ULTRASTRUCTURAL ANALYSIS OF SMALL BLOOD VESSELS IN SKIN BIOPSIES IN CADASIL

VESNA LAČKOVIĆ[1], M. BAJČETIČ[1], NADEŽDA ŠTERNIĆ[2], V. KOSTIĆ[2], JASNA ZIDVERC[2],
ALEKSANDRA PAVLOVIĆ[2], MAJA LAČKOVIĆ[3], and M. KOČICA[4]

[1]*Institute of Histology and Embryology, Faculty of Medicine,* 11100 Belgrade, Serbia
[2]*Institute of Neurology, Clinical Center of Serbia,* 11000 Belgrade, Serbia
[3]*Institute of Psychiatry, Clinical Center of Serbia,* 11000 Belgrade, Serbia
[4]*Institute for Cardiovascular Diseases, Clinical Center of Serbia,* 11000 Belgrade, Serbia

Abstract — Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited small- and medium-artery disease of the brain caused by mutation of the *Notch3* gene. Very often, this disease is misdiagnosed. We examined skin biopsies in two members of the first discovered Serbian family affected by CADASIL. Electron microscopy showed that skin blood vessels of both patients contain numerous deposits of granular osmiophilic material (GOM) around vascular smooth muscle cells (VSMCs). We observed degeneration of VSMCs, reorganization of their cytoskeleton and dense bodies, disruption of myoendothelial contacts, and apoptosis. Our results suggest that the presence of GOM in small skin arteries represents a specific marker in diagnosis of CADASIL.

Key words: CADASIL, GOM, skin biopsy, dermal blood vessel, ultrastructure, myoendothelial contacts, apoptosis

INTRODUCTION

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited, non-amyloid, and non-atherosclerotic disease of small and medium sized arteries, predominantly in the central nervous system, and is commonly overlooked or misdiagnosed (Tournier-Lasserve et al., 1993). Pathological changes are not limited to the cerebral arteries. The same pathological findings can be seen in medium-sized and small arteries of almost all organs (Chabriat et al., 1995).

CADASIL is characterized by transient recurrent subcortical ischemic strokes occurring at an average age of age of 45 (from 27 to 65) (Joutel et al., 1997) in the absence of risk factors such as hypertension and dislipidemia (Kalimo et al., 1999). Migraine attack (followed by ischemic attacks) with or without aura is commonly observed as an initial symptom in 30% of patients (Joutel et al., 1997). Afterwards, mood disorders, memory disturbances and progressive stepwise cognitive decline, and dementia appear in the course of the disease. All patients with symptoms have prominent symmetrical and extensive hypersignals within the cerebral white matter on brain magnetic resonance imaging (MRI) (Joutel et al., 1997).

CADASIL is caused by single mis-sense mutations or small deletions in *Notch3*, a gene located in chromosome 19q12 which encodes the large Notch3 transmembrane receptor (Kalimo et al., 2002). More than 70% of mis-sense mutations are in exons 3 and 4 (Joutel et al., 1996), which encode the first five EGF repeats of the Notch3 receptor. These mutations lead to an odd number of cysteine residues in the extracellular ligand-binding domain of the *Notch3* protein (Joutel et al., 1997). Mutations in the Notch3 gene lead to an accumulation of abnormal Notch3 ectodomains (Notch3[ECD]) on the cell membrane of VSMCs, leading to the loss of normal function of blood vessels. The Notch3 receptor

plays an essential role in the development, function, and maintenance of VSMCs. It is very important in determining the fate of VSMCs, enhancing their growth while inhibiting apoptosis and migration (S w e e n e y et al., 2004).

From the pathomorphological point of view, CADASIL is a generalized angiopathy characterized by degeneration of VSMCs and accumulation of GOM either within the thickened basal lamina or free between VSMCs. Also, CADASIL patients show abnormal accumulation of Notch3^{ECD} at the cell membrane of VSMCs in both brain vessels and peripheral small arteries.

