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# Are granular osmiophilic material deposits an epiphenomenon in CADASIL?

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## Abstract

*Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by mutations in the NOTCH3 gene. Pathophysiologically, there seems to be multimerization of the extracellular domain of the protein with a possible gain of function on vascular smooth muscular cells. However, the mechanisms and determinants of NOTCH3 multimerization are not completely understood, and it is not completely elucidated whether NOTCH3 multimerization contributes to the appearance of granular osmiophilic material (GOM) deposits, which are the pathological hallmark of CADASIL.*

*We recently reported a patient with parkinsonism and cognitive impairment and with evidence of diffuse white matter changes on imaging, carrying a NOTCH3 nonsense mutation in exon 3 (c.307C>T), and suggested that such a hypomorphic NOTCH3 mutation was likely to be pathogenic.*

*We further pursued ultrastructural examination of skin vessels in our case, and here we report the results, wishing to make a comment on whether GOM deposits should be considered the pathological hallmark for a definitive diagnosis of CADASIL in those patients whose mutations are predicted in the production of hypomorphic protein products.*

***Key words:*** *CADASIL, NOTCH3, granular osmiophilic material, GOM deposits.*

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common heritable cerebral small vessel disease, caused by mutations in the *NOTCH3* gene [2]. The gene encodes a single pass transmembrane protein, which is predominantly expressed in vascular smooth muscle cells (VSMC) [2]. *NOTCH3* mutations typically affect the extracellular domain ($N3^{ECD}$) within one of the 34 epidermal growth factor (EGF)-like repeat domains [2]. Each EGF-like repeat domain contains a highly conserved number of cysteine residues which seem to stabilize the $N3^{ECD}$ by the formation of disulfide bonds. Virtually, all CADASIL mutations hitherto described result

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in an uneven number of cysteine residues, leading to a multimerization of the $N3^{ECD}$ with a possible gain of function effect on VSMC [2,3]. However, mechanisms and determinants of *NOTCH3* multimerization are not completely understood.

We recently described a subject carrying a *NOTCH3* nonsense mutation in exon 3 (c.307C>T), who presented parkinsonism, cognitive impairment, and psychiatric features in his seventies. Despite the late onset, he had typical CADASIL imaging features and a positive family history for cerebral ischemic events in at least two different generations [4,13]. The variant is located in the EGF-like 2 region of exon 3 and causes the substitution of arginine with a stop codon at position 103 of the protein (p.R103X). The formation of such a premature stop codon results in the production of a truncated protein product lacking part of exon 3 and all the subsequent exons (4/33) and therefore characterized by the absence of all EGF-like repeat domains with the exception of EGF-like 1. A number of pieces of evidence (i.e., family history, MRI findings and the segregation of the mutation with the disease) led us to suggest that such a variant was likely to be pathogenic [4,13]. Concomitantly, Rutten *et al*. described another patient carrying the same variant [16]. They argued that it was a neutral polymorphism, based on immunohistochemical analysis and ultra-structural examination of skin vessels, which were found negative for $N3^{ECD}$ and granular osmiophilic material (GOM) deposits [16]. GOM deposits have been in fact described only in CADASIL patients and constitute a pathognomonic feature for the disease [1,10,15]. Prompted by their report, we further pursued ultrastructural examination of skin vessels in our case. Here, we report such results, aiming to make a comment on whether GOM deposits should be considered the pathological hallmark for a definitive diagnosis of CADASIL in those patients whose mutations are predicted in the production of hypomorphic protein products.

Skin biopsy samples were fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer, rinsed in cacodylate buffer and post-fixed in 1% osmium tetroxide/0.1 M cacodylate buffer, then rinsed again in buffer. Tissue samples were gradually dehydrated in a series of ascending concentrations of ethanol and, then, were immersed in propylene oxide before infiltration with the epoxy resin Epon 812. Ultrathin sections double stained with uranyl acetate and lead citrate were examined with a transmission electron microscope (Zeiss 900).

At the ultrastructural level, analysis of two skin biopsies performed in two different body sites (right and left arm) showed endothelial cells and smooth muscle cells with electron-lucent vacuoles and nuclear chromatin condensation (Figs. 1 and 2). Furthermore, smooth muscle cells presented irregular shape and electron-lucent vacuoles within the cytoplasm, as for degeneration or absence of cytoplasmic organelles (Fig. 3). Notably, such abnormalities were only observed in cells of the blood vessel walls and not in other regions of examined samples, arguing against fixation or orientation artefacts. Ultrastructural analysis of at least 20 vessels per skin biopsy did not show presence of GOM deposits (Figs. 1-3).

