Investigating APOE, APP-Aβ metabolism genes and Alzheimer’s disease GWAS hits in brain small vessel ischemic disease

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Alzheimer’s disease and small vessel ischemic disease frequently co-exist in the aging brain. However, pathogenic links between these 2 disorders are yet to be identified. Therefore we used Taqman genotyping, exome and RNA sequencing to investigate Alzheimer’s disease known pathogenic variants and pathways: APOE ε4 allele, APP-Aβ metabolism and late-onset Alzheimer’s disease main genome-wide association loci (APOE, BIN1, CD33, MS4A6A, CD2AP, PICALM, CLU, CR1, EPHA1, ABCA7) in 96 early-onset small vessel ischemic disease Caucasian patients and 368 elderly neuropathologically proven controls (HeX database) and in a mouse model of cerebral hypoperfusion. Only a minority of patients (29%) carried APOE ε4 allele. We did not detect any pathogenic mutation in APP, PSEN1 and PSEN2 and report a burden of truncating mutations in App-Aß degradation genes. The single-variant association test identified 3 common variants with a likely protective effect on small vessel ischemic disease (0.54>OR>0.32, adj. p-value <0.05) (EPHA1 p.M900V and p.V160A and CD33 p.A14V). Moreover, 5/17 APP-Aß catabolism genes were significantly upregulated (LogFC >1, adj. p-val<0.05) together with Apoe, Ms4a cluster and Cd33 during brain hypoperfusion and their overexpression correlated with the ischemic lesion size. Finally, the detection of Aβ oligomers in the hypoperfused hippocampus supported the link between brain ischemia and Alzheimer’s disease pathology.

Late-onset sporadic Alzheimer’s disease (LOAD) and small vessel ischemic disease (SVID) frequently influence each other and co-exist in the aging brain depicting a clinical, neuroradiological and neuropathological spectrum defined as 'mixed dementia'. Although mixed dementia represents the second common form of dementia in the elderly, as over 45% of LOAD patients neuropathologically diagnosed displayed significant cerebrovascular pathology1, the nature and the pathogenic ground at the basis of AD-SVID interaction is poorly understood2. APOE ε4 allele is the strongest risk factor for sporadic LOAD3–5, however its role in SVID has not been extensively investigated. Common hallmark in small vessel disease is cerebral amyloid angiopathy (CAA), which is caused by excessive deposition of Aβ 40 and 42 on the walls of small vessels6, responsible both for its ischemic and hemorrhagic manifestations (SVID and intracerebral hemorrhage [ICH])8. Both rare familial and common sporadic small vessel disease cases pointed to the potential role of APP-Aß dysmetabolism as key pathogenic mechanism underlying CAA small vessel disease subtype. First, autosomal dominant fully penetrant mutations in the secretase domain of APP, APP duplication, CST3 and TTR rare mutations cause familial CAA9–11. Second, common variants in IDE and LRP1 have been associated with increased risk of diabetes type 2 and migraine, respectively, that frequently are co-morbidities in SVID patients12,13. Third, perivascular and parenchymal Aß deposits have been reported in genetically diagnosed CADASIL patients and vascular dementia cases14–17. Despite the growing body of evidence supporting an imbalance between Aß production and degradation, APP-Aß metabolism role in SVID remains unknown.

Finally, in the last 10 years 9 main LOAD genome-wide association study (GWAS) loci have been identified and replicated by at least 2 independent GWASs and present the strongest effect sizes after APOE (BIN1, CLU,
CR1, PICALM, MS4A6A, ABCA7, EPHA1, CD33, CD2AP). They shed light on critical LOAD pathogenic pathways and these include: immune response (MS4A4A cluster, CD33, CR1, EPHA1, CD2AP, ABCA7), Aβ40–42 clearance (PICALM, BIN1, CD33 and ABCA7), lipid metabolism (CLU, ABCA7) and vesicles trafficking (PICALM, BIN1) (http://www.alzgene.org/). Among the genetic mechanisms underlying an increased susceptibility for LOAD at these loci, coding variability is emerging as a critical factor18–21.

Therefore, in this study we investigated APOE ε2, ε3 and ε4 alleles, APP-Aβ metabolism genes and the most replicated AD GWAS loci through a genetic screening in 96 early-onset independent familial and apparently sporadic SVID Caucasian patients and 368 elderly neuropathological proven controls (HEX database) and through a differential gene expression study during acute and subacute brain ischemia in a mouse model of vascular dementia and subcortical ischemic stroke. Moreover, we analysed whether brain hypoperfusion may have contributed to the generation of AD neuropathological hallmarks (Fig. 1).

We hypothesize that 1) coding variability together with significant differential gene expression in APP-Aβ metabolism genes and LOAD GWAS loci may play a role in SVID and brain ischemia and 2) acute severe hypoperfusion-ischemia may prime APP misfolding, toxic soluble oligomers formation that may in the long term accumulate in the stable form of amyloid plaques, as described in elderly patients with vascular dementia22,23.