The presence of GOM is a pathognomonic finding in CADASIL. Its detection had a sensitivity of 96% and specificity of 100% for diagnosis of this angiopathy (K a l a r i a et al., 2004). Diagnostic criteria for CADASIL also include immunohistochemical detection of the Notch3 ectodomain in skin blood vessels and the presence of mutation in the Notch3 gene. The diagnosis must therefore be established either by ultrastructural and immunohistochemical analysis of skin blood vessels and/or by genetic testing. Gene defects could be detected in only 80% of cases (J o u t e l et al., 1997).

METHODS

We present two patients (43- and 47-year-old brothers) admitted for treatment at the Institute of Neurology with diffuse white matter changes on MRI. Samples of skin biopsy were fixed in 3% glutaraldehyde and post-fixed in 1% osmium tetroxide. After dehydratation, tissue samples were embedded in Epon. In both cases, we analyzed semi-thin sections stained with toluidine blue to select best blood vessels. Ultra-thin sections where mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Phillips TEM 208S).

RESULTS

Semi-thin sections of skin in CADASIL patients stained with toluidine blue and analyzed under a light microscope showed numerous blood vessels with narrowed or obliterated lumen. Thickening of the vascular wall was present in most of the observed vessels (Fig. 1). Electron microscopy in both cases revealed numerous damaged blood vessels of the dermis with altered endothelial cells and destruction of VSMCs. Numerous deposits of GOM were observed Close to VSMCs (Fig. 2).

Shapes of endothelial cells were irregular, sometimes with clear vacuoles in their cytoplasm (Figs. 2, 5). Endothelial cells showed numerous basal processes protruding into the subendothelial space (Fig. 3). At the tip of these processes, we observed dark patches, which represent accumulation of actin filaments. Myoendothelial contacts were frequently absent and the subendothelial spaces were enlarged (Fig. 3).

Smooth muscle cells in the wall of examined dermal small arteries are irregular in shape with attenuated cytoplasm (Fig. 2). Detailed ultrastructural analysis in both cases showed degeneration of VSMCs including appearance of electron-lucent vacuoles in their altered cytoplasm (Figs. 4, 5). In addition, their cytoplasm showed disoriented cytoskeletal elements and the presence of irregularly arranged large and numerous dense bodies (Fig. 6). Smooth muscle cells appeared separated from their neighboring cells. Degeneration and loss of VSMCs leads to abnormal enlargement of the space between these cells (Fig. 2).

Further analysis revealed numerous accumulations of GOM located either between degenerating smooth muscle cells or in their indentations, often within the thickened basal lamina (Figs. 3, 4, 6). Deposits of GOM varied in size, shape, granular composition, and electron density (Fig. 6).

