# Heterozygous *NOTCH1* Variants Cause CNS Immune Activation and Microangiopathy

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NOTCH1 belongs to the NOTCH family of proteins that regulate cell fate and inflammatory responses. Somatic and germline *NOTCH1* variants have been implicated in cancer, Adams-Oliver syndrome, and cardiovascular defects. We describe 7 unrelated patients grouped by the presence of leukoencephalopathy with calcifications and heterozygous de novo gain-of-function variants in *NOTCH1*. Immunologic profiling showed upregulated CSF IP-10, a cytokine secreted downstream of NOTCH1 signaling. Autopsy revealed extensive leukoencephalopathy and microangiopathy with vascular calcifications. This evidence implicates that heterozygous gain-of-function variants in *NOTCH1* lead to a chronic central nervous system (CNS) inflammatory response resulting in a calcifying microangiopathy with leukoencephalopathy.

NOTCH proteins are conserved receptor proteins regulating cell fate in the development of many cell lineages. In humans, 4 NOTCH receptors are known.[1] Heterozygous loss-of-function *NOTCH1* (OMIM:90198) variants cause Adams-Oliver syndrome (AOS [OMIM:616028])[2] and cardiovascular defects, whereas somatic *NOTCH1* variants are associated with T-cell acute lymphoblastic leukemia (T-ALL) and different solid tissue cancers.[3] Heterozygous *NOTCH3* (OMIM:600276) variants cause cerebral autosomal dominant arteriopathy with

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subcortical infarcts and leukoencephalopathy (CADASIL; OMIM:125310).[4]

We report 7 patients with a leukoencephalopathy and calcifications with germline heterozygous gain-of-function *NOTCH1* variants.

## Materials and Methods

Institutional ethics approval and written informed consent from patients or guardians were obtained.

Trio genome sequencing was performed in patient 1 and trio exome sequencing in patients 3 to 7. *NOTCH1* Sanger sequencing was performed in patient 2. Patients were linked by GeneMatcher.[5] Clinical, radiological, and laboratory findings were reviewed.

Plasma was available from patients 2 and 3; cerebrospinal fluid (CSF) from patients 1, 2, 3, and 6. Eight plasma and 10 CSF control samples were available from healthy individuals and individuals with unrelated disease. Peripheral blood mononuclear cells (PBMCs) were obtained from patients 2, 3, and 6, and 9 healthy controls.

PBMCs were incubated with fluorescent-labeled monoclonal antibodies (Table S1) and immunophenotyped with fluorescence-activated cell sorting (FACS). To analyze in vitro activation of B- and T-cells, PBMCs were labeled with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE).[6] The B- and T-cell proliferation was assessed by measuring CFSE dilution with the same antibodies (see Table S1).

Culture supernatant was tested for IgM, IgG, and IgA secreted by mature B-cells with an in-house enzyme-linked immunosorbent assay (ELISA).[6] Plasma, CSF, and culture supernatants were assessed with multiplex Luminex assay and ELISA kits for different cytokines (see Table S1).

Patient 2 underwent autopsy 4 hours postmortem. Tissue was fresh frozen or fixed in 4% formalin. Antibodies for immunohistochemistry are listed in Table S2.

## Results

All patients (details in Table S3) were isolated cases. In patients 1 to 4, onset ranged from childhood to adulthood. They had slowly progressive spasticity, ataxia, and mild peripheral neuropathy, causing gait problems and wheelchair dependency. Intellectual decline and behavioral and mood issues occurred. Patients 5, 6, and 7 presented at birth, patients 5 and 6 presented with Hirschsprung disease, patient 6 also with presented multiple anomalies, and patient 7 presented with marked hypotonia. Patients 5, 6, and 7 had developmental delay. Patients 5 and 6 became spastic. Patient 7 developed epilepsy. Patient 1 died at 25 years of age due to neurologic decline. Patient 2 died at 37 years of age due to a central nervous system (CNS) *Pseudomonas aeruginosa* infection complicating intrathecal Baclofen. Patients 3, 4, 5, 6, and 7 are still living at 65, 28, 9, 6, and 6 years of age, respectively.