Materials and Methods

Gene selection. We studied APOE ε2, ε3 and ε4 genotype and 2 clusters of genes: 1) APP-Aβ metabolism genes: 31 genes involved in Aβ production (APP, PSEN1, PSEN2, ADAM9, ADAM10, ADAM17, BACE1, BACE2, NCSTN, PSENEN, APH1B, MEP1B, GPR3), APP stabilization (APLP1, APBA1), APP recycling (SORL1), Aβ deposition (TTR), intracellular degradation (EC1E1, EC2E2, IDE, CST3, CTSB, CTSD, LYZ, MME), extracellular degradation and clearance (ACE, MMP3, A2M, PLAT, KLK6, LRP1) and 2) LOAD GWAS mainly replicated loci: APOE, BIN1, CLU, CR1, PICALM, MS4A6A, ABCA7, EPHA1, CD33, CD2AP. Selection criteria for these genes has already been reported19,24. The pipeline followed in this study is described in Fig. 1.

Patient cohort. The cohort was composed of 96 independent familial and early-onset apparently sporadic SVID Caucasian non-Hispanic cases from the US, NINDS (National Institute of Neurological Disorders and Stroke), whose DNA was extracted and collected at the NINDS Repository.

All NINDS Repository samples were collected only after an IRB-approved, signed informed consent was secured by the submitter. All methods were carried out in accordance with relevant guidelines and regulations.

Inclusion criteria included small vessel ischemic disease diagnosis based on TOAST classification, early age at onset (<65 years [only 2 cases, whose age-at-onset was 68 and 71 years old have been included in the study because familial]), absence of known pathogenic mutations in Mendelian small vessel disease genes (HTRA1, NOTCH3, ACTA2 and COL4A1) and no enrichment for vascular risk factors except for hypertension, which generally plays a critical role in elderly people14. The mean age at disease onset was 51.5 years (range 34–71 years). 82.3% of the cases were male and 44.8% of the cases were positive for a familial history of cerebrovascular disorders. Among the comorbidities and possible risk factors for SVID, hypertension was reported in 60.4% of the patients, diabetes type 2 in 30.2%, myocardial infarction in 7.3%. The majority of the patients (at least 88.54%) did not present atrial fibrillation (AF), which is among the most important risk factors for embolic small vessel occlusion25. In 4.1% and 7.3% of the patients the presence of AF was reported and unknown, respectively. Given the prevalent role of hypertension and type 2 diabetes in SVID in the elderly people25 and the young age at onset...
of the cohort, these patients were considered enriched for genetic risk factors (Table 1). Finally, 368 controls > 70 years of age were selected from ‘HEALTHY EXOMES’, HEX, a publicly available database, which collects exome sequencing data from elderly neuropathologically proven controls (https://www.alzforum.org/exomes/hex)36.

### Exome sequencing in patients.
We performed whole exome sequencing on a cohort of 96 independent familial and early-onset sporadic SVID cases. DNA was extracted from blood using standard protocols. Library preparation for next generation sequencing used 50 ng DNA. Exome libraries were prepared using Nextera® Rapid Capture Exome Kit (4 rxn × 12 plex, FC-140-1002). The DNA library was then hybridized to an exome capture library (Nextera, Illumina Inc.) and precipitated using streptavidin-coated magnetic beads (Nextera, Illumina). Exome-enriched libraries were PCR-amplified, and then DNA hybridized to paired-end flow cells using a cBot (Illumina, Inc.) cluster generation system. Samples were sequenced on the Illumina HiSeq: 3000/4000 using 2 × 76 paired end reads cycles. We used exome sequencing data to identify common (minor allele frequency (MAF) >3%), rare (MAF <3%), and very rare (MAF <1%) coding variants in 31 genes involved in APP-{

| SVID | SEQUENCING STRATEGY | ORIGIN | AGE at onset (YRS) MEAN ± SD (RANGE) | MALE (%) | MALE Familial (%) | APOE e4 (%) | Hypertension | Diabetes | MI | AF +/− | NA | Hypercholesterolemia |
|------|----------------------|--------|-------------------------------------|----------|------------------|-------------|--------------|-----------|-----|-------|----|---------------------|
| 96   | WES                  | Caucasian, non-Hispanic (US) | 51.5 (34–71) | 82.3 | 44.8 | 29 | 60.4 | 30.2 | 7.3 | 4 | 2 | |

Table 1. Discovery cohort. WES, whole exome sequencing; YRS, years; MI, myocardial infarction; AF, atrial fibrillation; *at least one APOE ε4 allele; NA, not available.

### Biinformatics, exome sequencing.
The reads were aligned using BWAMEM v.0.7.1527 to the reference GRCh37 (hs37d5.fa), separate read groups were assigned for all reads from one lane, and duplicates were masked using Samblaster v0.1.24. Standard QC was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The variants were then called using GATK UnifiedGenotyper v3.729 and annotated using Samblaster v0.1.2428. Standard QC was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The variants were then called using GATK UnifiedGenotyper v3.729 and annotated using Jannovar v0.2430 using RefSeq v105 exons.

### APOE Genotyping.
APOE genotypes comprising the APOE ε2, ε3 and ε4 alleles, were assayed using LightCycler 480 Instrument II (Roche). SNP-specific primers and probes were designed by Thermo Fisher (TaqMan genotyping assays). The polymorphisms distinguish the ε2 allele from the ε3 and ε4 alleles at amino acid position 158 (rs7412) and the ε4 allele from the ε2 and ε3 alleles at amino acid position 112 (rs429358).

### Statistical analysis.
Power calculation was performed for Fisher’s exact test based on allelic association. We had 80% power for the detection of common variants (minor allele frequency (MAF) > 3%) with strong effect (OR <0.6 or >2), with a significance value of two-sided α = 0.05 (Fig. S1).

