Review paper

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Mysteries of CADASIL – the contribution of neuropathology to understanding of the disease

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Abstract

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a systemic vascular disease characterized by impairment of vascular smooth muscle cell (VSMC) structure and function related to NOTCH 3 *mutations. Clinically the syndrome is manifested as recurrent ischaemic strokes, migraine with aura, dementia and psychiatric symptoms. In spite of intensive investigations, there is relatively little insight into the underlying pathomechanisms that link VSMC with the Notch 3 signalling pathway, morphological changes and clinical symptoms. The introduction into neuropathology of novel immunohistochemical and molecular techniques opened new research and diagnostic perspectives in CADASIL studies. We present a review of current concepts regarding CADASIL pathogenesis, clinical picture and diagnosis in which neuropathological examinations played a key role.*

Key words: *CADASIL, GOM, Notch 3, ischaemic stroke, vascular dementia.*

Introduction

Although it is claimed sometimes that the "golden age" of neuropathology has passed, the introduction of novel immunohistochemical and molecular techniques has opened new research and diagnostic perspectives in an "old" discipline. Neuropathological studies in CADASIL are an example of such new possibilities that turned out to be very helpful in explaining the disorder's pathogenesis, clinical picture and diagnosis.

Over twenty years ago genetic studies and morphological examinations of brains from patients suffering from migraine, recurrent ischaemic strokes and progressing dementia allowed the diagnosis of a new syndrome called cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [3]. Numerous histopathological investigations revealed that CADASIL is a systemic vascular disease characterized by impairment of vascular smooth muscle cell structure and function related to *NOTCH 3* gene mutations on chromosome 19 [21]. These mutations lead to synthesis of abnormal Notch 3 receptor expressed in human blood vessels on vascular smooth muscle cells (VSMC) and pericytes.

On autopsy examination of CADASIL brain multiple small (lacunar) infarcts in the white matter and/or deep grey matter are found, whereas the cerebral cortex is usually well preserved. Lacunar infarcts are localized mainly in brain hemispheres, but they can

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also be seen in the brain stem and, occasionally, in the spinal cord. Sometimes, the microbleeds are visible but parenchymal brain haemorrhages are relatively uncommon. On light microscopy degeneration and loss of VSMC in middle and small-sized arteries, basophilic granular degeneration of vascular media, vessel fibrosis and hyalinization and enlargement of perivascular space are seen. In immunohistochemical examination, the most characteristic finding is the accumulation of Notch 3 receptor extracellular domain (N3-ECD) in the vessel wall. On electron microscopy, deposits of granular osmiophilic material (GOM) pathognomonic for the disease are observed in the tunica media. Deposits of GOM are located either free between degenerating VSMC within the thickening basal lamina or in indentations of VSMC cytoplasmic membrane.

Contribution of neuropathology in research on CADASIL

Pathogenesis

Understanding the regulatory mechanisms underlying CADASIL pathology is the most important area of the disease research. At present, there are a few theories trying to explain the pathogenesis of the disease but none of them has yet shown conclusive evidence.

Disturbances in Notch 3 signalling pathway

Although it has been a decade since *NOTCH 3* was found to be the causative gene for CADASIL, the molecular mechanism underlying the syndrome pathology remains unclear.

Notch 3 is an evolutionarily conserved signalling pathway. It is known that upon binding of ligands presented on a neighbouring cell, the receptor undergoes two proteolytic cleavages. First Notch 3 is cleaved on the extracellular side by the TNF-α converting enzyme (TACE), then within the plasma membrane by γ-secretase, which releases the intracellular domain (N3-ICD) of the receptor. After the second cleavage, N3-ICD translocates to the cell nucleus, interacts with DNA and triggers a cascade of events leading to gene expression (Fig. 1).