Laboratory studies, including work-up for brain calcifications, were unrevealing. Neurophysiology in patients 1 and 2 confirmed sensorimotor neuropathy. Sural nerve biopsy in patient 1 showed demyelination, without inflammation or calcium deposits.

Ages at magnetic resonance imaging (MRI) ranged from 11 months to 57 years. All MRIs demonstrated periventricular and deep cerebral white matter signal abnormalities, increasing over time (Fig 1). MRI and computed tomography (CT) showed numerous white matter calcium deposits, larger in patients 1, 2, 3, and 4, more numerous and smaller in patients 5, 6, and 7. All patients had subtle brain atrophy, most pronounced in patients 6 and 7. Chest and abdominal X-rays did not show calcifications elsewhere.

We detected de novo heterozygous missense variants or small in frame insertions or deletions in *NOTCH1* (GenBank:NM_017617.5), encoding *NOTCH1*, in all patients (Table S4), all affecting the extracellular negative regulatory region (NRR)-domain (Fig 2A–C). Genomic studies did not report other pathogenic/likely pathogenic gene variants.

The leukoencephalopathy with calcifications made us consider an inflammatory microangiopathy, similar to Aicardi-Goutières syndrome (AGS), a leukodystrophy with enhanced IFN-α signaling.[7] Total blood cell counts, lymphocyte subsets, and serum IgG, IgA, and IgM levels were normal. CSF from 3 of the 4 investigated patients contained increased IP-10 (Fig 2D), a cytokine secreted by astrocytes in response to IFN subtypes[8] downstream of NOTCH signaling.[9] IP-10 was normal in the plasma of patients and in the CSF and plasma of controls (Fig 2E). Additional analytes (see Table S1) were unremarkable (not shown).

The known impact of NOTCH signaling on T- and B-cell development, association of somatic *NOTCH1* variants with T- and B-cell leukemia, and increased CSF IP-10 levels prompted testing T- and B-cell phenotype and function. We found an imbalance in peripheral B-cell development, but T-cell phenotype and cellular activities appeared unaffected (Fig S1).

Brain microscopy (Fig 3) demonstrated calcifications in the periventricular and deep cerebral white matter, globus pallidus, cerebellar white matter, and dentate nucleus, in the walls of the arterioles and capillaries, outside CD34-positive endothelial cells, and inside the collagen IV-positive outer basal lamina, consistent with localization in smooth muscle cells and