Finally, ultrastructural analysis of small arteries in one of two cases revealed chromatin condensation and peripheral aggregation of nuclear material in several VSMCs, suggesting apoptotic cell death (Fig. 5). The presence of apoptotic VSMCs with marked destruction of the vascular wall in this case was in correlation with MRI findings and the severity of clinical manifestations.
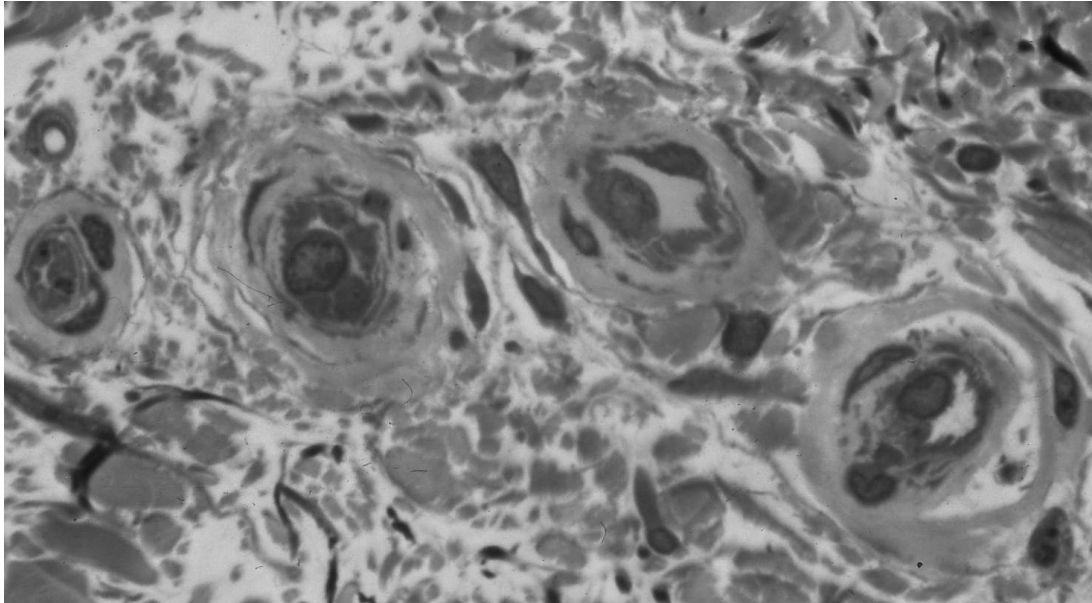
Fig. 1. Semi-thin sections of numerous blood vessels with thickened walls and narrowed or obliterated lumen (x 320).
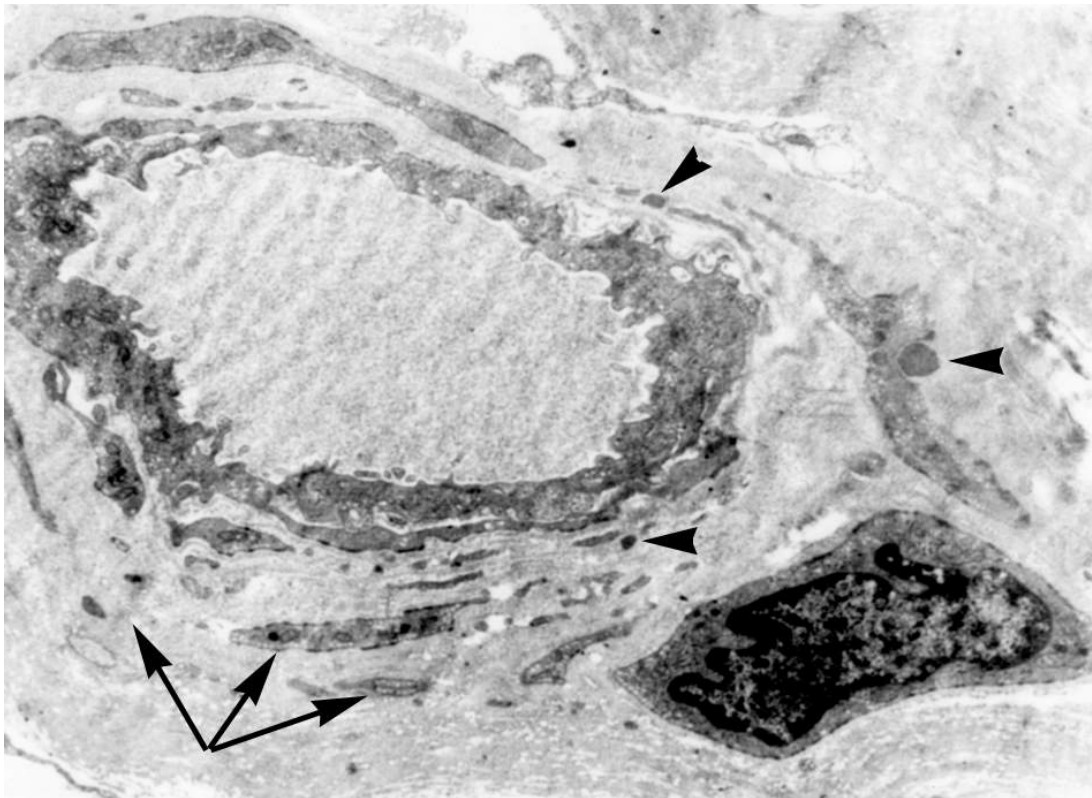