In the postnatal period, in the vascular system Notch 3 signalling is involved in many various processes such as cell-cell and cell-matrix communication, maturation of arterial vessel wall, phenotypic stability of VSMC, promotion of VSMC survival and protection from apoptosis, regulation of actin cytoskeleton, and response of VSMC to mechanical stretching of the vessel wall by blood pressure (references in [54]). So numerous and complex roles of Notch 3 as well as its context-dependent functions make investigations on CADASIL pathogenesis very complicated. Moreover, it has been reported that Notch 3 ligands can exert a concentration-dependent effect [39] that additionally makes investigations on CADASIL difficult.

Experimental studies demonstrated that young Notch 3 knockout mice develop normally and there are neither arterial abnormalities nor GOMs which appear in the adult animals [7] and in transgenic mice expressing mutated Notch 3 [44]. In a human fetus with *NOTCH 3* mutation, neither GOM deposits nor vascular defects were found [29]. In CADASIL patients, the first symptoms appear in adults in the third-fourth decade of life. These data indicate that in CADASIL disturbances in Notch 3 signalling appear in the postnatal period.

It is possible that as a result of mutations in the *NOTCH 3* gene, the receptor may gain or lose its function, resulting in disturbances in the Notch signalling pathway. Culture studies demonstrated that cells expressing mutated Notch 3 show normal signalling

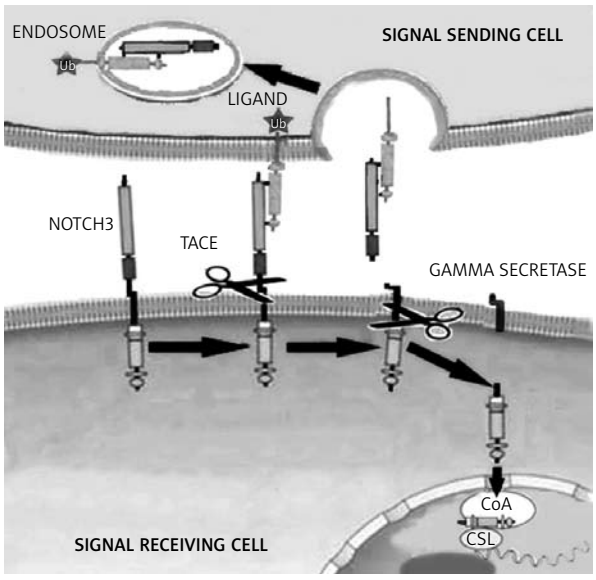

Fig. 1. Notch 3 signalling pathway

activity [41]. Diminished Notch 3 signalling was found only in a few mutations located at the ligand-binding site of the receptor. Moreover, in patients with mutation in the region of Notch 3 responsible for receptor dimerization, constitutive ligand-independent receptor activity was reported but neither GOM nor N3-ECD accumulation was found [10]. These findings imply that neither loss nor gain of Notch 3 function is directly responsible for CADASIL pathology.

Many studies have revealed that Notch 3 mutations in ligand-binding and non-ligand-binding sites cause similar symptoms. The phenotypic picture of CADASIL varies even in families with the same *NOTCH 3* mutation and clinical course in homozygous and heterozygous CADASIL patients is similar [52]. These clinical observations suggest that variations in the disease clinical picture can be due to a modifying effect of genetic factors distinct from the causative *NOTCH 3* mutations.

Since direct impairment of classical Notch 3 signalling seems to be not or not solely the cause of CADASIL, the pathomechanism of the disease may be connected with an alternative Notch 3-mediated pathway or an unknown factor(s) which modifies Notch 3 signalling. Since Notch 3 receptor can exist either as a heterodimeric form or as an intact colinear protein [16], the first hypothesis seems to be especially promising.

