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Regularities of plaque stabilization in various scenarios of neointimal calcification and vascularization

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Aim. To study the relationships between phenotypes of extracranial arteries' plaques (stable/unstable), their calcification and its causes, in particular, vascularization.

Material and methods. The study included 88 patients: patients (n=44) with ischemic stroke and those (n=44) with chronic brain ischemia. In all subjects, the parameters of systemic mineral homeostasis were assessed (total and ionized calcium, phosphate, total protein, albumin, and calcification propensity). Atherosclerotic plaques have been obtained during carotid endarterectomy, fixed in formalin, postfixed in 1% osmium tetroxide, stained in 2% osmium tetroxide, dehydrated in ascending ethanol series and acetone, stained with 2% alcoholic uranyl acetate and embedded into epoxy resin with its further polymerization. Epoxy resin blocks were grinded, polished, counterstained with Reynolds' lead citrate and sputter coated with carbon. Sample visualization was performed employing backscattered scanning electron microscopy. Number and area of calcium deposits and neointimal vessels were quantified using ImageJ. Statistical analysis was carried out using Mann-Whitney U-test and Spearman's rank correlation coefficient

Results. It was found that area of neointimal calcification, but not number of calcium deposits, was associated with the stable plaque phenotype. The stabilizing effect of calcification was manifested in retarding stenosis associated with plaque rupture and stroke. Calcification extent directly correlated with total and local plaque vascularization, which have been associated with unstable and stable plaque phenotype, respectively. In addition, plaque calcification negatively correlated with total protein and albumin, thereby reflecting the impaired systemic mineral homeostasis.

Conclusion. Atherosclerotic plaque calcification and active local vascularization reduce stenosis extent and stabilize plaque. In contrast, total plaque calcification contributes to the atherosclerosis progression and promotes major acute cardiovascular events.

Keywords: atherosclerosis, ischemic stroke, neointima, calcification, stenosis.

Relationships and Activities. The study (design, experiments, analysis and interpretation of data) was supported by a Complex Program of Basic Research of the Siberian Branch of the Russian Academy of Sciences within the Basic Research Topic of Research Institute for Complex Issues of Cardiovascular Diseases № 0546-2019-0002 "Pathogenetic basis for the development of cardiovascular implants from biocompatible materials using patient-oriented approach, mathematical modeling, tissue engineering, and genomic predictors".

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Extracranial artery (ECA) atherosclerosis, as it progresses, inevitably leads to hemodynamic disorders and insufficient cerebral blood supply [1]. In particular, plaque rupture in ECAs leads to an acute discrepancy between the blood delivery and oxygen demand (ischemic stroke), while permanent ECA stenosis is manifested by chronic cerebral ischemia (CCI). Thus, plaques can be subdivided into unstable (causing stroke) and stable (causing CCI) [2]. Despite the fundamental differences in the clinical phenotype of stable and unstable ECA atherosclerosis, the pathophysiological factors of plaque instability and especially the mechanisms of their regulation remain largely unknown. At the same time, predicting cardiovascular outcome in patients with multifocal atherosclerosis requires a clear understanding of regulating balance between plaque rupture and stabilization.

Despite the fact that the role of plaque calcification in its stabilization has been studied well, the reasons for development and progression of neointimal calcification, as well as the causal relationships between calcification and other determinants of unstable plaque phenotype, have not been adequately studied [3]. It is unclear why a number of plaques remain uncalcified until the cardiovascular event, and what factors are leading in calcification development. It also remains unclear which types of neointimal calcification stabilize the plaque and which, on the contrary, contribute to its rupture.

The study of this problem is complicated by the fact that sample preparation of tissues with extra skeletal mineralization for histology is extremely difficult due to impaired tissue integrity due to significant differences in the density of calcium deposits and surrounding tissues. As a result, the analysis of the interaction of calcification with other pathological processes occurring in the neointima becomes almost impossible. Our group previously developed an original method for preparing calcified plaques for electron microscopy, which consists in staining formalin-fixed tissues with osmium tetroxide and uranyl acetate, further embedding dehydrated tissues in epoxy resin, grinding and polishing polymerized epoxy blocks, followed by backscattered-electron imaging [4-6]. This method allows one to preserve the integrity of calcified plaques and investigate their colocalization with neointimal vessels (*vasa plaquorum*) [7].