Presence of multiple deposits of GOM at ultrastructural examination of brain or skin vessels is the pathognomonic hallmark of CADASIL [1,8,11], with 100% specificity and 96% sensitivity [7]. Nevertheless, the origin, chemical nature and function of GOM deposits are still not clear. There is a suggestion that $N3^{ECD}$ constitutes a component of GOM deposits [5]. If the latter hypothesis is true, then it is not surprising that our patient did not show GOM deposits. His genetic variant is indeed characterized by a premature stop codon, which results in a truncated protein product lacking almost all the $N3^{ECD}$. On the other hand, it is still unknown whether GOM accumulation is necessary for development of the disease. In fact, analysis of vessels from transgenic

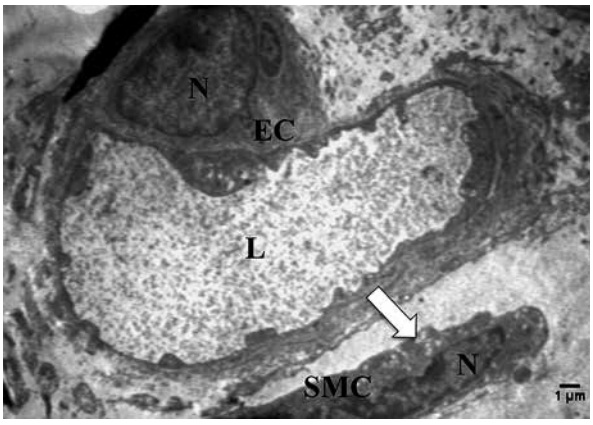


**Fig. 1.** Endothelial cell (EC) and smooth muscle cell (SMC) with electron-lucent vacuoles (arrow) and nuclear chromatin condensation. L – lumen, N – nucleus.

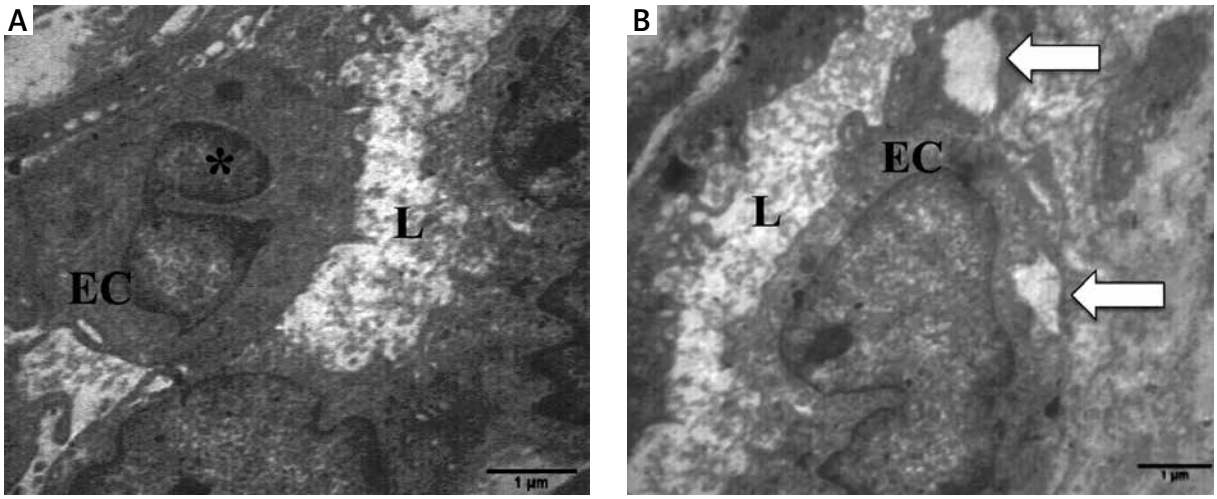


**Fig. 2.** Endothelial cells (EC) with irregularly shaped nuclei (asterisk) and clear areas located in the cytoplasm (arrows). L – lumen.