Recently, a novel mechanism of VSMC regulation by Notch 3 through platelet-derived growth factor receptor-β (PDGFR-β) has been suggested. PDGFR-β is a key determinant of VSMC biology both in ontogenesis and the postnatal period. In the postnatal period, PDGFR-β is released during vascular injury and decreases Notch 3 expression and signalling, induces loss of contractile phenotype of VSMC, their dedifferentiation, proliferation and migration. Culture study demonstrated that stimulation of human CADASIL VSMC by Notch 3 ligand resulted in diminished expression of PDGFR-β in comparison to control cells [20]. The authors postulated that PDGFR-β can be a direct target of activated Notch 3 receptor and a potential function of *NOTCH 3* in the postnatal period may be to temper the pro-migratory and anti-differentiation effect of platelet-derived growth factor.

Endocytosis of Notch 3 receptor

Like many other membrane receptors, Notch 3 undergoes endocytosis in which a substantial amount of the receptor is targeted for degradation. After cleavage by TACE, N3-ECD is shed from the surface of the signal receiving cell and as a complex with ligand is internalized by the signal sending cell. This process requires binding to ubiquitin. In CADASIL, increased expression of ubiquitin in the vessel wall was found [9]. Since experimental data proved that intracellularly located mutated Notch 3 protein is not bound to ubiquitin [26], increased ubiquitin expression in CADASIL may be a consequence of malfunction of other cellular processes requiring ubiquitination such as endocytosis.

Both in CADASIL patients and the animal model of the disease, GOM are often located in indentations of degenerating VSMC which resemble forming endocytic vesicles (Fig. 2). In VSMC indentations beneath GOM, abundant numerous calveolae are observed. These vesicular structures participate in endocytosis and may be related to the formation of GOM [24]. Notch receptors mutated only in the extracellular N-terminus, but not in the intracellular C-terminal domain, accumulate to abnormal levels and exhibit a reduced rate of internalization [2]. It is noteworthy to stress that in CADASIL, all known mutations are located in the extracellular part of Notch 3. All the above-mentioned findings suggest that disturbed endocytosis of the mutated N3-ECD may be responsible for the accumulation visible in CADASIL of Notch 3 protein in the vessel wall. But the consequences of disturbed N3-ECD endocytosis go much further. Abnormal endocytosis in ligand-expressing cells means that altered Notch signalling

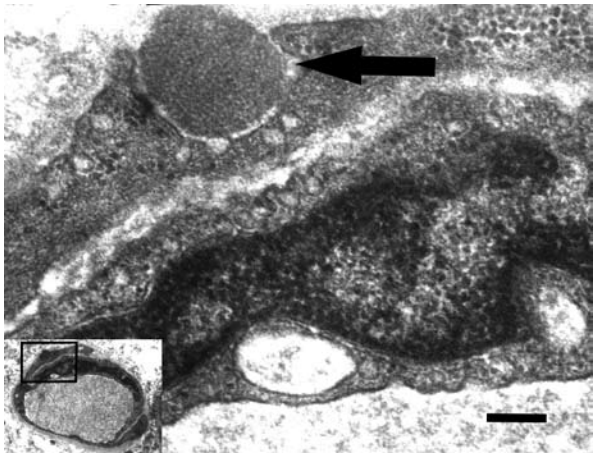

Fig. 2. GOM located in indentations of the degenerating VSMC (arrow). Around GOM visible numerous caveolae; bar 200 nm. Insert: small skin artery with GOM; bar 1 µm

is in signal-sending, not in signal-receiving cells. Our hypothesis can also explain why Notch 3 signalling is preserved in cells with the mutated receptor independently of location of the mutation.

Accumulation of N3-ECD in vessel wall

It is not clear whether CADASIL pathology occurs as a direct consequence of perturbed Notch 3 signal regulation or as an indirect consequence of the abnormal accumulation of the Notch 3 protein.

Like in other degenerative diseases, accumulation of N3-ECD may be a result of misfolding and aggregation of the mutated Notch 3 protein. Mutations in the *NOTCH 3* gene lead to synthesis of the protein with a changed number of cysteine residues and, in consequence, abnormal three-dimensional structure. Since misfolded proteins have revealed increased propensity to aggregate and form insoluble proteinaceous deposits, GOM, which is composed of N3-ECD [19], can be such a deposit responsible for the cytotoxic effect in VSMC in CADASIL [6].