The aim was to study the relationships between the phenotypes of ECA plaques (stable/unstable), their calcification, and the causes of calcification. Ultimately, this made it possible to identify the calcification types characteristic of stable and unstable plaques, as well as the pathomorphological determinants of calcifying and non-calcifying phenotype in ECAs.

Material and methods

The study included 88 patients hospitalized in Neurosurgery department of the L. S. Barbarash Kuzbass Clinical Cardiology Dispensary with ECA stenosis verified by ultrasound (44 patients with stroke and 44 patients with CCI). Plaques were considered unstable in stroke and stable in CCI. The study was carried out in accordance with Good Clinical Practice and Declaration of Helsinki. The study protocol was approved by the local ethics committee. All patients signed written informed consent. Cerebrovascular diseases (CCI and ischemic stroke), as well as concomitant diseases (hypertension, heart failure, chronic obstructive pulmonary disease, asthma, chronic kidney disease, diabetes, overweight and obesity) were diagnosed and treated according to current clinical guidelines and standards developed by European Society of Cardiology, Global Initiative for Chronic Obstructive Lung Disease, Global Initiative for Asthma, Kidney Disease: Improving Global Outcomes, American Diabetes Association and European Association for the Study of Obesity). Glomerular filtration rate was estimated according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. Left ventricular ejection fraction was assessed using echocardiography (Sonos 2500 ultrasound system, Hewlett Packard). The percentage of ECA stenosis in patients with cerebrovascular disease was assessed using color Doppler ultrasound (Vivid 7 Dimension Ultrasound System, General Electric Healthcare). Data on age, sex, smoking status and pharmacological history were collected at admission time.

In all patients participating in the study, upon hospital admission, the parameters of systemic mineral homeostasis (concentration of total and ionized blood calcium, phosphorus, total protein and albumin) were determined on a biochemical analyzer (Konelab 60i, Thermo Scientific). All patients underwent carotid endarterectomy, during which a number of them (21 patients with stroke and 27 patients with CCI) received ACB for further ultrastructural examination. After 24-hour fixation in formalin (B06-003, BioVitrum), each biomaterial was postfixed with 1% osmium tetroxide (19110, Electron Microscopy Sciences) in 0,1 M phosphate buffer solution for 12 h, then stained with 2% osmium tetroxide in bi-distilled water for 48 h. Then the samples were dehydrated through a series of ethanol solution in ascending concentration (50, 60, 70, 80, and 95%, all in two changes, each change for 15 min), stained with 2% uranyl acetate