In the single-variant analysis, allele frequencies were calculated for each coding variant in cases and controls and Fisher's exact test on allelic association was performed. Fisher’s exact test was also used for the statistical analysis of the truncating mutations in APP-Aβ metabolism genes. Low frequency and rare variants were defined as having a 1% < MAF < 3% and MAF < 1%, respectively, either in cases or controls. MAF was based either on the HEX database for elderly controls > 70 years of age or EXac database version 0.3.1 database (http://exac.broadinstitute.org/). A P-value of 0.05 was set as a nominal significance threshold, after false discovery rate (FDR) correction. We report the complete list of coding variants detected in the APP-Aβ metabolism genes and LOAD GWAS loci in the supplementary tables (Table S1 and S2).

T-test performed was used to detect the statistical significance of the number of neurons and glial cells positive for Aβ oligomers in hippocampus during acute (2d) and subacute (7d) hypoperfusion in BCCAS and naive mice.

### BCCAS mouse model, experimental design and exclusion criteria.
All experiments and experimental protocols were approved by the Landesamt für Gesundheit und Soziales and conducted according to the German Animal Welfare Act and institutional guidelines. 22 male C57BL/6J mice, purchased at 8 weeks of age, Charles River, Germany, were housed in a temperature (22 ± 2°C), humidity (55 ± 10%), and light (12/12-hour light/dark cycle) controlled environment. As previously described19, the animals underwent hypoperfusion between 9 and 13 weeks of age. Hypoperfusion was achieved by bilateral common carotid artery stenosis (BCCAS).
BCCAS mice were imaged before surgery, 24 hours and 1 week post-surgery. At 2 days and 7 days tissue was processed for immunohistochemistry and RNA sequencing.

The BCCAS surgery is further described in the supplementary.

**RNA sequencing data: acute (2d) and subacute (7d) hypoperfusion in BCCAS mouse model.** To study APP-Aβ metabolism and LOAD GWAS genes during brain acute and subacute hypoperfusion, we used a mouse model of vascular dementia, where brain hypoperfusion is achieved through the placement of microcoils around both common carotid arteries leading to a ≈70% stenosis (bilateral common carotid stenosis [BCCAS] mouse model)\(^1\). The main features of the model during severe acute and subacute hypoperfusion have been already described\(^1\).

In this study, 8 BCCAS mice, 8 sham and 4 naive mice were sacrificed with cervical dislocation 2 days and 7 days post coil insertion surgery, followed immediately by post-mortem dissection of the prefrontal cortex, striatum and hippocampus from one hemisphere. The other hemisphere was preserved for immunohistochemistry. The dissected tissues were immersed in RNA later and stored at −80°C for later use for mRNA-Sequencing. Total RNA was extracted using miRNeasy Kit (Qiagen, Cat # 217004). Total RNA quality was assessed with the use of Bioanalyzer. Average RIN (RNA Integrity Number) of our samples was 9. Next Generation Sequencing mRNA libraries were prepared with Illumina TruSeq RNA Library Preparation Kit (Illumina, Cat # RS-122-2001).

**Bioinformatics, RNA sequencing.** Processing, quality assessment and analysis of RNAseq data was carried out using a custom pipeline. We aligned paired end reads with STAR\(^3\) against the GRCm38.p4 genome using gencode.vM12 annotation\(^4\) (http://www.gencodegenes.org/mouse_releases/12.html), excluding alternative scaffolds and patches. Gene counts were determined using HTSeq.\(^3\) Testing for differential gene expression and cerebral blood flow and gene-expression correlation was done using DESeq.\(^2\,3\) Genes were counted as differentially expressed where they had a moderated fold change of 2 or more, contrasting coil to shame samples and where their false discovery rate (FDR) adjusted p-value was below 0.05.

**BCCAS mouse model, histology.** The staining protocol for the mouse brain histological sections has been already described\(^3\). Briefly, for the 20 mice subjected to gene-expression study (8 BCCAS mice, 8 Sham mice and 4 naive mice) one hemisphere was used for RNA sequencing and the contralateral for histology. Fresh frozen hemispheres were cut into 20-µm-thick sections on a cryostat. Moreover, to histologically study both hemispheres, 2 mice were deeply anaesthetized with ketamine and xylazine and perfused through the heart. µfrozen hemispheres were cut into 20-µm-thick sections on a cryostat. Moreover, to histologically study both hemispheres, 2 mice were deeply anaesthetized with ketamine and xylazine and perfused through the heart. µfrozen hemispheres were cut into 20-µm-thick sections on a cryostat. Moreover, to histologically study both hemispheres, 2 mice were deeply anaesthetized with ketamine and xylazine and perfused through the heart.

**Oligomers detection and counts.** ImageJ version 1.52 A was used to count neuronal and glial cells positive for Aβ oligomers.

**Methods to prevent bias, statistics.** Mice were randomized to receive hypoperfusion. RNA library preparation and pooling were randomized and blinded, respectively. All methods were carried out in accordance with relevant guidelines and regulations.