Another possible cause of accumulation of GOM and N3-ECD may be disturbed/insufficient cellular mechanisms responsible for protein degradation. Proteome analysis of VSMC from a CADASIL patient in whom four differentially expressed proteins involved in protein degradation and folding were found [17] supports the hypothesis of dysfunction of cellular "cleaning" mechanisms in CADASIL.

Mechanism of VSMC degeneration and loss

VSMC apoptotic death was demonstrated in vascular diseases such as arteriosclerosis and aneurysm formation. Numerous studies have suggested that apoptosis is also responsible for VSMC death in CADASIL, but these suggestions are based only on the results of the terminal transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) method. However, lack of specificity of the TUNEL assay for apoptotic cells is well documented (references in [49]) and now it is believed that the TUNEL method reveals cells with damaged DNA rather than cells really undergoing apoptosis.

In transgenic mice with *NOTCH 3* mutation, apoptotic VSMC were not found [7]. In CADASIL patients, morphological evidence for VSMC apoptosis such as chromatin condensation, membrane blebbing or apoptotic bodies were not observed. Moreover, an immunohistochemical study revealed that in CADASIL brains expression of caspase 3, one of the apoptosis markers, was observed mainly in endothelial cells and pericytes [13]. In our detailed morphological study on CADASIL tissue we found expression of pro-apoptotic BAX protein (Fig. 3) and caspase 3 (Fig. 4) on single VSMC and vascular media. These findings indicate that in CADASIL, VSMC can die via caspase 3-dependent programmed cell death, although we also did not find any morphological features characteristic for apoptosis, either at light or electron microscopic level.

Numerous experimental studies have demonstrated that Notch 3 protects from apoptosis and promotes VSMC survival, but there is still a missing link between Notch 3 activation and up-regulation of the survival signals. It is also unknown what factor(s) directly triggers VSMC death.

In the transgenic mouse model of CADASIL, lost anchorage and functional impairment of VSMC were observed before GOM and N3-ECD accumulation [44]. It is known that normal cell detachment leads to its death via anoikis. Anoikis (homelessness) is a subtype of apoptotic death involving cells that have lost contact with the extracellular matrix, or that interact with the matrix in an inappropriate way. This phenomenon is observed both physiologically in normal skin keratinocytes or interstitial epithelium as well as in pathology, when neoplastic cells lose their susceptibility to anoikis and metastasize.

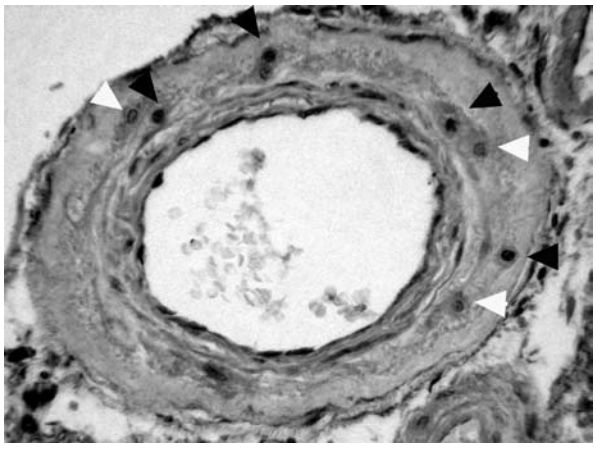

Fig. 3. Expression of pro-apoptotic BAX protein on VSMC in brain arterioles. Visible immunopositive (black arrowheads) and immunonegative (white arrowheads) VSMC nuclei; ×200