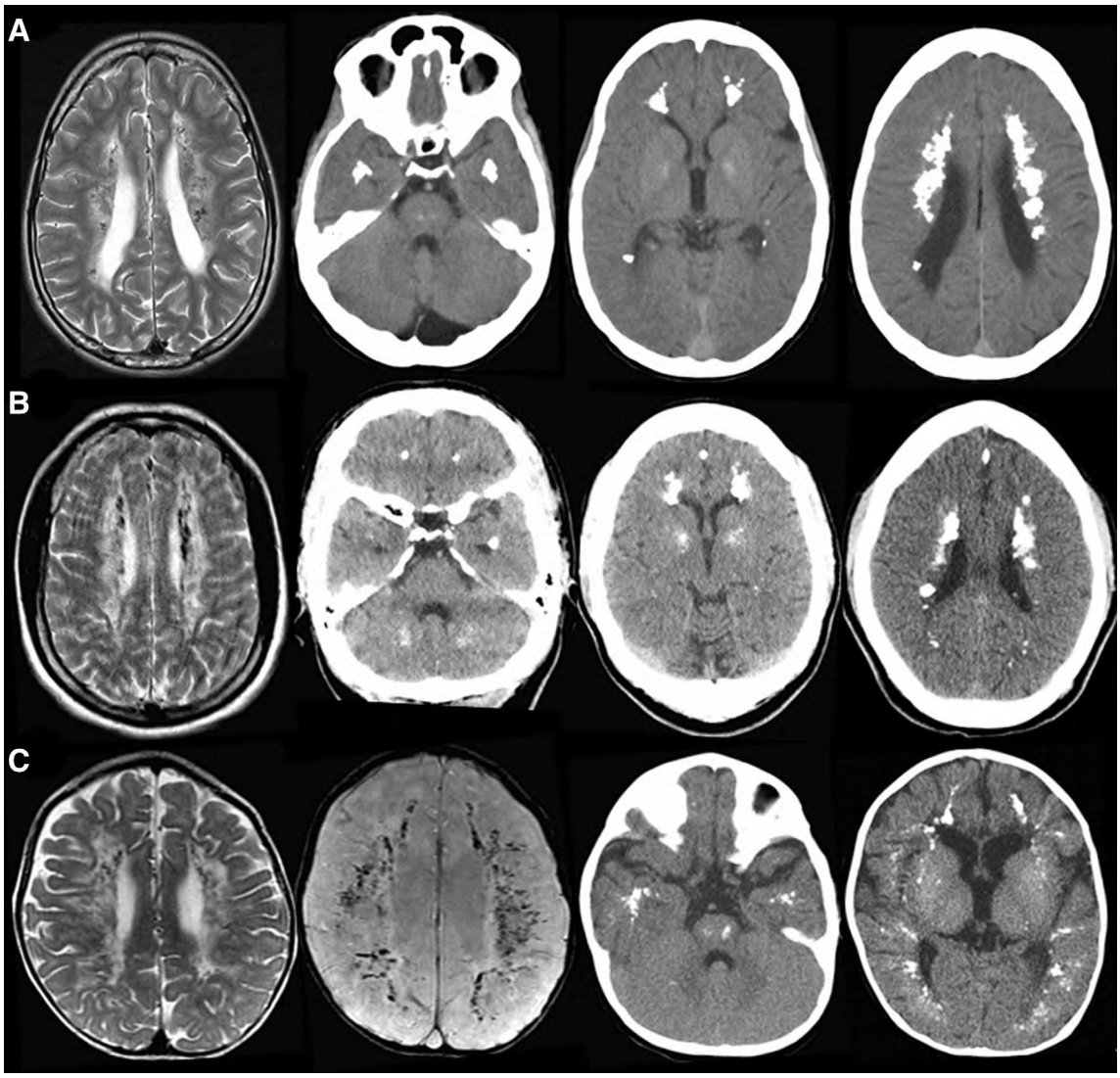


**FIGURE 1: Neuroimaging pattern. (A) Patient 1, T2-weighted magnetic resonance imaging (MRI) (*left*) at 18 years of age shows hyperintensity of the periventricular and deep cerebral white matter, sparing the directly subcortical white matter. Within the abnormal white matter, numerous black dots are present, suggestive of calcium deposits. Computed tomography (CT) at 18 years of age (3 pictures on the *right*) shows numerous small and larger calcifications in the periventricular and deep cerebral white matter, including the temporal lobes. Subtle calcium deposits are present in pons and globus pallidus. The lateral ventricles are slightly dilated. (B) In patient 2, T2-weighted MRI (*left*) and CT (3 pictures on the *right*) at 33 years of age show the same pattern. In this patient, CT also shows calcium deposits in the dentate nucleus, but not in the pons. (C) In patient 5, T2-weighted MRI (*left*) at 20 months of age shows extensive signal abnormalities in the cerebral white matter, containing many black dots, which are suggested to be calcium deposits by susceptibility weighted images (on the *left*, second). The volume of the cerebral white matter volume is reduced with mildly enlarged lateral ventricles and subarachnoid spaces. A CT at 14 months of age confirms numerous small calcifications spread over the cerebral white matter, with also some calcium deposits in the basal nuclei, brain stem, and cerebellum.**