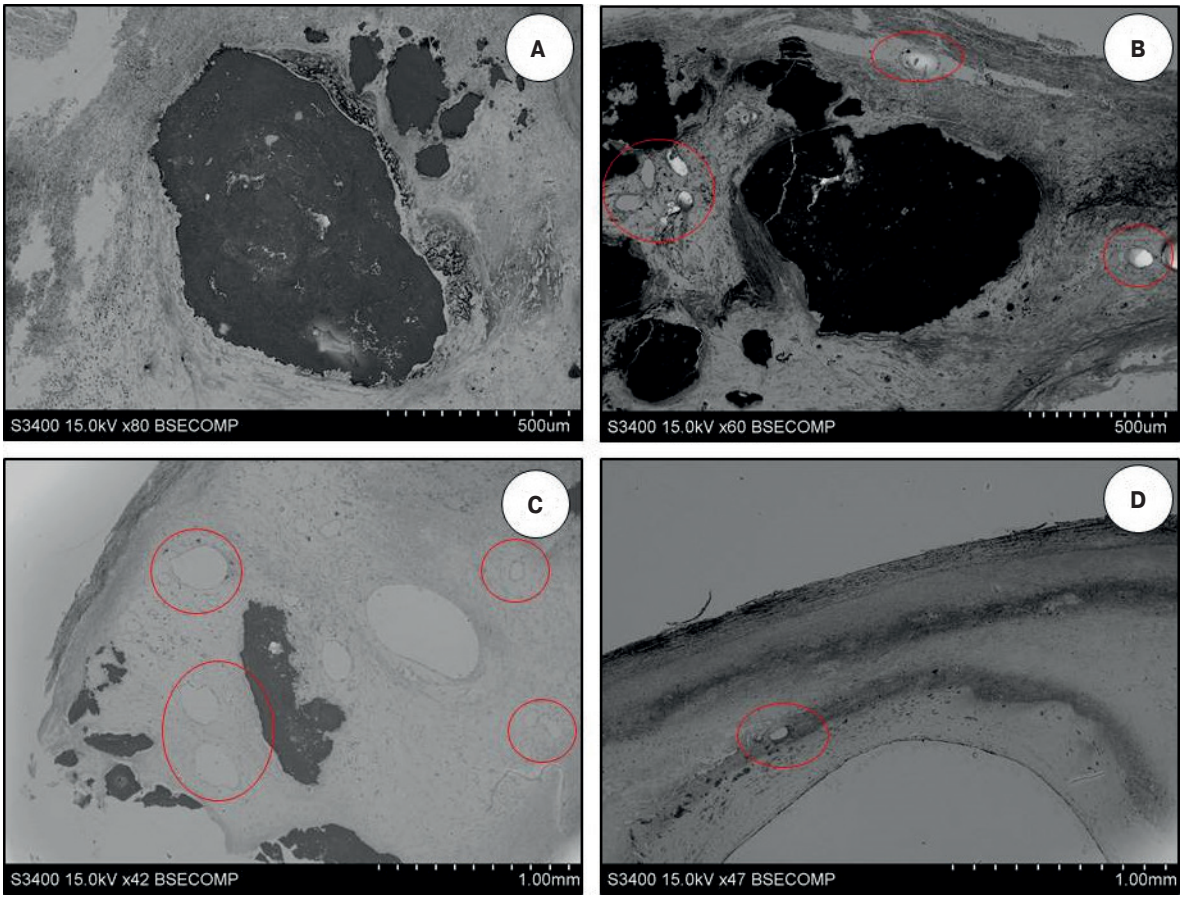

Figure 1. Interposition of neointimal vessels and calcifications in plaques. **A.** No blood vessels around calcifications; **B.** Large number of vessels around the calcification; **C.** Large number of vessels both around the calcification and in the plaque; **D.** No calcium, in the presence of newly formed vessels. New neointimal vessels are marked in red.

(22400-2, Electron Microscopy Sciences) in 95% ethanol (5 h), dehydrated with 99m7% isopropanol (06-002, BioVitrum) for 5 h and acetone (150495, LenReaktiv) for 1 h, impregnated with acetone mixture and epoxy resin Epon (14120, Electron Microscopy Sciences) in a ratio of 1:1 (6 h), after which it was transferred into new epoxy resin portion (for 24 h) and then polymerized in FixiForm tools (40300085, Struers) at 60° C. After that, the samples in epoxy blocks were grinded and polished on the TegraPol-11 system (Struers). Contrasting with lead citrate (17810, Electron Microscopy Sciences) was performed according to Reynolds methods for 7 min by applying the solution to polished sample surface, followed by washing it with bi-distilled water. Then, epoxy carbon blocks were sprayed onto the polished surface using a vacuum coater (EM ACE200, Leica). The structure of samples was visualized by backscattered electron microscopy using a S-3400N (Hitachi) electron microscope in the BSECOMP mode at an accelerating voltage of 10 kV.

The number and area of neointimal calcifications and vessels were analyzed both over the entire plaque and immediately around calcifications (one per plaque). Area of neointimal calcifications and vessels were estimated using the ImageJ software (National Institutes of Health). Statistical processing and graphical presentation of the results were performed using the GraphPad Prism 7 software (GraphPad Software). Due to the insufficient distribution of sample size for assessing the distribution normality, the data were described by nonparametric criteria (median and interquartile range), while intergroup comparison was performed using the Mann-Whitney U test. Correlation analysis was performed using the Spearman's rank correlation coefficient. Differences were considered significant at $p \leqslant 0,05$.