**Results**

**Genetic screening.** APOE ε4 allele is not associated to increased risk for SVID. The majority of SVID cases were homozygous for ε3 allele (58.3%), around one third of the patients carried in heterozygosity the ε4 allele (27% and 1%, genotype frequencies for ε4/ε3 and ε4/ε2, respectively), whereas a minority of cases (1%) were homozygous either for APOE ε2 or ε4 allele (Table 2). Average age at onset for carriers was 51 years, which did not differ significantly compared to patients homozygous for ε3 allele (52 years). Finally, familial cases displayed a moderately higher APOE ε4 carrier frequency compared to sporadic ones (32.5% and 26.4%, respectively). APOE ε2 allele was detected in 13/96 (13.5%) patients, 10/13 (77%) with a very young age at onset (<55 years).

**APP-Aβ metabolism genes.** To study a possible role of APP-Aβ metabolism genes in SVID, we focused on 1) possible enrichment for pathogenic mutations in Mendelian genes (APP, PSEN1 and PSEN2), underpinning autosomal dominant AD (http://www.molgen.ua.ac.be/ADMutations/) and 2) burden of damaging mutations in APP-Aβ catabolism genes upon APP-Aβ production genes, underlying sporadic LOAD\(^7\).

We screened protein coding variability in 31 genes involved in APP-Aβ metabolism and we identified 130 coding variants: 21 common and 88 rare. ADAM10 and PSENEN did not harbour any coding variant. Among the
rare variants we report 21 novel variants, and 10 truncating mutations. The majority of the variants detected were singletons (86/130 [66.15%]). BACE1, BACE2, CST3, CTSB and CTSD did not harbour any rare damaging variant. The majority of patients, 75/96 (78.12%), carried at least one rare likely damaging variant and almost half of them, 43/96 (44.8%), harboured multiple likely pathogenic alleles in the studied genes.

**AD Mendelian genes: APP, PSEN1, PSEN2.** We report a total of 5 rare coding variants in APP, PSEN1 and PSEN2. None of these are likely to be deleterious. APP p.V576L and p.T280del do not cluster in the conserved secretase domain; PSEN1 p.E318G and PSEN2 p.L2F and p.R62H map outside the alpha helix surface of the transmembrane domains (TMs), where all the pathogenic mutations have been reported (alpha-helix rule)38 (Table 3).

**Other genes playing a key role in APP-Aβ metabolism.** CST3 p.A25T in homoyzgosy and SORL1 variants clustering in VPS domain are known risk factors for LOAD and may influence SVID susceptibility. Among the variants detected in the other genes involved in APP-Aβ metabolism, 3 missense mutations in genes playing a role in APP-Aβ degradation were of particular interest: one polymorphism reported as pathogenic in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), CST3 p.A25T, and SORL1 p.E270K and p.A528T (Table 1).

Importantly, homoyzgosy for CST3 p.A25T has been significantly associated with AD39 and other neurodegenerative conditions such as macular degeneration40. In our cohort, we report 4/96 [4.16%] patients homoyzgosy for the minor allele A, a frequency which was 2.55 times higher when compared with HEX controls (6/368 [1.63%]). Notably, the homoyzgosy carriers, 3 sporadic and 1 familial cases, displayed an average age at onset of 49.75 years (range: 39–60 y), 3/4 (75%) were homozygous for p.A25T, and 1/4 (25%) patients presented 3 moderate risk factors for SVID: hypertension, myocardial infarction and type 2 diabetes, 3/4 (75%) displayed hypertension and 1/4 (25%) did not present any risk factor for SVID (Table S3).

Interestingly, SORL1 p.E270K and p.A528T clustering in the vacuolar protein sorting (VPS10) domain (Aa 124–757) have been found in 2/96 SVID patients homozygous for APOE ε3 allele and have been reported pathogenic and to segregate within AD families41.

**APP-Aβ genes, pooled variants.** Analogously to LOAD, SVID patients are enriched for LoF mutations in genes involved in APP-Aβ degradation rather than production. We then compared coding genetic variability between 14 genes mainly involved in APP-Aβ production (ADAM10, ADAM17, ADAM9, APBA1, APOE, APP, BACE1, BACE2, GPR3, NCSTN, PSEN1, PSEN2, PSENEN) and 17 genes taking part in APP-Aβ degradation (A2M, ACE, CST3, CTSB, CTSD, ECE1, ECE2, IDE, KLK6, LRP1, LYZ, MEF1B, MME, MMP3, PLAT, SORL1, TTR). We report a significant enrichment for loss of function (LoF) mutations (stop gain/loss, singletons, splice-site mutations) in genes regulating Aβ degradation in SVID patients (9/96 [9.4%]), both when compared to APP-Aβ production genes (1/96 [1%]) (Fisher p-value= 0.01837), and Aβ degradation genes in the HEX cohort (6/368 [1.6%]) (Fisher p-value= 3.496e-14) (Fig 2, Table 4).

**LOAD GWAS loci (BIN1, CD33, MS4A6A, PICALM, CLU, CR1, EPHA1, ABCA7).** Single coding variant association test. EPHA1 p.V160A, CD33 p.A14V, ABCA7 p.G1527A are LOAD GWAS hits or in LD with LOAD GWAS hits and may play a modest protective effect in SVID.

To investigate the role of the LOAD GWAS loci with the strongest effect sizes after APOE (BIN1, CD33, MS4A6A, PICALM, CLU, CR1, EPHA1, ABCA7), we focus on a possible significant association between coding variants detected in these loci and SVID.