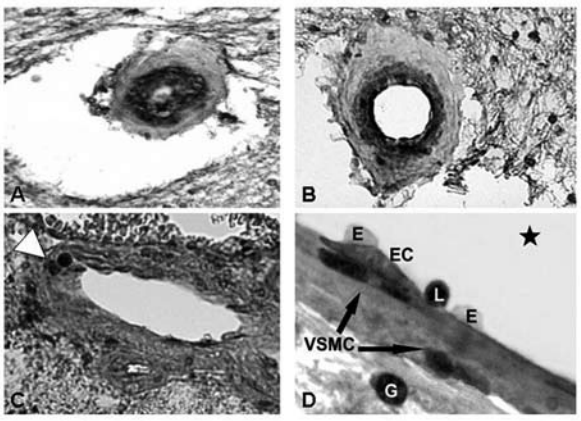

Fig. 4. Expression of caspase 3 on VSMC. A. Small white matter artery with immunoreactive vascular media; visible severe enlargement of the perivascular space; ×100. B. Small meningeal artery with immunoreactive vascular media; ×100. C. Two caspase 3-immunoreactive cells (white arrowhead) in the vascular media of the white matter small artery; ×400. D. Fragment of the wall of the middle-sized white matter artery with two caspase-3 immunoreactive VSMC. E – erythrocyte, EC – endothelial cell, G – glia, L – lymphocyte, asterix – vessel lumen; ×1000

It is known that VSMC can, under certain conditions, express tissue plasminogen activator (t-PA) [37]. Cell-associated t-PA converts plasminogen into plasmin, which may proteolyze the pericellular matrix, leading to cell detachment and subsequent anoikis.

Vessel wall and blood-derived cells can also release matrix metalloproteinases (MMP) – enzymes degrading components of the extracellular environment. It was shown that collagen fragments released by MMP might propagate VSMC apoptosis [57].

Another possible cause of VSMC damage in CADASIL can be oxidative stress. Morphological and biochemical alterations found in skeletal muscle in CADASIL patients indicate mitochondrial pathology [11]. VSMC are classified as type II cells, in which apoptosis is mainly associated with the mitochondrial-dependent signalling pathway [36]. Our finding of Bax expression on CADASIL VSMC, the protein involved in the mitochondrial-dependent apoptotic pathway, is in line with this classification. Using proteome analysis of VSMC from a CADASIL patient three differentially expressed proteins involved in cellular stress were identified [17], which supports the hypothesis of the role of oxidative stress in CADASIL. Taken together, the data indicate that oxidative stress seems to be involved in CADASIL pathogenesis, but as a secondary phenomenon due to arteriopathy and chronic hypoxia.

Involvement of other cellular elements of the vessel wall in CADASIL

Apart from VSMC, also other cellular elements of the vessel wall seem to be affected in CADASIL. Endothelial cells showed increased cytoplasm density, presence of compact bundles of microfilaments within the cytoplasm, destruction of endothelial tight junctions, irregularly shaped nuclei and widening of the subendothelial space [31,45]. Apart from morphological changes, endothelial cells also revealed functional abnormalities: impaired permeability [45], disturbed endothelium-depended vasodilatation in resistance arteries [48] and altered protein expression. Our preliminary immunohistochemical study showed that in CADASIL, endothelial expression of platelet endothelial cell adhesion molecule 1 (PECAM-1) is disturbed (Fig. 5). PECAM-1 is an adhesion molecule expressed on endothelial cells at the intercellular junctions. It suppresses "mitochondrial" Bax-dependent apoptosis, mediates transmigration of white blood cells through the endothelium, stabilizes intercellular contacts, and works as a mechanoresponsive molecule which is chemically modified when a direct mecha-

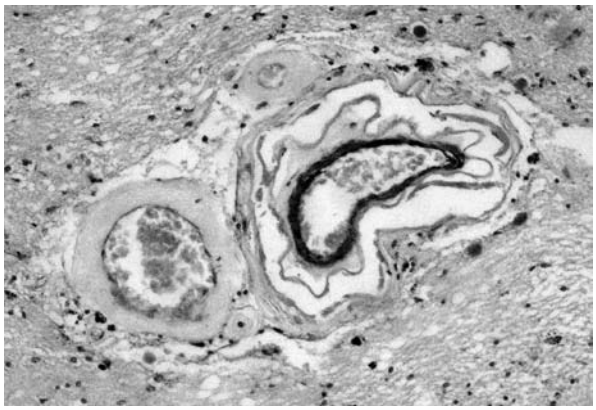

Fig. 5. Disturbances in endothelial PECAM-1 expression in the white matter vessels. Visible lack of the protein expression in two small arteries while vessel on the right side reveals increased immunostaining; ×100