Results

At the initial phase, when studying the plaque ultrastructure by scanning electron microscopy, the main interposition types of neointimal calcifications and vessels were established (Figure 1). This made it possible to further reveal the features of plaque calcification and vascularization in different phenotypes. First of all, the hypothesis about the stabilizing effect

Table 1

Sex and age characteristics, comorbidities and pharmacological history of the included subjects

Patient group/studied cofactor	Patients with chronic cerebral ischemia	Patients with ischemic stroke	P
Sex and age characteristics			
Male gender	26/44 (59,09%)	31/44 (70,46%)	0,37
Age	67,0 (61,0-73,7)	64,50 (59,25-70,0)	0,07
Comorbidities or pathological conditions			
Hypertension	41/43 (95,3%)	39/41 (95,1%)	0,64
Heart failure	36/43 (83,7%)	37/41 (90,2%)	0,57
Chronic obstructive pulmonary disease or asthma	3/43 (7,0%)	7/41 (17,1%)	0,27
Smoking	2/43 (4,6%)	6/41 (14,6%)	0,24
Chronic kidney disease	4/43 (9,3%)	4/41 (9,8%)	0,76
Diabetes	10/43 (23,2%)	13/41 (31,7%)	0,53
Overweight	25/43 (58,1%)	20/41 (48,8%)	0,52
Obesity	5/43 (11,6%)	9/41 (22,0%)	0,33
Quantitative parameters			
Body mass index, kg/m^2	27,6 (24,2-32,0)	26,3 (24,6-32,8)	0,88
Glomerular filtration rate, ml/min/1,73 m^2	73,0 (60,0-82,0)	77,0 (66,0-91,5)	0,13
Left ventricular ejection fraction, %	64,0 (60,5-65,5)	65,0 (64,0-67,0)	0,10
Percentage of extracranial artery stenosis	75,0 (70,0-83,5)	86,0 (75,5-95,0)	0,01
Medical history before hospital admission			
Antiplatelet agents	25/43 (58,1%)	15/41 (36,6%)	0,08
Beta-blockers	16/43 (37,2%)	13/41 (31,7%)	0,76
Angiotensin-converting enzyme inhibitors	7/43 (16,3%)	3/41 (7,3%)	0,35
Statins	29/43 (67,4%)	14/41 (34,1%)	0,01
Nitrates	0/43 (0,0%)	0/41 (0,0%)	0,91
Angiotensin II receptor blockers	16/43 (37,2%)	5/41 (12,2%)	0,02
Aldosterone antagonists	2/43 (4,7%)	1/41 (2,4%)	0,96
Calcium channel blockers	14/43 (32,5%)	5/41 (12,2%)	0,05
Diuretics	0/43 (0,0%)	1/41 (2,4%)	0,97
Anticoagulants	5/43 (11,6%)	1/41 (2,4%)	0,23