pericytes, confirmed by markers SMA and PDGFRβ (see Fig 3I, J). These blood vessels had a narrowed or occluded lumen and were surrounded by ischemic to necrotic tissue, with loss of neuropil, foamy macrophages, astrogliosis, and capillary proliferation. Noncalcified arteriolar walls were massively thickened with a small eccentric lumen (see Fig 3D). In the mentioned brain areas, virtually all blood vessels were affected. The normal tunica intima and severely thickened tunica media of affected blood vessels contrasts with Moya Moya disease, in which the intima shows fibrous thickening and the media is atrophic; in addition, no abnormal blood vessels were found in the leptomeninges. The nonischemic white matter had diminished myelin and increased oligodendrocyte precursors, approaching 20% of all cells, suggesting a defect in their maturation and function. No evidence of cell cycle arrest or caspase 3-mediated apoptosis was found (not shown).

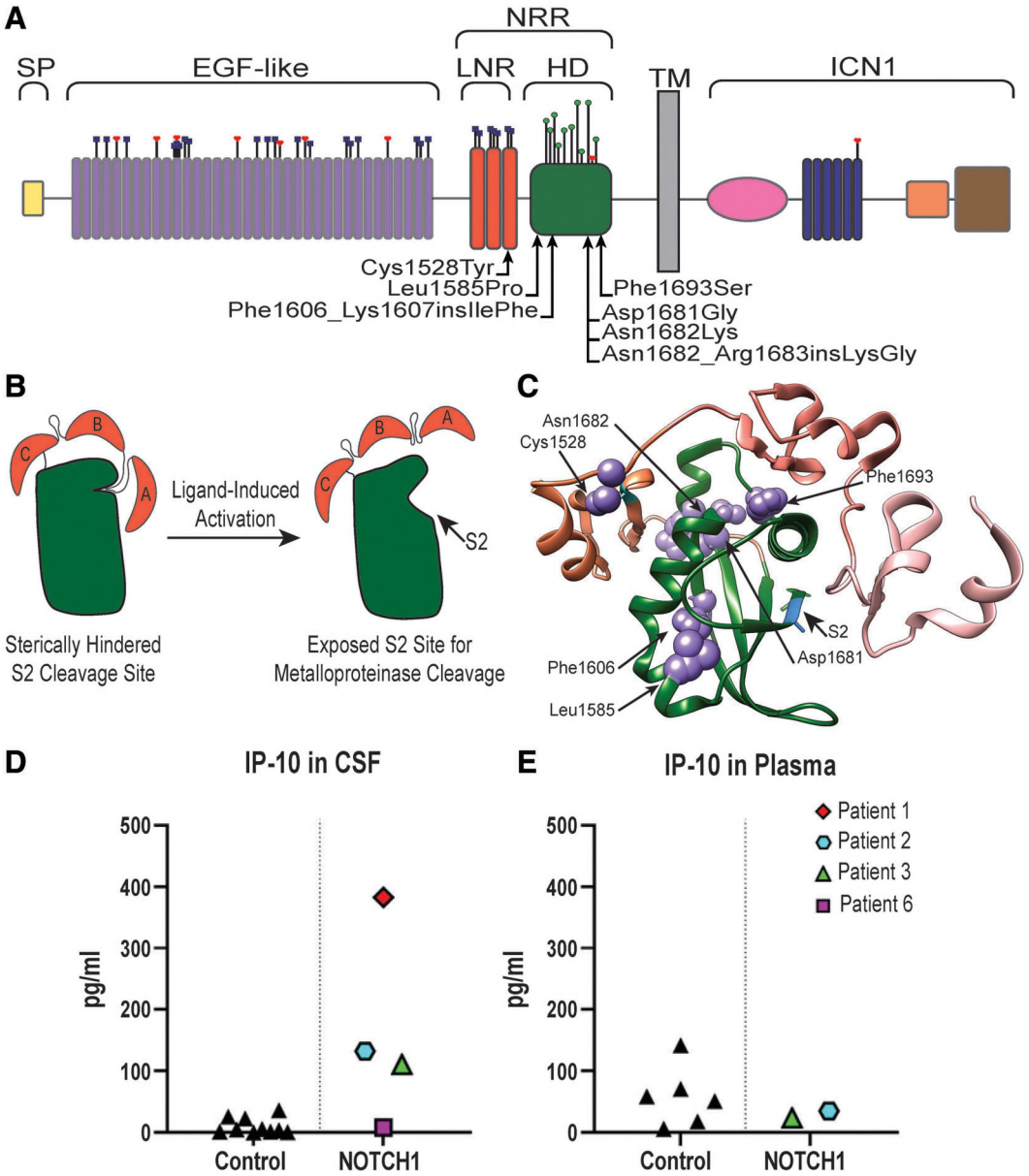


FIGURE 2: Overview of NOTCH1 protein, structure, function, and genomic variants and elevations of CSF IP-10. (A) NOTCH1 is a single-pass transmembrane protein with an intracellular signaling domain (ICN1), a transmembrane domain (TM), and extracellular domain with regulatory function in ligand binding. The negative regulatory region (NRR) in the extra-cellular domain is responsible for preventing ligand-independent activation of NOTCH1. NRR consists of 3 Lin12/NOTCH (LNR) domains and a heterodimerization domain (HD). Upon cleavage, ICN1 is transferred into the nucleus to induce a transcriptional response. Lollipop plots show the position of the 10 most frequently affected residues across NRR, found in COSMIC for T-cell acute lymphoblastic leukemia (T-ALL), with height of lollipops representing variant frequency (*green circle lollipop*). The position of variants associated