We screened protein coding variability in 9 highly replicated LOAD GWAS loci in 96 SVID patients and we identified 69 coding variants: 26 common, 4 rare and 39 very rare. Among these, 24 were singletons, 6 were novel and 6 were truncating mutations. PICALM and CD2AP did harbour the lowest number of variants (0,005 variants per Kb of coding sequence). By contrast, MS4A6A harboured the highest number of coding variants (7,4 variants per Kb of coding sequence). The majority of patients, 81/96 (84.3%), carried at least one rare likely damaging variant and almost half of them, 45/96 (46.8%), harboured multiple likely pathogenic alleles in the studied genes.

Among these variants, 36/69 (52.2%) have also been detected in the 368 controls >70 years of age in the HEX database and were selected for the single-variant-based analysis (Table 4).

The single variant association test identified 3 coding variants whose allelic frequency significantly differed between SVID cases and controls (EPHA1 p.M900V and p.V160A and CD33 p.A14V) (adj. p-value < 0.05). Importantly, all of these are common variants, with a modest to no damaging effect (15.46 > CADD score > 5.284) with moderate to strong likely protective effect size (0.3 < OR < 0.6) and only a minority of carriers are at least heterozygous for the APOE ε4 or ε2 alleles (up to 28.2% and 21.2%, respectively) (Table 5).

| COHORTS           | N   | ε4/ε4  | ε4/ε3 | ε3/ε3  | ε3/ε2  | ε2/ε2  |
|-------------------|-----|--------|--------|--------|--------|--------|
| Caucasian SVID    | 96  | 1      | 27     | 58.3   | 11.45  | 1      |
| Caucasian controls | 6262 | 1.8    | 21.3   | 60.9   | 12.7   | 2.6    | 0.8    |
| Caucasian LOAD    | 5107 | 14.8   | 41.1   | 36.4   | 4.8    | 2.6    | 0.2    |

Table 2. APOE genotype. N, number; SVID, small vessel ischemic disease; LOAD, late-onset Alzheimer’s disease. *Data for Caucasian controls and LOAD are taken from a previous publication (Farrer et al., 1997)
Interestingly, EPHA1 p.V160A, CD33 p.A14V and 2 missense mutations nominally associated with SVID (ABCA7 p.G1527A and CR1 p.Q1022H) have been already reported either as functional, as LOAD GWAS hit, in linkage disequilibrium (LD) with LOAD GWAS hits or significantly linked to different complex traits (blood protein levels and haematological traits) further supporting the critical effect of the amino acid substitution in these positions.

### Table 3. Coding variants detected in APP, PSEN1 and PSEN2 in the SVID cohort.

| Gene   | Position | rsID       | Ref/Alt | Genomic change | Aa change | PROVEAN    | SIFT       | Polyphen2   | Carrier freq (%) | MAF(%) | Carrier APOE ε4 (%) |
|--------|----------|------------|---------|----------------|-----------|------------|------------|-------------|------------------|---------|------------------|
| APP    | 21:27284236 | rs200769792 | C/T     | c.1726G > A    | p.V576I   | Neutral    | Tolerated  | probably damaging | 1/96 (1) | 0.5   | e3/e2            |
| APP    | 21:27394188 | rs764406483 | TGTG/T  | c.837_839del   | p.T280del | NA         | NA         | NA          | 2/96 (2) | 1     | e3/e3 e3/e3     |
| PSEN1  | 14:73673178 | rs17125721  | A/G     | c.953 A > G    | p.E318G   | Deleterious| Damaging   | benign       | 5/90 (5.5) | 2.7   | e3/e3 e3/e3     |
| PSEN2  | 1:227069612 | NOVEL      | C/T     | c.4C > T      | p.L2F     | Neutral    | Damaging   | probably damaging | 1/93 (1) | 0.5   | e3/e3            |
| PSEN2  | 1:227071449 | rs58973334  | G/A     | c.185 G > A   | p.R62H    | Neutral    | Tolerated  | benign       | 1/93 (1) | 0.5   | e3/e3            |

Figure 2. Number of loss of function (LoF) mutations in APP-\(\beta\)-degradation and production genes detected in the HEX and SVID cohorts and number of individuals per each cohort. The SVID cohort presents a burden of truncating mutations, compared to the HEX cohort. SVID, small vessel ischemic disease.
Table 4. Loss of function mutations detected in the SVID and HEX cohort. Aa, amino-acid change. Freq, frequencies; MAF, minor allele frequency; SVID, small vessel ischemic disease.

Gene expression screening during acute and subacute hypoperfusion in the BCCAS mouse model

APP-Aβ metabolism. We have used RNA sequencing data from prefrontal cortex, hippocampus and striatum of a mouse model of ischemia characterized by watershed and mainly subcortical infarcts (Fig. 3A–G), therefore a reliable model to study vascular dementia.

We detected a selective significant overexpression (up to 8-fold change and adj. p-value<0.05) of 5/17 genes (29.4%) involved in APP-Aβ degradation (A2m, Plat, Ctsd, Ctsb and Klk6) and none of the genes controlling APP-Aβ production during brain acute or subacute hypoperfusion (2d and 7d post-surgery, respectively). A2m was ubiquitously overexpressed both in prefrontal cortex, hippocampus and striatum, both 2 and 7 days post-surgery. By contrast, Plat, Ctsd and Klk6, displayed a specific time and region pattern of overexpression: Plat was overexpressed in prefrontal cortex and striatum 2 days post-ischemia and Ctsd and Klk6 in striatum and hippocampus 7 days post-surgery, both tissues characterized by the highest degree of tissue remodelling and overall differential expression (Table 6).