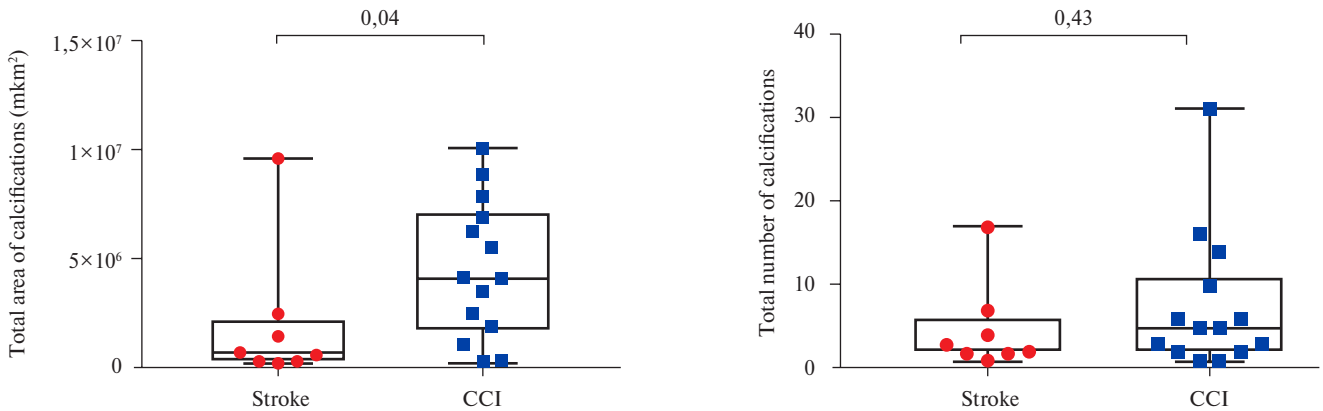

Figure 2. Total area of calcifications (left) and total number of calcifications (right) in the plaque of patients with stroke and CCI. Mann-Whitney test. P values are shown above the graphs.
Abbreviation: CCI — chronic cerebral ischemia.

Table 2

Correlation matrix for assessing the relationship between plaque volume, its blood supply and calcification

Spearman's rank correlation coefficient (r; P)							
	Stenosis percentage	Neointimal vessels number	Neointimal vascular area	Neointimal vessels number around Ca	Neointimal vascular area around Ca	Total Ca area	Number of calcifications
Stenosis percentage		0,41; 0,13	0,33; 0,23	-0,08; 0,83	-0,21; 0,54	-0,41; 0,18	-0,27; 0,39
Neointimal vessels number	0,41; 0,13		0,94; 0,0001	0,70; 0,0005	0,65; 0,001	0,53; 0,02	0,58; 0,007
Neointimal vascular area	0,33; 0,23	0,94; 0,0001		0,70; 0,0004	0,68; 0,001	0,58; 0,008	0,60; 0,006
Neointimal vessels number around Ca	-0,08; 0,83	0,70; 0,0005	0,70; 0,0004		0,96; 0,0001	0,63; 0,003	0,54; 0,01
Neointimal vascular area around Ca	-0,21; 0,54	0,65; 0,001	0,68; 0,001	0,96; 0,0001		0,56; 0,01	0,52; 0,02
Total Ca area	-0,41; 0,18	0,53; 0,02	0,58; 0,008	0,63; 0,003	0,56; 0,01		0,69; 0,0004
Number of calcifications	-0,27; 0,39	0,58; 0,007	0,60; 0,006	0,54; 0,01	0,52; 0,02	0,69; 0,0004	

Note: significant correlations are highlighted.
Abbreviation: Ca — representative calcification.

of calcification was tested, given the high prevalence and severity of comorbidities in patients (Table 1). In some works, it was shown that calcification in general helps to stabilize plaque, protecting it from rupture, however, the predominance of microcalcifications over macrocalcifications leads to the opposite effect [8]. Electron microscopy revealed that an increase in the total area, but not in calcifications number (Figure 2), has a stabilizing effect on plaques in patients with ECA atherosclerosis, which confirms this data [9-11]. Thus, it can be concluded that severe calcification prevents plaque rupture and specifies its stable phenotype.

However, the mechanism of plaque stabilization by calcification remains unclear. Quantitative image analysis found that the total area (but not the total number) of calcifications negatively correlated with vascular stenosis degree (r=-0,41) (Table 2), which was higher in unstable plaques (stroke) than in stable (CCI) (Figure 3). It should be noted that the stenosis degree also positively correlated (r=0,41) (Table 2) with the number of neointimal vessels, which reflects the volume of plaque blood delivery and, according to the literature, also contributes to its growth and rupture [12]. One may conclude that the morphological substrate of calcification stabilizing effect is the containment of plaque from growing into the vessel lumen, which sooner or later will lead to neointimal instability and fibrous cap rupture.