Fig. 2. An arteriole in the dermis. Degeneration and loss of VSMCs (arrows) and presence of numerous GOM deposits (arrowheads). TEM x 3200.
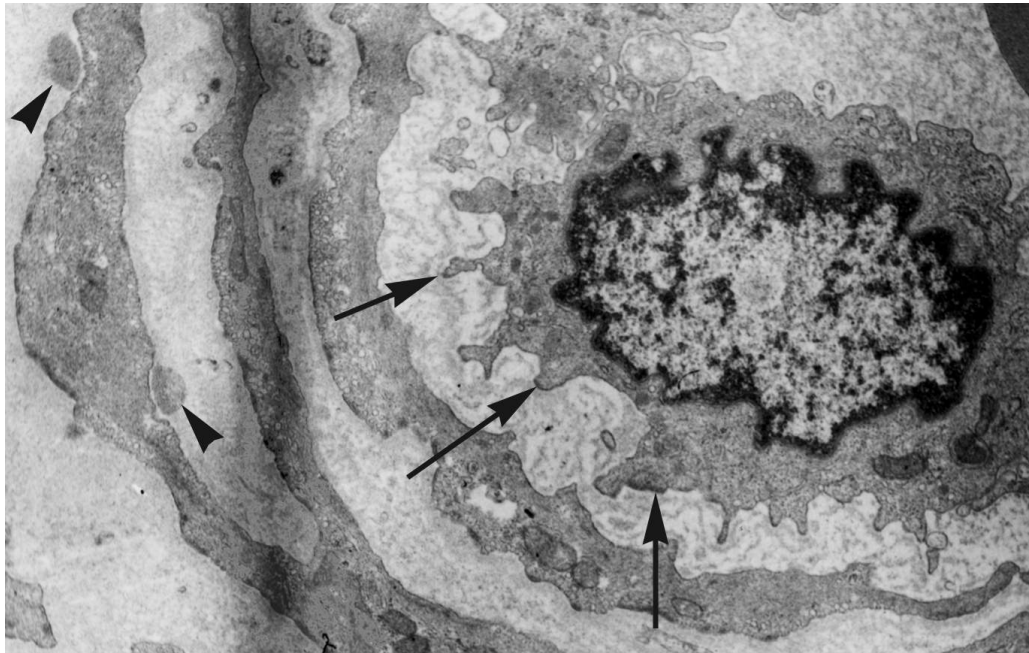
Fig. 3. Disruption of myoendothelial junctions (arrows) and presence of GOM deposits (arrowhead). TEM x 16000.
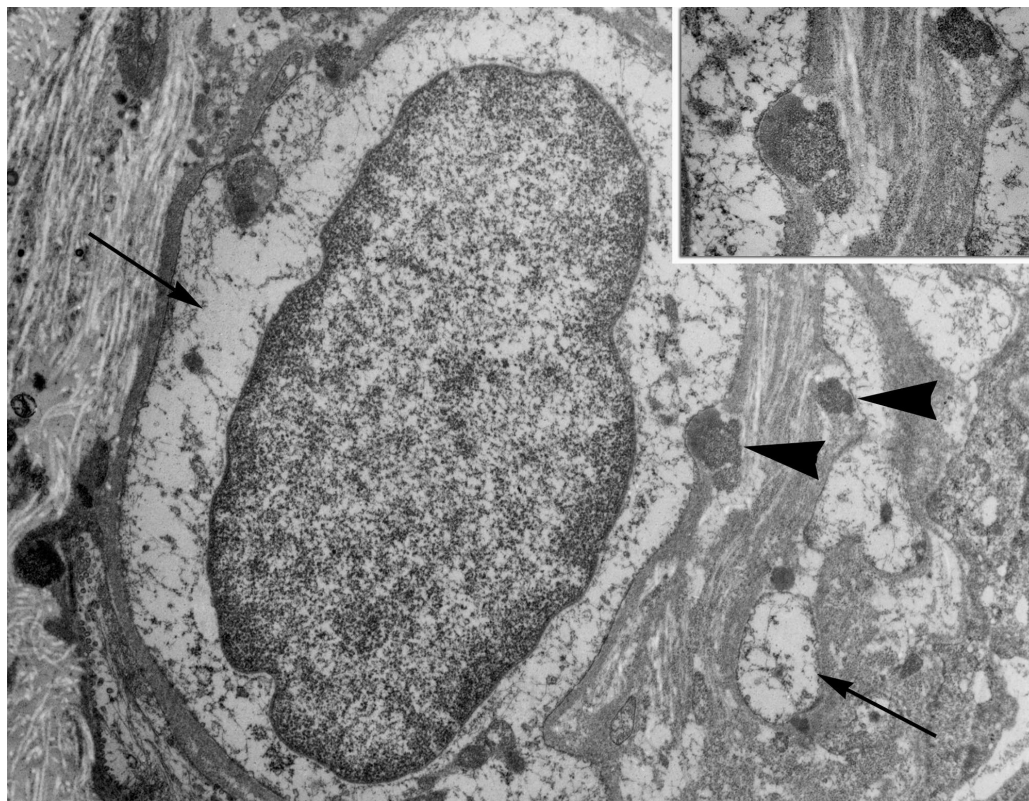