LOAD GWAS loci. We report a significant upregulation (up to 7-fold change, and adj. p-value<0.05) of Aproc, Cd33, Msda cluster (Msda4a, Msda4c, Msda1c, Msda1d, Msda14, Msda4b, Msda6b, Msda7), particularly in the most affected brain areas during subacute hypoperfusion (hippocampus and striatum, d7) (Fig. 3). Together with APP-Aβ degradation genes (A2m, Plat, Ctsd, Ctsb and Klk6), the overexpression of Aproc, Cd33, Msda likely relied on microglia infiltration in the infarct and peri-infarct area at day 7 as these genes shared the same expression pattern of other microglia markers such as Aif1 and Cd68 (up to 6-fold upregulation in hippocampus and striatum), was proportional to the hippocampal lesion volume detected at day 7 on T2-weighted MRI and caused by a severe drop of brain cerebral blood flow (CBF) (≈60–70% brain CBF reduction compared to naive mice) (Fig. 4). Thus strongly arguing for consequential rather than causal upregulation of these genes and a possible role in ischemic lesion resolution. This was further supported by the concomitant significant co-expression of 7 different matrix metallo proteases (Mmp2, Mmp8, Mmp11, Mmp12, Mmp13, Mmp19, Mmp23) and 51 lysosomal genes at day 7 (Tables S5 and S6, Fig. S2C,D).

Aβ oligomers detection during brain acute-subacute hypoperfusion in BCCAS mice. We investigated the hypothesis that acute-subacute ischemia may have triggered the de novo misfolding of APP with a consequent generation of toxic Aβ oligomers. We indeed identified Aβ oligomers mostly in CA1 region in the hippocampus of BCCAS mice 7 days post-surgery (Fig. 5A,B). These were found both in pyramidal neurons (mainly axonal processes) (Fig. S5B–F) and particularly in reactive astrocytes (Fig. 5C,D), analogously to those detected in APPPS1 mice at the age of 2 months (Fig. 3). These were present to a significant lower degree in the hippocampus of BCCAS mice 2 days post-surgery (t-test p-val=0.0321), characterized by a very moderate microglia/macrophage infiltration as suggested by Cd68 and Aif1 expression (Table 6), a modest overall tissue remodelling and gene differential expression (Fig S2) and almost absent in the hippocampus of naive mice (t-test p-val=0.0006) (Fig. 5E). Thus supporting the hypothesis that severe subacute brain hypoperfusion (60–70% CBF reduction), which however may not cause ischemic lesions detectable on T2-weighted MRI was necessary and sufficient to prime APP misfolding (Fig. 3A,B).
### Table 5. Alzheimer’s disease GWAS hit single-variant association test in the SVID cohort. ID, identification number; Aa, amino acid; MAF, minor allele frequency; SVID, small vessel ischemic disease; CADD, combined annotation dependent depletion; HEX, Healthy Exomes; p-val, p-value; adj, adjusted; OR, Odds Ratio; CI, Confidence Interval. LD, linkage disequilibrium, LOAD, late-onset Alzheimer’s disease. GWAS, genome-wide association study.

| Gene | Position | rsID | Allele | cDNA change | AA change | MAF HEX_70 | MAF SVID | CADD score | SVID allele count/allele number | HEX allele count/allele number | p-val | p-val adj | OR | CI | APOE ε4/ ε2 (%) | Comment |
|------|----------|------|--------|------------|------------|------------|-----------|------------|-------------------------------|-------------------------------|-------|-----------|-----|----|----------------|---------|
| EPHA1 | 7:143088867 | rs6967117 | T/C | c.2698 A > G | P | 0.919 | 0.786 | 15.46 | 151/192 | 658/716 | 1.41e-06 | 5.0e-05 | 0.325 | 0.025–0.517 | 27.5/15.5 | LD with LOAD GWAS hit rs3865444 (Raj et al., 2014, p.33) Blood Protein Level GWAS hit (Sahure et al., 2017) Hematological trait GWAS hit (Astle et al., 2016) |
| CD33 | 19:51728477 | rs12459419 | A/T | c.4828 A > G | P | 0.917 | 0.843 | 11.84 | 162/192 | 675/736 | 3.9e-03 | 0.0468 | 0.829–0.810 | 28.2/12.9 | Blood Protein Level GWAS hit (MacArthur et al., 2015) |
| ABCA7 | 7:143097100 | rs4725617 | A/G | c.205 A > G | P | 0.936 | 0.036 | 15.51 | 79/192 | 38/406 | 0.012 | 0.086 | 0.135–0.852 | 16.6/50 | protecting against immunocomplex deposition (Birmingham et al., 2003) |
| CR1 | 1:207726161 | rs200082366 | G/T | c.3066 G > T | P | 0.936 | 0.036 | 15.51 | 79/192 | 38/406 | 0.012 | 0.086 | 0.135–0.852 | 16.6/50 | |
| CD33 | 19:51728641 | rs2450509 | A/G | c.205 A > G | P | 0.407 | 0.442 | 0.209 | 85/192 | 299/734 | 0.4108 | 1 | 0.826–1.611 | 32.3/15.38 | Blood Protein Level GWAS hit (Astle et al., 2016) |
| CR1 | 1:207782916 | rs4444469 | A/T | c.4828 A > T | P | 0.974 | 0.994 | 6.589 | 191/192 | 717/736 | 0.095 | 0.570 | 0.793–211.22 | 29.1/13.5 | |
| CR1 | 1:207795320 | rs2296160 | A/G | c.5905 A > G | P | 0.834 | 0.880 | 0.001 | 169/192 | 599/718 | 0.145 | 0.745 | 0.893–2.469 | 29.78/13.8 | |
| CR1 | 1:207787503 | r66691117 | A/G | c.4843 A > G | P | 0.1943 | 0.239 | 8.406 | 46/192 | 143/736 | 0.190 | 0.820 | 0.873–1.931 | 25/10 | |
| ABCA7 | 19:1055319 | rs3745842 | G/A | c.4046 G > A | P | 0.354 | 0.357 | 0.367 | 68/192 | 217/536 | 0.228 | 0.820 | 0.562–1.149 | 31.5/15.78 | |