Further, the task was set to investigate neointimal calcification progression. The number and area of

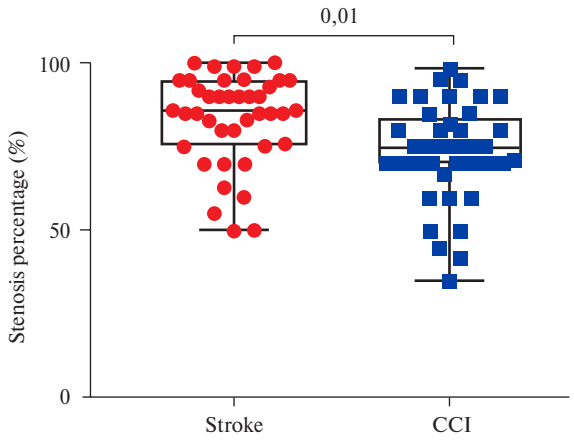

Figure 3. Stenosis percentage in patients with stroke and CCI. Mann-Whitney test. P value is shown above the graph.
Abbreviation: CCI — chronic cerebral ischemia.

calcifications, regardless of its phenotype, correlated with the total blood supply to neointima (r=0,53-0,60) (Table 2) and with the direct blood supply near the representative calcification (r=0,52-0,63) (Table 2). The total number and area of neointimal vessels strongly correlated with each other (r=0,94) (Table 2) and with the number and area of vessels around calcifications (r=0,65-0,70) (Table 2), which confirms the validity of using both of these measures to assess the plaque blood delivery. Based on the above, one may assume that the general and local (near the calcification) plaque blood circulation causes the active calcification progression (Figure 4).

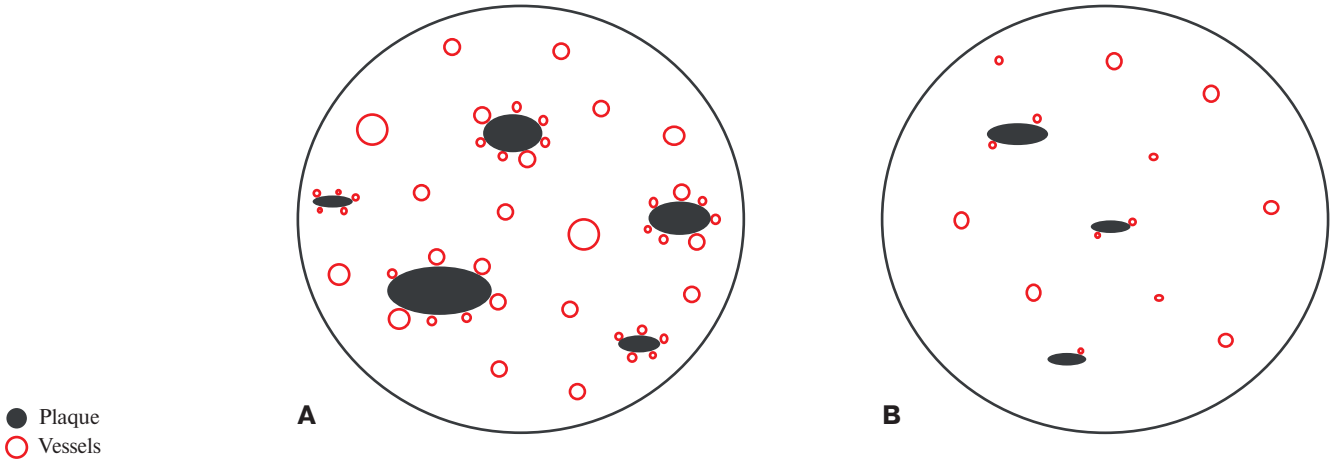

Figure 4. Arrangement of neointimal vessels in the plaque. **A** — calcifying phenotype, **B** — non-calcifying phenotype.