Fig. 4. An arteriole with degenerated VSMCs (arrows) and GOM in basal lamina (arrowheads). TEM x 5000. The inset shows GOM in higher magnification. TEM x 25000.

DISCUSSION

CADASIL is a general angiopathy characterized by recurrent subcortical ischemic strokes. All clinically affected patients exhibited signal abnormalities of the white matter on MRI. These findings agree with recent studies and demonstrate that cranial MRI is a sensitive tool for screening patients for CADASIL, even when the initial symptoms are unusual (B o u s s e r and T o u r n i e r - L a s s e r v e, 2001). However, these signal alterations on MRI - often characteristic of advanced stages of CADASIL - are uncertain and non-specific. On the other hand, genetic analysis sometimes may be difficult due to the large size and various mutations of the *Notch3* gene.

Our study demonstrates that skin biopsy is reliable for establishing a diagnosis of CADASIL. Even clinically asymptomatic patients with minimal MRI abnormalities show the characteristic pathological changes of the small arteries, with GOM deposits near or within the thickened basal lamina of VSMCs. The specific finding of GOM deposits so far appears to be unique and pathognomonic for CADASIL (E b k e et al., 1997). It is very important in differential diagnosis of a wide spectrum of diseases presenting MRI-detectable white matter lesions such as MELAS (mitochondrial encephalopathy with lactic acidosis and stroke-like episodes), amyloid angiopathy, familial hemiplegic migraine, antiphospholipid antibody syndrome, Sneddon's syndrome, and subcortical arteriosclerotic encephalopathy (Binswanger's disease) in patients without family history (K a l i m o et al., 1999).

Until recently, the biochemical nature, origin, and function of GOM deposits were unknown. The material usually stains with PAS, consistent with acid polysaccharide. Other histochemical stains have shown that the material does not contain amyloid, elastin, chromatin, calcium, or iron (L a p o i n t et al., 2000). Also, analysis by electron energy loss spectrometry showed no metal or mineral material within the GOM (S c h r ö d e r et al., 2000). Immunohistochemical studies have also shown the absence of immunoglobulins or complement proteins, as well as HSP 70, cystatin C, transthyretin, gelsolin, fibrinogen, ubiquitin, cathepsin D, and α1-antichymotrypsin (L a p o i n t et al., 2000). Some results from other immunohistochemical studies suggest that the GOM may come from VSMCs or be debris of degenerated muscle cells or basal lamina (B e r g m a n n et al. 1996; R u b i o et al., 1997). However, a recent study showed that the GOM is composed predominantly of accumulation of Notch3^{ECD} (I s h i k o et al., 2006).