with Adams-Oliver Syndrome (*blue square lollipop*) and cardiovascular abnormalities (*heart-shaped red lollipop*) is depicted based on ClinVar pathogenic/likely pathogenic variants. In this case, no frequency data were available and the height is used to show multiple variants near these sites and are fully listed in Table S5. (B) Schematic representation, based on Gordon et al.,[10] of NOTCH1 autoregulation by NRR. Upon ligand binding, the LNR domains relax their steric inhibition of HD, exposing the S2 site, which is then cleaved by a metalloprotease, initiating the NOTCH1 signaling cascade. (C) Predicted structure of NOTCH1 (PDB:3ETO). Most variants identified in this study reside in HD (variants annotated in *purple*, and HD in *green*). The HD adopts a configuration characterized by a 4-stranded β-sheet base with packing of 3 α-helices creating a hydrophobic core. Variants p.(Asn1682Lys), p.(Asn1682_Arg1683insLysGly), and p.(Phe1693Ser), observed in patients 2, 4, and 5, respectively, reside in α-helices near the top of HD, whereas p.(Leu1585Pro), found in patient 6, affects a residue in an α-helix near the base of HD. Variant p.(Phe1606_Lys1607insIlePhe in patient 1, disrupts a strand of the β-sheet structure forming the domain's base. Variant p.(Cys1528Tyr) in patient 3, situated in LNR-C, the pivotal LNR, which controls the hinge mechanism of the NRR, disrupts a disulfide bond between Cys1528 and Cys1554 and is likely to affect stability of the calcium-binding pocket of LNR-C and disrupt the interface between LNR-C and HD. (D) IP-10 presence in CSF of patients with *NOTCH1* variants compared with controls, in pg/ml measured by Luminex. As a control group, healthy individuals and individuals with unrelated disease are used. (E) IP-10 presence in the plasma of patients with *NOTCH1* variants compared with controls, in pg/ml measured by Luminex.