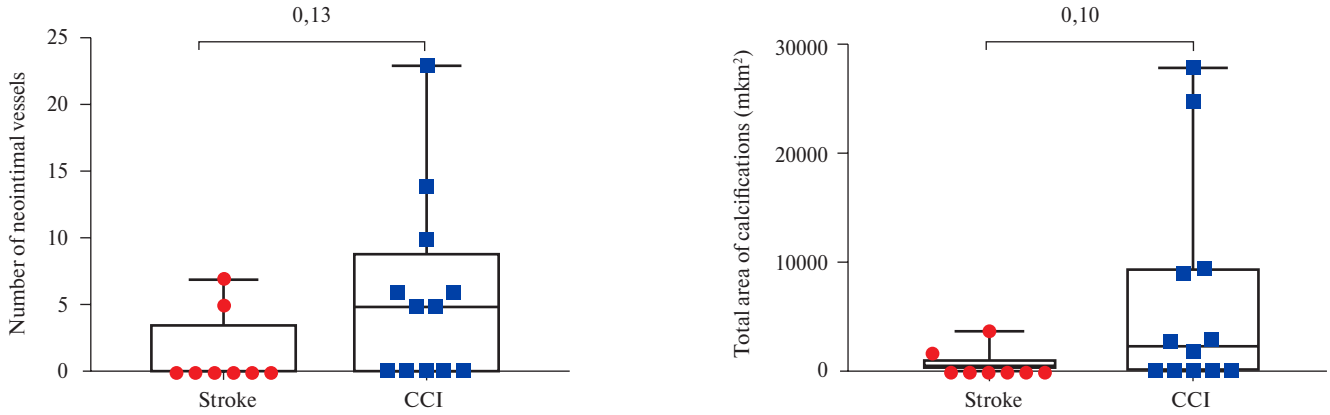

Figure 5. Total number of neointimal vessels around the representative calcification (left) and the total area of neointimal vessels around the representative calcification (right). Mann-Whitney test. P values are shown above the graphs.
Abbreviation: CCI — chronic cerebral ischemia.

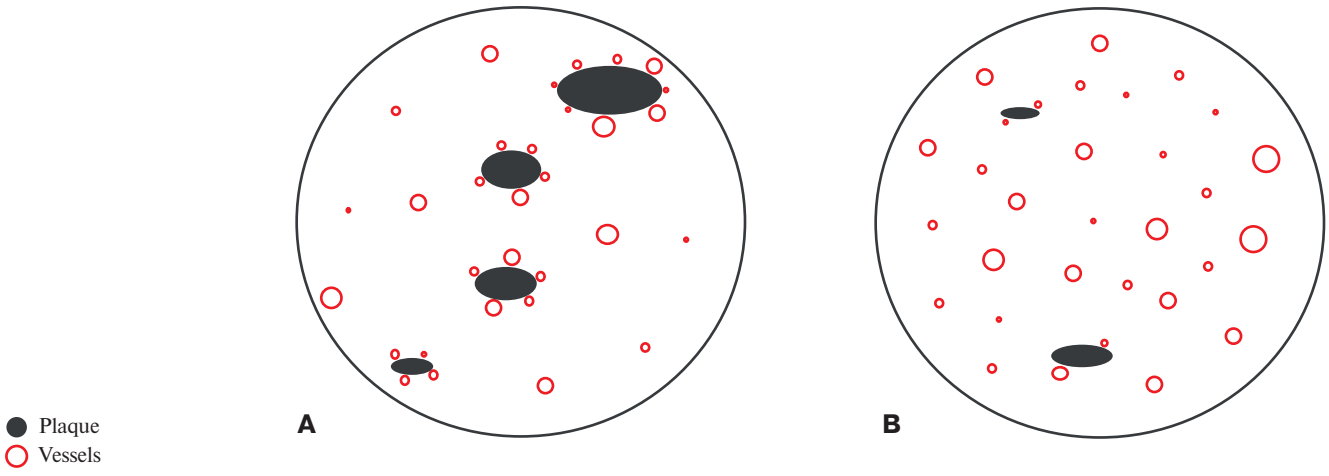

Figure 6. Arrangement of neointimal vessels in the plaque. **A.** Stable phenotype. **B.** Unstable phenotype.

Thus, the following paradox arises: the calcification contributing to plaque stabilization progresses due to vascularization, which, however, is associated with an unstable plaque phenotype. To explain this paradox, we studied the association of the extent of plaque general and local blood supply with its stability/instability. It was found that active blood delivery near calcification is associated with a stable plaque phenotype (Figure 5), in contrast to the general one, which is associated with plaque growth

leading to fibrous cap rupture. Therefore, satisfactory local blood supply to neointimal calcifications contributes to its enlargement and plaque stability, while poor general blood supply contributes to plaque instability (Figure 6).

