA). The DFA and the LAD correlated consistently in all three parameters, confirming the previous observations. The external carotid artery showed consistent correlations with all three examined arterial territories while the common carotid artery did not.

Subgroup analysis of 25 patients with coronary heart disease resulted in the same correlations (CCA-coronary, ICA-femoral, femoral-coronary, ECA-all regions) as observed in the whole cohort. Multiple linear regression analysis was performed to investigate the effect of different vascular risk factors (diabetes, hypercholesterolaemia, smoking and hypertension) and the other relevant clinico-pathological data (gender, peripheral artery disease, symptomatic carotid stenosis, coronary heart disease, TOAST classification of the patient's stroke). The correlations remained closely similar except for the CCA-LAD and ICA-ECA pairs. We found no significant differences between the severity indices of the subgroups of patients with and without left ventricular hypertrophy in the autopsy report.

5. DISCUSSION

5.1. Studying the connection between IMT, Chlamydia infection and carotid atherosclerosis

The novelty of this study is the comprehensive clinico-pathological approach with the application and comparison of *in vivo* and *post mortem* markers of both carotid atherosclerosis and *Chlamydia pneumoniae* infection. IMT characterizes the early phase of atherosclerosis. *Post mortem* measured average wall thickness was supposed to represent the cumulative effect of intima-media thickening and atherosclerotic plaques, while irregularity was used to assess the effect of atherosclerotic plaques themselves on the vessel. While good correlation was observed between in vivo IMT and post mortem measured average wall thickness, IMT and wall irregularity data did not correlate well. This observation supports the hypothesis that these different parameters describe different attributes of atherosclerotic wall changes.

Of the classical risk factors, hypertension and age were found to have a significant effect on IMT. This is consistent with the results of previous studies.

The prevalence of IgG seropositivity (73%) was similar to that of the general population of this age. However, 67% of the IgG seropositives (49% of the whole cohort) were IgA positives, indicating that a high proportion of the *Chlamydia pneumoniae* exposure coincided with the stroke. As we found no correlation between IgA seropositivity and CRP levels (highly sensitive systemic marker of inflammation), the role of *Chlamydia pneumoniae* infection in pneumonia and death could not be proven in our in acute stroke patients.

IgG or IgA seropositivity had no significant effect on IMT. Neither did IMT irregularity correlate with the presence of either IgA or IgG antibodies. In summary, the systemic markers of *Chlamydia pneumoniae* infection were not associated with the examined *in vivo* markers of common carotid artery atherosclerosis.

The local presence of *Chlamydia pneumoniae* was proven by detecting the specific Chlamydial DNA sequences in the characteristic plaques of the carotid arteries in 54% of the examined patients. This prevalence is similar to other studies from the field. The agreement between the antibody tests and the PCR results was not strong, because occurrence of IgA is a systemic response to active infection at any site and IgG is a marker of lifetime infection. IgA seropositivity was in partial agreement with PCR results, suggesting the biological validity of the applied PCR detection.

The presence of Chlamydial DNA correlated neither with the *in vivo* nor with the *post mortem* morphological markers of atherosclerosis (IMT, wall thickness and irregularities).

Chlamydia pneumoniae is a frequent pathogen that can infect both vascular and inflammatory cells. A meta-analysis revealed that disease association of *Chlamydia pneumoniae* was found in cross-sectional but not in longitudinal studies, which indicates the lack of causality. In the present cross-sectional study both local and systemic markers of *Chlamydia pneumoniae* infection were tested and both of them were frequently positive in acute ischemic stroke patients. In spite of its frequent occurrence, *Chlamydia pneumoniae* infection did not correlate with the severity of carotid atherosclerosis. This way our examination does not support the link between atherosclerosis and *Chlamydia pneumoniae* infection. So we do not recommend serological screening for *Chlamydia pneumoniae* or antibiotic treatment of patients with atherosclerotic disease in clinical practice until such time as clear evidence supports the link between atherosclerosis and *Chlamydia pneumoniae* infection.

5.2. Examination of artery wall calcium content

As far as we know, this is the first study to compare IMT measured *in vivo by* ultrasound and the calcium content of the same pieces of the CCA determined on calcium elemental maps using the PIXE analysis. Correlation between the total calcium content of the samples and IMT was not significant (Spearman r = 0.545, p = 0.066). In sight of the very uneven distribution of calcium on the elemental maps, we examined whether IMT and calcium content of the most expressly calcified areas correlate or not. The *Lower limit of the upper quartile$_i$* of calcium content of the pixels in each calcium distribution map correlated significantly with IMT (Spearman r = 0.69, p = 0.01). Corroborating this result, significant correlation was found between IMT values and the number of pixels in the *Pooled Upper Quartile$_{a\ 12}$* (Spearman r= 0.66, p= 0.02).