nical force is applied to it [12]. As a consequence of abnormalities in PECAM-1 expression, increased vessel permeability and perivascular microbleeds can develop. Penetration of different factors from the blood plasma may also influence the structural integrity of the vessel wall, leading to VSMC detachment and anoikis.

Also pericytes seems to be involved in the pathological process in CADASIL. In human vessels, Notch 3 expression is found only on VSMC and pericytes. Pathognomonic for the disease accumulation of GOM on the vessel wall was found in capillaries, small vessels deprived of VSMC and composed of endothelial cells and pericytes [43]. Experimental studies on *NOTCH 3* knockout mice [53] and in CADASIL patients revealed vessels lacking pericytes [32] or their degeneration [49]. Moreover, in CADASIL tissue pericytes showed expression of caspase 3 [13]. So, VSMC is not the only target in the pathological process in CADASIL. Although VSMC injury dominates, all cellular compounds of the vessel wall are affected.

Mechanisms of brain tissue damage in CADASIL

Gradual destruction of VSMC leads to progressive wall thickening, fibrosis, luminal narrowing in small and medium-sized penetrating arteries and, in consequence, to ischaemia and lacunar infarcts. But the mechanism of brain tissue injury in CADASIL is more complicated. An experimental study demonstrated that in knockout *NOTCH 3* mice the ischaemic area induced by the middle cerebral artery occlusion was larger than in control animals [1], which suggests increased susceptibility of brain tissue to stroke in CADASIL. In CADASIL patients, impaired vasodilatation of the vessel wall and reduced cerebral blood flow (CBF) was found [42,48] that additionally increased tissue ischaemia. Reduced CBF is also a very powerful inducer of VSMC apoptosis [5]. Apoptotic cells can release membrane-bound pro-coagulant microparticles into the circulation [34]. In CADASIL patients, an increased level of fibrin degradation products in the plasma and thrombosis of small cerebral arteries was detected [18]. Apoptotic VSMC can also release inflammatory cytokines responsible for recruitment of monocytes/macrophages to the surrounding tissue [46]. In CADASIL brain tissue, numerous perivascular macrophages were found (Fig. 6). Macrophages, in turn, are a source of enzymes responsible for the extracellular matrix degradation. It was also demonstrated that phagocyting macrophages can release soluble death ligands such as Fas-L, resulting in bystander death of adjacent cells [4] that could contribute to progress of VSMC damage.

Neuropathological background of CADASIL clinical picture

Migraine is present in about 40% of CADASIL patients and usually precedes other clinical symptoms of the disease. Migraine occurrence is followed by the presence of GOM and other vascular abnormalities (Fig. 7). Despite conflicting findings, migraine,

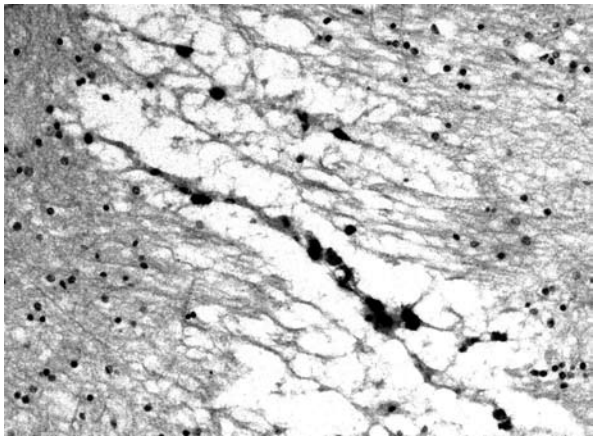

Fig. 6. Severe perivascular spongiosis and numerous CD-168 immunoreactive macrophages alongside small white matter penetrating arterioles; ×200