### Discussion

In this study we aimed at investigating the role of AD known pathogenic alleles and pathways: APOE ε4 allele, APP-β metabolism genes and LOAD most replicated GWAS hits both in terms of genetic variability in a cohort of 96 familial and early-onset SVID patients and differential gene expression during acute and subacute hypoperfusion in the BCCAS mouse model resembling vascular dementia (Fig. 1).

In our cohort, around one third of the patients (29%) carried APOE ε4 allele. E3/ε4 and ε4/ε4 genotype frequency (27% and 1%, respectively) approximated the one reported in Caucasian controls (21.3% and 1.8%, respectively) and was significantly lower compared to the frequency reported in Caucasian LOAD patients (41.1% and 14.8%, respectively) (p-value = 0.05192 and 0.0003155 for ε3/ε4 and ε4/ε4 genotypes, respectively) (Table 2) (25). Therefore suggesting, in concert with previous studies, that APOE ε4 allele may not critically influence the susceptibility to SVID (26).

Moreover we report the prevalent role of APP-β degradation genes upon genes involved in APP-β production as well as Mendelian genes causative for Alzheimer’s disease (APP, PSEN1 and PSEN2). We detected an enrichment for truncating mutations in genes playing a key role in APP-β catalysis, both when compared to genes controlling APP-β production in SVID patients or APP-β degradation genes in 368 neuropathologically confirmed elderly controls (9.4% and 1.6%, respectively) (Fisher p-value = 3.496e-14) (Fig. 2, Table 4).

In addition, we report a common polymorphism in CST3 (p.A25T), whose homozygous carrier frequency was significantly higher compared to HEX controls (4.16% and 1.63%, respectively). Interestingly, this polymorphism is supposed to influence CST3 intracellular processing with a reduced extracellular secretion (30,31), leading to increased amyloid fibril formation and Aβ deposition (32). A similar effect to what has been described for the pathogenic mutation CST3 p.L68Q, causative for hereditary cerebral haemorrhage with amyloidosis, Icelandic type (HCHWA-I), resulting in increased intracellular localization of the mutant Cystatin C (33).

Furthermore, we detected 2 rare coding variants (p.E270K and p.A528T) in the SORL1 VPS10 domain, reported to interact with Aβ and harbouring SORL1 pathogenic mutations (39, Table S1). The carrier frequency...
of SORL1 variants in the VPS10 domain detected in our SVID cohort was similar to the frequency detected in a Caucasian British and American LOAD cohort, where 8 variants in 323 patients have been identified (2% and 2.4%, carrier frequency in SVID and LOAD, respectively) whereas only 5 SORL1 variants in the VPS10 domain have been reported in 676 elderly controls in the same cohort (0.7%)34. This suggests that SORL1 mutations may influence the susceptibility also for SVID and may support the previously reported role of SORL1 in vascular dementia35.

By contrast, none of the 5 rare coding variants detected in APP, PSEN1 and PSEN2 are likely to be risk factors for SVID (Table 3). First, they have already been described as benign polymorphisms (PSEN1 [p.E318G] and PSEN2 [p.R62H]) (www.molgendatabase). Second, they all cluster outside the reported pathogenic domains (APP [p.V576I and p.T280del], PSEN1 [p.E318G] and PSEN2 [p.L2F and p.R62H]). This further shows that rare variants in APP, PSEN1 and PSEN2 are not common pathogenic factors in familial and early-onset apparently sporadic SVID cases. In line with this observation, 11/50 (22%) pathogenic mutations in APP have been reported as causative for AD and CAA, and only 2/50 (4%) lead exclusively to CAA. A smaller fraction of PSEN1 pathogenic mutations (4/219 [1.8%]) has been described as causative for both AD and CAA and none exclusively for CAA.

The predominant role of APP-Aβ degradation genes was further confirmed by RNA sequencing data in a mouse model of mainly subcortical ischemia, mimicking small vessel ischemic disease, where only genes belonging to the APP-Aβ degradation path (A2m, Plat, Ctsd, Ctsb and Klk6) were significantly overexpressed in hippocampus and striatum during acute and subacute hypoperfusion (Table 6). Among these, Klk6 expression has been already reported restricted to endothelial cells and increased of approximately 2-fold in the frontal cortex of patients with vascular dementia36.

Importantly, we showed that genetic and gene expression variability in LOAD GWAS genes are also likely to influence the susceptibility to SVID and acute-subcute ischemia.