The pathogenesis of CADASIL remains unclear at present (K a l a r i a et al., 2004). Despite the appearance of GOM in the wall of small and medium arteries in numerous organs (kidney, heart, lung, liver), clinical manifestations are only observed in the central nervous system, suggesting that topographic differences in the structure of the blood vessels as well as the blood-brain barrier may play a role in the pathogenesis of this disorder (H a g e l et al., 2004). Brain tissue, as no other human tissue, depends on a continuous blood supply. Thus, defective mechanotransduction to shear and tensile stress might reduce cerebral blood flow sufficiently to cause ischemic cell injury (D u b r o c a et al., 2005).

Our study showed the absence of myoendothelial contacts and enlargement of subendothelial spaces (Fig. 3). Disruption of these contacts, with consequent impairment of structure and function of their gap junctions, may result in breakdown of signal transduction between endothelial and smooth muscle cells, influencing vascular wall homeostasis and vasomotor response (H a e f l i g e r et al., 2003).

According to our findings, certain modifications of cytoskeletal arrangement, particularly of actin filaments in both endothelial and smooth muscle cells, point to a possible role for altered focal adhesions (FAs) within the vascular wall in the pathogenesis of CADASIL. Focal adhesions ensure that endothelium and medial smooth muscle cells act as contiguous tissue, with high spatial and functional coordination in response to various influences including shear force, cyclic stretch, angiogenetic signals, and proinflamatory stimuli (R o m e r et al., 2006). These influences alter adhesive relationships between endothelial and smooth muscle cells, as well as the relationship between these cells and
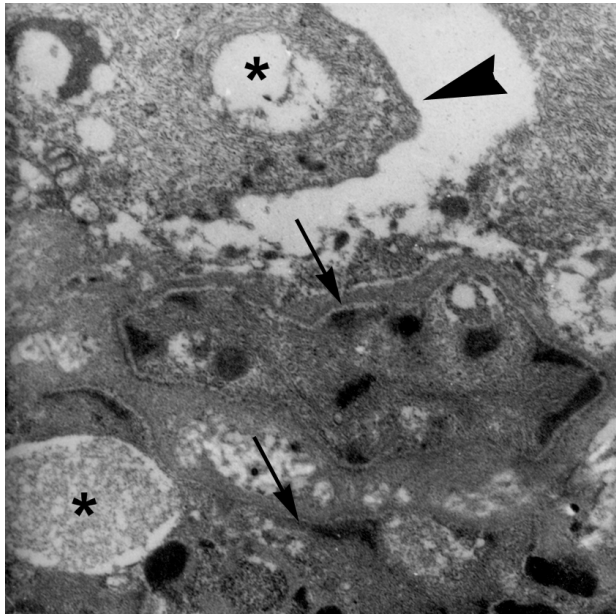
Fig. 5. An arteriole from skin biopsy showing apoptotic nuclei of VSMCs (arrows) and degenerated endothelial cells (arrowhead) with clear vacuoles in both types of cell (asterisks). TEM x 8000.

Fig. 6. Vascular smooth muscle cells with numerous irregularly arranged large dense bodies (arrowhead) and GOM (arrows). TEM x 13000.

the extracellular matrix, by affecting the transcellular actin meshwork and rearrangement of FAs to the cell periphery (I n g b e r, 1997). This supports the enhancement of a peripheral cortical actin rim that is essential to endothelial function (B i r u k o v et al., 2002) and may explain our observation of actin accumulation in the basal cytoplasmic processes of endothelial cells. Presumably altered FAs in CADASIL patients may be in relationship with both the disoriented cytoskeletal structures and the appearance of irregularly arranged and large dense bodies that we observed (Fig. 6).