In another work, significant correlation was found between CCA IMT and atherosclerotic calcification of the carotid arteries measured by electron beam CT. The authors also suggested that carotid IMT is a good predictor of the atherosclerotic calcification.

The localization of the calcification within the artery wall was examined on 8 specimens divided into 16 layers each. There was a significant difference between the median calcium content of the different layers, and calcium accumulated mainly between layers 4 and 11, which were identified to represent the tunica media of the artery wall. This finding was strengthened by the number of pixels in the *Pooled Upper Quartile $_{a8}$* as well. Similar results

were found in another trial examining the thoracic aorta, where calcification occurred first in the tunica media and intimal involvement was detected only in older ages.

During the selection of the samples, we excluded the arteries with macroscopic atherosclerotic plaques. In spite of this, the PIXE method showed early calcium accumulation in the vessel wall. Our patients had only mild internal carotid artery stenosis and each plaque had hyperechogenic and homogeneous structure on the ultrasound images. It is known that CCA IMT is associated with the occurrence of carotid plaques. Because of the small size of our sample and the low variability of plaque morphology, we could not correlate the CCA IMT and the degree of internal carotid artery stenosis. Further prospective investigations need to examine these correlations.

Analysis of vessel wall components helps to understand the pathogenesis of atherosclerosis. The details of calcification are still not fully understood, but it seems a well organized and regulated process that is similar to the ones found in bone tissue. Non-differentiated vascular smooth muscle cells have a central role. Some factors induce them to differentiate into calcium secreting cells and others inhibit this type of differentiation. Of the factors regulating bone formation, matrix vesicula, BMP-2, osteopontin, osteocalcin, collagen I, matrix glia protein and sialoprotein are proven to be present in vascular calcification as well. Presence of oxidized lipids (which inhibit bone formation in bone tissue) is very important in atherosclerotic calcification, providing a further piece of evidence for the connection between bone metabolism and atherosclerosis. One can come across opinions as extreme as to suggest that a bone-like mineral homeostasis is present in the artery walls and calcium accumulation is the consequence of the disequilibrium between the function of osteoblast and osteoclast-like cells due to pathological regulation. Thicker IMT can be the consequence of the proliferation of smooth muscle cells in the media layer of the artery wall. Differentiation of the smooth muscle cells into calcium-secreting cells leads to early calcium accumulation in the media layer. In our cross-sectional study, using the PIXE method and ultrasound images, we could demonstrate this early calcium accumulation in the media layer. Our results have also shown a significant relationship between calcium content of distributional maps measured by PIXE analysis and corresponding IMT on B-mode ultrasound images of human CCAs.

In our opinion, PIXE analysis can be a suitable method for the precise quantitative and spatial follow-up of the calcification in studies dealing with the early stage of atherosclerosis. PIXE analysis should be used preferably in animal experiments studying the effect of drugs on the

atherosclerotic process, while in human atherosclerosis research, non-invasive methods like electron-beam CT are the optimal techniques.

5.3. Pathological comparison of the atherosclerosis in different arterial regions

Common Carotid Artery

Atherosclerosis of the CCA and its role in the estimation of the atherosclerotic burden of the entire vasculature has been most frequently examined, as the CCA is the most easily accessible artery. The prognostic value of CCA IMT was also confirmed. The Cardiovascular Health Study (almost 6000 subjects) found that a thicker CCA IMT is associated with higher risk of myocardial infarction and stroke in adults without history of cardiovascular disease. Others reported that IMT and the Framingham risk score were significantly correlated in more than 6400 subjects. Therefore, based on observational and interventional studies, CCA IMT has been accepted as a surrogate marker for the severity of cardiovascular disease.

In autopsy analysis of neurological patients, a French group found a strong association between the frequency of coronary atherosclerosis and the severity of atherosclerosis in the carotid or any of the intracranial brain feeding arteries. In other studies, carotid plaque area was shown to be a better indicator for coronary heart disease than IMT. After correction for risk factors, our results did not show significant correlation between the atherosclerotic parameters of the CCA and the coronary arteries. This discrepancy between previous results and ours can be explained by differences in the approach of atherosclerosis assessment. IMT shows early changes of the artery wall while our parameters (wall thickness, irregularities, wall index) measure the more advanced stage of atherosclerosis (severe wall changes). Therefore, our results do not contradict but complement the previous observations demonstrating a correlation between IMT and coronary atherosclerosis. Some authors found a correlation between CCA wall thickness and lower extremity arterial occlusive disease. Our data did not show significant correlations between CCA and femoral arteries, supporting the findings of a previous work where only a weak correlation was found between plaque load of the CCA and other peripheral arteries. Similarly to a previous IMT based study, we could not observe close correlations between the atherosclerotic changes of CCA and ICA.