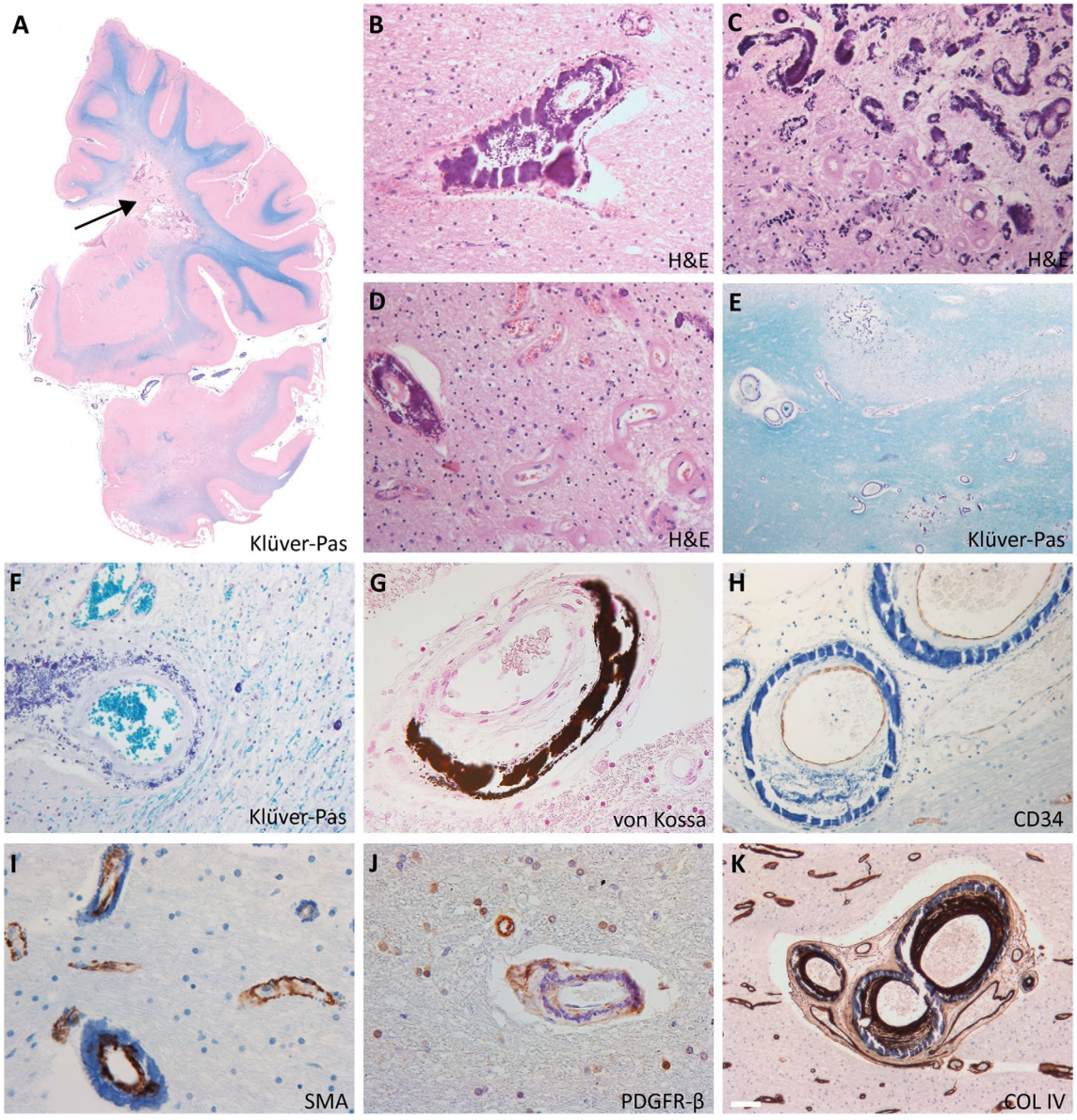


**FIGURE 3: The white matter in *NOTCH1*-related leukoencephalopathy: A calcifying microangiopathy. (A) Kluver-PAS stained whole mount scan of the left cerebral hemisphere at the level of the neostriatum shows lack of myelin in the periventricular and deep white matter, with relative preservation of the U-fibers. Note presence of tissue destruction with calcification in the periventricular and deep frontal regions (*arrow*). (B, C, D) At high magnification, Hematoxylin and eosin (H&E) stain of the periventricular frontal white matter (B, D) and cerebellar hemisphere (C) shows abnormally mineralized arterioles and capillaries; the non-mineralized blood vessels are also affected, with thickened walls due to increased tunica media and small eccentric lumina (D). (E, F) Kluver-PAS stain of the deeper frontal white matter shows reduction to virtual lack of myelin and mineralized blood vessels. (G) A von Kossa stain confirms arterial mineralization with calcium phosphate deposition in the tunica media of the vascular wall. (H, I, J, K) Immunohistochemical stains against different component of the blood brain barrier demonstrate that in the larger vessels mineralization occurs outside the endothelial layer (CD34, H). The mineralizations are localized in proximity of the tunica media (smooth muscle Actin, SMA, I) and, in the capillaries, the pericytes (platelet-derived growth factor β, PDGFRβ, J). Mineralization is contained within the inner endothelial and outer glial basement membrane (collagen IV, Col IV, and K). Of note, the findings consistent with the meningoencephalitis caused by *Pseudomonas aeruginosa* are not described or illustrated. C, D and E: Scale bar 20 µm. B, F, G, H, I, J, K: Scale bar 10 µm.**

## Discussion

Inactive NOTCH1 resides in the cell membrane. The extracellular domain is composed of epidermal growth factor (EGF)-like repeats, responsible for ligand binding, and the NRR (see Fig 2A). The NRR is made up of a stem-like heterodimerization (HD)-domain, capped by 3 Lin12/NOTCH (LNR)-domains that prevent premature NOTCH1 signaling in the absence of a ligand[10] (see Fig 2B, C). Upon ligand binding, LNR-domains relax their steric inhibition of the HD-domain, exposing the S2-site, which is then cleaved

(see Fig 2B). The active intracellular fragment (ICN1) is transferred into the nucleus, inducing a transcriptional response. The NOTCH1-signaling pathway plays an important role in differentiation, proliferation, and apoptosis of normal cells and is involved in pathological processes, like cancer and inflammation.