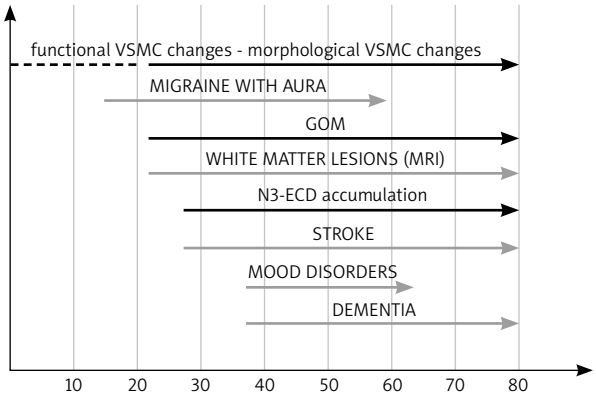

Fig. 7. Time-dependent clinico-pathological correlations in CADASIL

especially migraine with visual aura, appears to be generally associated with transient decreases in regional CBF. In a mouse model of CADASIL, impaired autoregulation and decreased relaxation (or increased resistance) of cerebral vessels was demonstrated [8,28]. In CADASIL patients, impaired endothelium-dependent vasodilatation in resistance arteries was found [48]. Also, functional impairment of VSMC was observed very early, before GOM deposits and N3-ECD accumulation [44]. So, vascular dysfunction seems to be an early pathogenic event in CADASIL that may be related to migraine development.

The main clinical presentation of CADASIL is iterative strokes in young adults despite the lack of cardiovascular risk factors. Early vascular dysfunction can promote morphological changes and development of brain ischaemia. Lacunar infarcts, mainly in the basal ganglia and fronto-temporal white matter, lead to cognitive deficits and dementia of the subcortical vascular type. But not all individuals with cognitive decline and dementia are "victims" of ischaemic strokes. Moreover, subcortical lesions alone, without affecting the main cholinergic structures, seem to be insufficient to cause dementia. A single case report suggesting cholinergic denervation in CADASIL was published [38]. Morphological evidence for cholinergic neuronal impairment was provided three years later by Keverne et al. [27], who discovered loss of cholinergic neurons in the nucleus basalis of Meynert and damage to cholinergic fibres in the external capsule. Recently, cholinergic dysfunction of motor cortex has also been confirmed by electrophysiological tests [35].

Neuropathology as a key tool in CADASIL diagnosis

The diagnosis of CADASIL has far-reaching implications not only for the patient, but also for his family members. Unfortunately, the disease is often overlooked or misdiagnosed.

CADASIL may be diagnosed well before the first stroke on the basis of genetic analysis of the *NOTCH 3* gene and presence of characteristic morphological changes in vessels in biopsy material. Since CADASIL is a generalized angiopathy, pathomorphological changes are observed not only in CNS vessels but also in the skin, muscles and internal organs. The most commonly used material in diagnostic procedures is skin biopsy; it is rarely muscle or nerve biopsy. Brain biopsy, although conclusive [55], should be avoided because of its invasiveness and risk of complications.

Differential diagnosis in CADASIL involves diseases with similar clinical picture, especially in their early stages, and disorders producing similar changes in neuroimaging (Table I). But the true challenge for neuropathologists is diseases with not only similar clinical and MRI manifestations but also with similar morphological changes (Table II).

As genetic screening of all exons of the *NOTCH 3* gene is a time-consuming process, skin biopsy offers an alternative rapid approach to molecular genetic diagnosis. However, the diagnostic value of skin biopsy varies and its negative result does not automatically exclude CADASIL.