Internal Carotid Artery

In our study there was no close correlation between the atherosclerotic scores of ICA and coronary arteries. This is in contrast to previous observations. On the other hand, the parameters of the ICA showed a good correlation with those of femoral arteries (DFA and CFA) as was reported in other *in vivo* studies.

Femoral Arteries

Our correlations between femoral and coronary arteries are interesting in view of the previous observations with different results. One group did not find significant correlation between IMT or plaques of the CFA and coronary artery stenosis. Others observed significant correlations between CFA-IMT and the risk of coronary artery disease. Moreover, CFA-IMT was the only independent parameter to predict the Gensini score, which measures coronary atherosclerosis. Coronary calcium content also correlated well with common femoral artery plaques. Other studies found that femoral plaque load and femoral IMT are stronger predictors for significant coronary artery disease than those of the common carotid arteries. Unfortunately, these studies used only the proximal part of the femoral artery for comparisons. Our data showed unequivocal femoral-coronary correlations mainly for the DFA. This discrepancy between previous studies and ours may have resulted from different measuring methods and different evaluation parameters. Most of the aforementioned studies used the IMT of the CFA, while others calculated the number of plaques in the femoral arteries. We used seven parameters in order to quantify more precisely the vessel wall abnormalities. Furthermore, we investigated systematically the total vessel length (instead of measuring the IMT of a circumscribed, plaque-free vessel segment). This approach provides much more reliable estimation of atherosclerosis burden than IMT measurement.

Regional differences

There was a significant difference in atherosclerosis severity between different examined arteries. The coronary arteries are the most severely involved, followed by the femoral arteries. Severe atherosclerosis parameters in the carotids, especially CCA, are less frequent. ECA shows similar burden to the coronaries, which also supports our observations.

The explanation of these differences between vessel regions is still lacking, but hemodynamic phenomena in particular could play a role. Geometric factors (the angle of origin of the ICA, curves of the vessels) were observed to influence the place of atherosclerotic plaque formation. Regions in the arteries with disturbed flow patterns are more susceptible to

atherosclerosis than segments with laminar flow. Plaques tend to be formed in areas of low and oscillating shear stress, which is closely related to turbulent, disturbed flow. This may explain our observation that ECA and DFA are better predictors of coronary atherosclerosis than the CCA, because ECA and DFA have several branches similarly to coronaries, while CCA does not. Flow pattern may be responsible for the similar extent of atherosclerosis in these arteries.

In conclusion, our findings suggest that besides the IMT measurement of common carotid artery, which is frequently used as an early indicator of coronary disease, other vessels should also be considered as indicators of global atherosclerosis. External carotid (ECA) and femoral arteries (DFA and CFA) are as or even more sensitive atherosclerosis markers than CCA. In everyday practice it means that the extent of the external carotid artery atherosclerosis (which is mostly marginal during the carotis ultrasound examinations) becomes important information and, together with the extent of lower extremity atherosclerosis, should be considered as a risk marker of coronary atherosclerosis and cardiovascular morbidity. We can imagine the application of these data in a scoring system based on observations on a large patient population.

6. SUMMARY, NEW RESULTS

Our examinations were performed on patients with severe acute ischemic stroke. A comparative clinico-pathological approach was applied. Cardiovascular risk factor screening, serological marker analysis of *Chlamydia pneumoniae* infection, and common carotid artery intima-media thickness measurements were performed *in vivo*. The presence of *Chlamydia pneumoniae* in the carotid plaques, localisation and quantity of calcium accumulation were examined *post mortem*. Further on, the atherosclerotic changes of the carotid, femoral and coronary arteries were analyzed and compared.

➔ In the comparison of IMT, Chlamydia infection and carotid atherosclerosis, good correlation was found between in vivo common carotid IMT and post mortem measured average wall thickness data of the common and external carotid arteries. Systemic markers of *Chlamydia pneumoniae* infection (IgG and IgA antibodies) did not show correlation with the in vivo markers of the common carotid artery atherosclerosis (IMT and its irregularity). Local presence of the Chlamydial DNA correlated neither with presence of IgA nor with that of IgG antibodies. Further on, presence of Chlamydial DNA was independent of either in vivo or post mortem morphological markers of atherosclerosis. *In spite of a high detectability rate, Chlamydia pneumoniae infection did not correlate with the severity of atherosclerosis. Therefore our study does not support the link between Chlamydia infection and atherosclerosis.*