Somatic *NOTCH1* variants occur in many cancer types[11] and over 50% of T-ALL cases.[3] Most affect the HD-domain, destabilizing the auto-inhibitory conformation of the HD-domain with the LNR-domain and causing ligand-independent constitutive NOTCH1 activation.[10] Variants p.(Cys1528Tyr), p.(Leu1585Pro), and p.(Phe1693Ser), identified in patients 3, 7, and 6, respectively, have been associated with T-ALL. Notably, somatic variants at all loci affected in our patients result in ligand-independent increased signaling activity.[3] The somatic *NOTCH1* variants commonly present in T-ALL promote proliferation, but are not the primary oncogenic drivers.[12] It has been shown they do not induce T-ALL, but accelerate T-cell transformation in hematopoietic progenitors expressing the *KRAS* oncogene.[12]

The neuroimaging pattern of leukoencephalopathy with calcifications most closely resembles that of AGS, distinct from other such patterns.[13] We therefore considered a similar inflammatory microangiopathy. In 3 of the 4 patients tested, CSF but not plasma IP-10 was elevated. We did not find T-cell abnormalities. In mice, NOTCH1 enhances B-cell activation and antibody secretion,[14] but we observed less efficient proliferation and early differentiation to plasmablasts with low-normal antibody secretion in vitro. This B-cell programming imbalance did not lead to autoimmune phenomena or increased infection rates. The elevated CSF and normal plasma IP-10 in our patients indicates a chronic inflammatory state of the CNS only, in line with a CNS-confined disease. In AGS, a systemic disease, IP-10 is elevated in CSF and serum.[8]

Germline heterozygous loss-of-function *NOTCH1* variants across the gene cause AOS,[2] characterized by aplasia cutis congenita of the scalp, limb defects, and often vascular anomalies. Brain calcifications are common.[15] Periventricular white matter abnormalities on MRI have been mentioned, but not illustrated.[16] Germline heterozygous *NOTCH1* variants have also been associated with isolated congenital heart disease and aortic valve anomalies, some with valve calcifications.[17]

The effects of loss- and gain-of-function variants in *NOTCH1* overlap. Vascular calcifications in AOS result from decreased NOTCH1 signaling, in our patients from enhanced signaling. Hirschsprung disease has been associated with NOTCH1 downregulation[18] and was observed in 2 of our patients. The observed decreased in vitro B-cell proliferation, differentiation, and antibody secretion suggest loss-of-function effects. Whether and how the mechanisms of NOTCH1 gain- and loss-of-function effects are, in part, shared, also dependent of the cellular context, is unknown.