Table I. Differential diagnosis in CADASIL

Atypical or early stage of multiple sclerosis
Familial hemiplegic migraine
MELAS syndrome (mitochondrial encephalopathy, lactic acidosis, stroke-like episodes)
Alzheimer's disease
Microangiopathies: • cerebral vasculitis • cerebral amyloid angiopathies • subcortical arteriosclerotic encephalopathy (Binswanger's disease) • hereditary small vessel diseases

Table II. Hereditary small vessel diseases

CARASIL (cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy)
Portuguese-French type familial small vessel disease [53]
Swedish type hereditary multi-infarct dementia [33]
PADMAL (pontine autosomal dominant microangiopathy and leukoencephalopathy) [14]
Autosomal dominant leukoencephalopathy [15]
Retinal vasculopathy with cerebral leukodystrophy caused by mutations in the TREX1 gene on chromosome 3 including: HERNS – (hereditary endotheliopathy with reticulopathy, nephropathy and stroke syndromes), CRV (cerebroretinal vasculopathy) and HVR (hereditary vascular retinopathy)
Hereditary systemic angiopathy with cerebral calcifications, retinopathy, progressive nephropathy and hepatopathy [56]

Accumulation of N3-ECD

Accumulation of N3-ECD in the vessel wall is detected by antibodies to N3-ECD applied in immunohistochemical methods. In dermal arteries the first deposits of N3-ECD are already detectable before the age of 20 years [22,23]. The diagnostic criterion is the presence of at least 2 immunopositive vessels in a tissue specimen. There is neither a difference in immunostaining between symptomatic and asymptomatic carriers of the *NOTCH 3* mutations nor a correlation between immunostaining and clinical or MRI parameters.

In the literature, sensitivity of immunohistochemical examination of N3-ECD in biopsy material is 85.4-96%. Sensitivity can be dependent on deepness of the skin biopsy [25]. If the biopsy is taken too superficially it contains mainly capillaries, while GOM are mostly present in deeply located small arteries. The diagnostic value of N3-ECD accumulation is also influenced by the fact that vasculopathic changes in skin biopsy can be focal [47].

Specificity of N3-ECD accumulation in skin biopsy is 95.2-98%. Lack of standardized immunostaining method and commercially available antibodies to N3-ECD impacts specificity of the reaction. Moreover, there is one report demonstrating N3-ECD accumulation in a non-CADASIL patient [29]. So, N3-ECD immunostaining of biopsy material is a supportive but not definitive diagnostic tool in CADASIL.

Accumulation of GOM

In a human fetus with a mutated *NOTCH 3* gene, deposits of GOM were not found [30]. In CADASIL patients, GOM were observed at the age of 20 years [29]. There were no correlations between the presence, number and size of GOM and severity of damage of VSMC [31].

Sensitivity of GOM examination in electron microscopy is 45-50%. The main cause of such low sensitivity is the small size of the examined specimen. Focal localization of GOM additionally influences sensitivity of the method. Detection of GOM by electron microscopy is only useful when positive. If the results of examination are negative, CADASIL cannot be ruled out.

GOM specificity is 100%, which means that this morphological change is pathognomonic for CADASIL. But GOM should be distinguished from the non-specific granular debris present between degenerative VSMC in some other diseases [52]. In the literature, there is a single report demonstrating GOM presence in a 13-yr old girl with negative family history, genetic examination of the *NOTCH 3* gene and N3-ECD accumulation [40]. Probably this case report is an example of the above-mentioned GOM-like changes.

Summary

1. The pathological process in CADASIL vessels is not limited to VSMC.
2. Loss of VSMC in vascular media is due to apoptosis.
3. Disturbances in the Notch 3 pathway probably are present in signal sending, not signal receiving cells.
4. The gold diagnostic standard in CADASIL is still genetic analysis and electron microscopy.
5. GOM are pathognomonic for CADASIL but should be distinguished from the non-specific granular debris present between degenerative VSMC in some other diseases.
6. N3-ECD immunostaining of biopsy material is a supportive but not definitive diagnostic tool. Confirmation of DNA analysis is requisite both for positive and negative results of Notch 3 immunostaining.

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