➔ In the study comparing arterial wall calcium content and IMT, we were the first to compare ultrasonographically measured IMT and elementary calcium distributional maps in post mortem specimens determined by the PIXE method. *Early calcium accumulation was detected in the media layer of the carotid arteries and significant correlation was found between the calcium content of the arterial wall and IMT measured on the identical arterial segments.*

➔ Results of the study comparing the atherosclerotic burden of the different arterial regions suggest that besides the IMT measurement of common carotid artery, which is frequently used as an early indicator of coronary disease, other vessels should also be considered as indicators of global atherosclerosis. *In our opinion, external carotid and femoral arteries are as or even more sensitive atherosclerosis markers than the common carotid artery.*

7. PUBLICATIONS

7.1. In extenso publications underlying the doctoral thesis

1. **Molnár S,** Kerényi L, Ritter MA, Magyar MT, Ida Y, Szöllősi Z, Csiba L. Correlations between the atherosclerotic changes of femoral, carotid and coronary arteries A post mortem study. *J Neurol Sci.* 2009;287:241-5. **IF2008: 2.359**

2. Kónya J, **Molnár S,** Magyar MT, Szekeres CC, Kerényi L, Csiba L. Severity of carotid atherosclerosis unrelated to Chlamydia pneumoniae infection in acute ischemic stroke patients: a clinicopathological study. *Cerebrovasc Dis.* 2008;25:170-175. **IF: 3.041**

3. Magyar MT, Szikszai Z, Kertész Z, **Molnár S,** Uzonyi I, Szíki GA, Csiba L. Calcium distribution in the vessel wall and intima-media thickness of the human carotid arteries. *Ultrasound Med Biol.* 2007;33:1171-8. **IF: 1.922**

7.2. Abstracts in the topic of the doctoral thesis

1. **Molnár S,** Kerényi L, Ritter M, Magyar T, Ringelstein EB, Csiba L. Comparison of in vivo carotid IMT and carotid wall thickness measured with a novel method (comparison of in vivo and post-mortem data). *Cerebrovasc Dis.* 2003;16(Suppl 4):75

2. Kerényi L, **Molnár S**, Ritter M, Magyar T, Ringelstein EB, Csiba L. Correlation of atherosclerotic changes between carotid, coronary and femoral arteries. Comparison of in vivo findings with autopsy. *Cerebrovasc Dis.* 2003;16(Suppl 4):54

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7.3. Other publications

1. Molnar MJ, Valikovics A, **Molnar S,** Tron L, Dioszeghy P, Mechler F, Gulyas B. Cerebral blood flow and glucose metabolism in mitochondrial disorders. *Neurology.* 2000;55:544-8. **IF: 4.781**

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3. Kemény V, **Molnár S**, Andrejkovics M, Makai A, Csiba L. Acute and chronic effects of vinpocetine on cerebral hemodynamics and neuropsychological performance in multi-infarct patients. *J Clin Pharmacol.* 2005;45:1048-54. **IF:2.889**

4. Szikszai Z, Kertész Z, Uzonyi I, Sziki GA, Magyar MT, **Molnár S**, Ida Y, Csiba L. Regional calcium distribution and ultrasound images of the vessel wall in human carotid arteries. *Nuclear Instruments and Methods in Phisics Researh B.* 2005; 231:263-267. **IF: 1.181**

5. Rosengarten B, Paulsen S, **Molnar S**, Kaschel R, Gallhofer B, Kaps M. Acetylcholine esterase inhibitor donepezil improves dynamic cerebrovascular regulation in Alzheimer patients. *J Neurol.* 2006;253:58-64. **IF: 2.984**

6. Rosengarten B, **Molnar S**, Trautmann J, Kaps M. Simultaneous VEP and transcranial Doppler ultrasound recordings to investigate activation-flow coupling in humans. *Ultrasound Med Biol.* 2006;32:1171-80. **IF: 2.011**

7. Rosengarten B, Paulsen S, **Molnar S**, Kaschel R, Gallhofer B, Kaps M. Activation-flow coupling differentiates between vascular and Alzheimer type of dementia. *J Neurol Sci.* 2007;257:149-54. **IF: 2.315**

8. Oláh L, Raiter Y, Candale C, **Molnár S**, Rosengarten B, Bornstein NM, Csiba L. Visually evoked cerebral vasomotor response in smoking and nonsmoking young adults, investigated by functional transcranial Doppler. *Nicotine Tob Res.* 2008;10:353-8. **IF: 2.539**

9. **Molnár S**, Csiba L. Magyar stroke-betegek vaszkuláris rizikófaktorainak alakulása 1 éves követés során. *Háziorvos továbbképző szemle.* 2008;13:118–123

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