In AGS, calcium deposits occur in the smooth muscle fibers of the brain arterioles.[19] We previously showed that IFN-α enhances calcification in a dose-dependent manner in cultured human vascular smooth muscle cells.[7] In *NOTCH1*-related calcifying leukoencephalopathy, calcifications also occur in the vascular tunica media, in smooth muscle cells of the arterioles, and pericytes of the capillaries. The prevalent view is that medial calcification is related to phenotypic changes of smooth muscle cells and pericytes into cells that promote mineralization, resulting in a calcifying microangiopathy. Inflammatory cytokines are known to stimulate such phenotypic changes,[20] suggesting that AGS and *NOTCH1*-related disease share this mechanism. However, it is possible that *NOTCH1*-related vascular calcifications are mediated in different ways or are multifactorial, inflammation being one factor.

In conclusion, we show that heterozygous gain-of-function *NOTCH1* variants lead to a chronic CNS inflammatory response with elevated CSF IP-10, resulting in a calcifying microangiopathy with leukoencephalopathy. This association identifies new roles of post-developmental NOTCH1 signaling, especially in relation to CNS inflammatory responses.

## Acknowledgments

The authors thank the patients and their families for participation in this study. We also thank Marjolein Breur and Machiel H. Jansen, research technicians, for their excellent work. We thank Joanna Crawford at the University of Queensland Institute for Molecular Bioscience for her efforts on the genome sequencing in patient 1. We thank Edoardo Della Sala and Paola Di Martino, PhD students at the Department of Medical Sciences, University of Torino, Italy, for their contributions to the laboratory work for patient 3. For the evaluation and diagnosis of patient 6, we thank Rong Mao and Pinar Bayrak-Toydemir from ARUP Laboratories and the University of Utah; and Dr Steven Bleyl, Dr James Bale, Dr Matt Velinder, Dr Steven Boyden, Dr Joseph Yost, Dr Charles Murtaugh, Dr John C. Carey, and Ms Abbey Bentley, all at the University of Utah for their help in the clinical, molecular, and bioinformatic evaluation. G.H. was supported by the Ochsner MD-PhD Scholarship. This study was in part financed by the Australian National Health and Medical Research Council (NHMRC 1068278) and the Medical Research Future Fund (ARG76368). The research conducted at the Murdoch Children's Research Institute was supported by the Victorian Government's Operational Infrastructure Support Program. P.Z. and M.B. are supported by the ZonMw VENI grant number 016.196.107.

## Potential Conflicts of Interest

Nothing to report.

## Author Contributions

G.H., P.Z., S.A.M.T., C.S., M.B., T.W.K., and M.S. vdK. contributed to the conception and design of the study. G.H., P.Z., S.A.M.T., A.A., L.D.B., J.L.B., A.C., E.G., T.P., W.S., C.S.H., G.V., M.A.A.P.W., M.S., K.W., F.S., T.K., M.L., T.R., R.J.T., C.S., M.B., T.W.K., and M.S.vdK. contributed to the acquisition and analysis of data. G.H., P.Z., S.A.M.T., C.S., M.B., T.W.K., and M.S.vdK. contributed to drafting the text and preparing the figures. All authors reviewed and approved the manuscript prior to submission.

## Data Availability

The sequencing methods will be clarified on request. The raw data used in preparation of figures and tables will be made available or shared in anonymized format by request of a qualified investigator.

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