Although previous studies did not reveal apoptotic VSMCs in CADASIL patients (D o m e n g a et. al., 2004), we observed several smooth muscle cells of the arteriolar wall in the apoptotic process (see Fig. 5). W a n g et al. (2002) demonstrated that Notch3 signaling is a critical determinant of VSMC survival. They showed that Notch3 receptor activation induced an anti-apoptotic phenotype in VSMCs. Therefore, a defective Notch3 receptor may lead to apoptosis and degeneration of VSMCs, suggesting the existence of a vascular mechanism that leads to ischemic blood flow and its consequences on surrounding white matter. Our study demonstrates that findings of GOM deposits in skin biopsy is sufficient for confirmation of a diagnosis of CADASIL, inasmuch as ultrastructural analysis of the skin is a useful tool for screening and differential diagnosis of patients suffering from migraines, subcortical strokes, and dementia in the absence of vascular risk factors. However, R u b i o et al. (1997) reported a patient without positive family history in whom a brain biopsy demonstrated neuropathological evidence of CADASIL, whereas blood vessels in muscle and skin biopsies failed to show the presence of GOM deposits. This is likely due to the fact that changes in the skin may be focal, or else the biopsy may not have been deep enough. The possibility of false negative results therefore exists. In these cases, molecular genetic testing is required, but in comparison with genetic analysis electron microscopy is easier, less expensive, and probably equally sensitive (F u r b y et al., 1998).

In conclusion, our results confirm the role of vascular dysfunction in the pathogenesis of CADASIL

and correlation of structural abnormalities in the wall of small arteries and arterioles with clinical manifestations of this disease. We were able to demonstrate disruptions of myoendothelial contacts and apoptosis of VSMCs, suggesting that these events may play an important role in the pathogenesis of CADASIL. In addition, we propose that an impaired FA signaling mechanism could also play an important role in the pathogenesis of this disease. Our study supports the view of CADASIL as a systemic vascular disease. Electron microscopic examination of skin biopsy is a highly specific and relatively sensitive diagnostic method for establishing a diagnosis of CADASIL. As it is easy to perform and cost-effective, skin biopsy can be considered the method of first choice.

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УЛТРАСТРУКТУРНА АНАЛИЗА МАЛИХ КРВНИХ СУДОВА У БИОПСИЈИ КОЖЕ КОД CADASIL-А

ВЕСНА ЛАЧКОВИЋ[1], М. БАЈЧЕТИЋ[1], НАДЕЖДА ШТЕРНИЋ[2], В. КОСТИЋ[2], ЈАСНА ЗИДВЕРЦ[2], АЛЕКСАНДРА ПАВЛОВИЋ[2], МАЈА ЛАЧКОВИЋ[3] и М. КОЧИЦА[4]

[1]*Институт за хистологију и ембриологију, Медицински факултет,* 11000 Београд, Србија
[2]*Институт за неурологију, Клинички центар Србије,* 11000 Београд, Србија
[3]*Институт за психијатрију, Клинички центар Србије,* 11000 Београд, Србија
[4]*Институт за кардиоваскуларна обољења, Клинички центар Србије,* 11000 Београд, Србија

Церебрална аутозомно доминантна артериопатија са субкорталним инфарктима и леукоенцефалопатијом (CADASIL) је наследно обољење малих артерија мозга чији је узрок мутација *Notch3* гена. Веома често ова болест се погрешно дијагностикује. У овом раду испитиване су биопсије коже два члана породице код које је први пут дијагностикован CADASIL у Србији. Електронска микроскопија је показала да крвни судови дермиса оба пацијента садрже бројне депозите гранулираног осмиофилног материјала (GOM) око васкуларних глатких мишићних ћелија код којих је уочена дегенерација, реорганизација цитосклета и густих тела, прекиди миоендотелних спојева и апоптоза. Наши резултати указују да налаз GOM-а у малим артеријама дермиса представља специфичан маркер за дијагностиковање CADASIL-а.