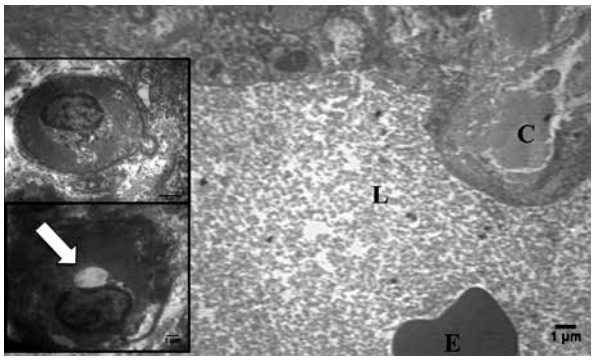


**Fig. 3.** A blood vessel. Insets showing smooth muscle cells irregular in shape and size, with a few degenerated cytoplasmic organelles and clear variable areas (arrows). L – lumen, C – collagen fibrils, E – erythrocyte.

mice expressing mutant *NOTCH3* shows that VSMC damage precedes $N3^{ECD}$ and GOM accumulation [6,12]. Moreover, there is no apparent correlation between the presence and number of GOM deposits and severity of VSMC damage [9,15]. In addition, it is interesting that even though GOM deposits are detected along the vasculature throughout the body, the symptoms of CADASIL are almost exclusively restricted to the central nervous system [12]. Although a gain of function effect on VSMC triggered by multimerization of the mutated protein (and possibly by presence of GOM deposits) is the most supported pathophysiological mechanism in CADASIL, certain naturally occurring mutations unambiguously result in abolished *NOTCH3* signaling and function [14]. In addition, several studies have revealed that mutations leading to *NOTCH3* over-expression dominantly suppress Notch signaling rather than increase it [12]. However, it has also been argued that the archetypal Arg169Cys mutation in *NOTCH3* does not drive the pathogenesis through a loss-of-function mechanism [16]. Overall, it is conceivable that different mechanisms can contribute to the pathophysiology of CADASIL, among which one holds that accumulation of $N3^{ECD}$/GOM in the brain vessels would promote the abnormal recruitment of functionally important extracellular matrix proteins that may eventually cause multifactorial toxicity. Unfortunately, the lack of appropriate instruments to directly assess, *in vivo*, the consequence of mutations on *NOTCH3* transcriptional activity in the brain arteries leaves opens the question of whether hypomorphic *NOTCH3* can drive CADASIL-like symptoms, regardless of the presence of the GOM.

More consistent data on the role of *NOTCH3* in VSMC have been obtained from animal models, even though both knock-out and knock-in mice are not entirely considered robust models of CADASIL [6]. On the one hand, Notch3–/– mice exhibit abnormalities in the cerebrovascular patterning [6] and show marked defects in distal muscular arteries, particularly in the cerebral ones, in the absence of $N3^{ECD}$ and GOM accumulation, even if they do not develop the disease [6]. On the other hand, in some of the knock-in models overexpression of *NOTCH3* up to

4-fold does not lead to CADASIL features or to GOM accumulation (for an extensive review, see [6]).

Such a pattern (i.e., vascular abnormalities in the absence of GOM accumulation) might resemble what we observed in our patient (Figs. 1-3), and we would argue that another mechanism, which is not mediated by $N3^{ECD}$ and GOM accumulation, might underlie such vascular abnormalities, at least for hypomorphic *NOTCH3* mutations. We acknowledge that a higher vascular abnormality burden may have been expected in our case. However, it is conceivable that a brain vessel biopsy would have shown more extensive damage. Moreover, we acknowledge that we did not use another technique (e.g. immunogold staining), but, as stated above, we only found abnormalities in the VSMC and consistently within different samples, rendering the chance of artefacts unlikely.

In summary, there seems to be a spectrum of disorders associated with different *NOTCH3* mutations, with hypomorphic *NOTCH3* presumably causing CADASIL-like symptoms via loss-of-function mechanisms, in line with other studies showing that common *NOTCH3* variants may increase the cerebrovascular risk in the elderly [13,17].

A number of lines of evidence support the hypothesis that *NOTCH3* haploinsufficiency can be clinically relevant, and further neuropathological data will be crucial to define the spectrum of CADASIL-like disorders and to definitively elucidate the role of GOM deposits.

## Disclosure

Authors report no conflict of interest.

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