Finally, it was investigated whether plaque calcification was associated with above mineral homeostasis parameters. Both the total area and the number of plaque calcifications negatively correlated with levels of total protein (-0,37 and -0,38, respectively) and albumin (-0,34 and -0,40, respectively), reflecting the neointimal calcification during depletion of calcium ion depot. At the same time, the total calcification area also negatively correlated with phosphorus level (-0,48), and the number of calcifications — with the total calcium level (-0,38), which may indicate a partial transition of these ions from serum to ectopic calcifications (plaques).

Discussion

It has been proven that neointimal calcification is a long-term, complex and multifactorial process that plays one of the key roles in atherogenesis, while certain calcification phenotypes are a predictor of plaque progression [13]. A in-depth study of the determinants of stable and unstable atherosclerotic phenotypes made it possible to reveal the factors contributing to plaque stabilization. According to the literature, an increase in the total calcification area contributed to stabilizing effect [9-11, 14]. This was revealed in this study as well. Therefore, one may assume that the calcification severity determines the stable plaque phenotype and prevents its rupture. The stabilizing effect of calcification is manifested in a decrease in stenosis degree by restraining plaque from growing into the vessel lumen.

New neointimal vessels saturate the plaque with oxygen and nutrients necessary for its metabolism, but at the same time they also contribute to plaque proliferation, thereby increasing the stenosis degree. Some authors showed that active blood supply to the carotid plaque aggravates the clinical prognosis and leads to an increased risk of ischemic stroke [12]. As the plaque grows, the ECA stenosis degree increases. With a decrease in the vessel lumen, the transmural pressure in new neointimal vessels increases, which destabilizes the hemodynamic response of the plaque to blood pressure changes and leads to its even greater expansion during systole [12]. According to literature data, the network of newly formed vessels occupies up to 14% of neointimal tissues [12]. These vessels are characterized by frequent intraplaque hemorrhage and an increased pressure gradient in the neointimal tissues due to a lumen decrease and transverse pulsating movements of the neointimal vessels [12].

A detailed study of neointimal blood delivery made it possible to establish that its vascularization contributes not only to plaque growth, but also to calcification progression. When studying the role of vascularization in plaque calcification, attention should be paid to location of intraplaque vessels. The active general neointimal blood supply is the most characteristic of the unstable phenotype (stroke). At the same time, active local blood supply promotes the calcification growth and, consequently, the plaque stabilization and the development of a stable phenotype (CCI).

At a certain stage of plaque development, neointimal cells die due to impaired homeostasis and a lack of oxygen and nutrients with a critical extracellular matrix thickening. Plaque phagocytes cease to efficiently process dead cells [15]. The starting point for calcification onset can be the deposition of microscopic calcium granules on the remains of dead cells and necrotic nucleus of plaques. Other important aspects of calcification are osteogenic differentiation of vascular smooth muscle cells and high activity of phosphate-generating enzymes, in particular, alkaline phosphatase [14]. It can be assumed that the proliferation of neointimal vessels in the area of newly formed calcification contributes to constant delivery of mineral ions and bioactive substances necessary for calcification.

Conclusion

An in-depth study of the relationships between the ECA plaque phenotypes revealed that the stable phenotype (CCI) is characterized by neointimal calcification (contributing to a decrease in stenosis degree) and active local blood supply directly in the area of macrocalcification. The active general neointimal blood supply contributes to a non-calcifying unstable phenotype (stroke), which contributes to the rapid plaque proliferation into the vessel lumen, the progression of atherosclerosis and development of adverse events. Neointimal blood supply specifics significantly effect the calcification processes, and various types of neointimal calcification make a significant contribution to plaque phenotype formation.

Relationships and Activities. The study (design, experiments, analysis and interpretation of data) was supported by a Complex Program of Basic Research of the Siberian Branch of the Russian Academy of Sciences within the Basic Research Topic of Research Institute for Complex Issues of Cardiovascular Diseases № 0546-2019-0002 “Pathogenetic basis for the development of cardiovascular implants from biocompatible materials using patient-oriented approach, mathematical modeling, tissue engineering, and genomic predictors”.

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