We reported 3 common coding polymorphisms significantly associated to SVID and likely to play a mild protective role (adj. p-value <0.05 and 0.325<OR<0.54): EPHA1 p.M900V and p.V160A, CD33 p.A14V (Table 5). Among these, CD33 rs12459419-T has been reported to be in high LD with LOAD GWAS hit rs3865444-A and was suggested to explain its effect, such as the alternative splicing of CD33 with increased production of isoforms lacking exon 2, which encodes the IgV domain that typically mediates binding of sialic acid in SIGLEC family members. This CD33 isoform counteracts the inhibitory effect of CD33 on TREM2 in microglia and would ultimately reduce amyloid deposition and thus exert a moderate protective effect on Alzheimer’s disease and likely SVID susceptibility37. In addition, the critical role of this polymorphism (CD33 rs12459419) was further reinforced by its significant association, together with EPHA1 p.V160A, with blood protein levels, and haematological traits in two different GWASs37.44.

Moreover, Apoe, Cd33 and the Ms4a cluster were significantly upregulated in hippocampus and striatum particularly during subacute hypoperfusion (up to 7-fold change at day 7) (Table 6). Together with APP-Aβ degradation genes, overexpression of Apoe, Cd33 and Ms4a cluster correlated with hippocampal lesion size at day 7.
(Fig. 4B,C) and likely microglia infiltration of the infarct and peri-infarct areas (co-expression of microglia markers \textit{Cd68} and \textit{Aif1}), suggesting that upregulation of these genes was tightly driven by and consequential to the severity of hypoperfusion, moreover, arguing for an active role of these genes in tissue remodelling and ischemic lesion resolution. This is further supported by the fact that significant overexpression of \textit{Cd33}, \textit{Ms4a6d} and \textit{Apoe} (>1.5 fold change and adj \textit{p}-value <0.05) markedly correlated with AD pathology in 2 mouse models of Alzheimer's disease characterized by severe A\textsubscript{B} plaques and tau tangle deposition (HOTASTPM and TAU mice, 18 months of age\textsuperscript{62}). Therefore implying that \textit{Cd33}, \textit{Ms4a} cluster and \textit{Apoe} are not A\textsubscript{B} or tau specific.

Finally, the shared pathogenic pathway between LOAD-SVID-ischemic stroke was supported by histological findings of neurons and reactive astrocytes positive for A\textsubscript{B} oligomers in the main hypoperfused areas such as hippocampus at day 7 in the BCCAS mouse model (Fig. 5).

### Table 6. Differential gene expression during acute and subacute hypoperfusion in BCCAS mouse model.

| Gene   | AD path       | Region_Time | Log2FC | \textit{p}-value | FDR     |
|--------|---------------|-------------|--------|-----------------|---------|
| A2m    | A\textsubscript{B} catabolism | Hippocampus\_2d | 3.48 | 2.28E-14 |         |
|        |               | Prefrontal cortex\_2d | 3.07 | 2.10E-11 |         |
|        |               | Striatum\_2d | 2.57 | 3.56E-08 |         |
|        |               | Hippocampus\_7d | 2.9 | 3.98E-11 |         |
|        |               | Prefrontal cortex\_7d | 2.52 | 2.43E-07 |         |
|        |               | Striatum\_7d | 2.94 |            |         |
| Plat   | A\textsubscript{B} catabolism | Prefrontal cortex\_2d | 1.2 | 1.46E-09 |         |
|        |               | Striatum\_2d | 1.07 | 8.95E-08 |         |
| Klh6   | A\textsubscript{B} catabolism | Hippocampus\_7d | 2.37 | 5.52E-08 |         |
| Ctsb   | A\textsubscript{B} catabolism | Hippocampus\_7d | 1.4 | 2.50E-10 |         |
|        |               | Striatum\_7d | 1.3 | 8.37E-09 |         |
| Ctsd   | A\textsubscript{B} catabolism | Hippocampus\_7d | 2.52 | 3.03E-14 |         |
|        |               | Striatum\_7d | 2.16 | 1.69E-10 |         |
| Apoe   | GW AS hit     | Hippocampus\_7d | 1.35 | 4.20E-10 |         |
| Ms4a4a | GW AS hit     | Hippocampus\_2d | 1.8 | 2.93E-04 |         |
|        |               | Hippocampus\_7d | 1.85 | 6.08E-05 |         |
|        |               | Striatum\_7d | 1.61 | 6.80E-04 |         |
| Ms4a4c | GW AS hit     | Hippocampus\_2d | 2.56 | 3.65E-07 |         |
|        |               | Hippocampus\_7d | 2.08 | 1.48E-05 |         |
|        |               | Striatum\_7d | 2.27 | 1.89E-06 |         |
| Ms4a6c | GW AS hit     | Hippocampus\_2d | 1.76 | 2.86E-04 |         |
|        |               | Hippocampus\_7d | 2.8 | 4.64E-11 |         |
|        |               | Striatum\_7d | 2.32 | 8.45E-08 |         |
| Ms4a6d | GW AS hit     | Hippocampus\_2d | 2.53 | 1.43E-09 |         |
|        |               | Hippocampus\_7d | 2.33 | 3.98E-09 |         |
|        |               | Striatum\_7d | 1.98 | 9.37E-07 |         |
| Ms4a14 | GW AS hit     | Hippocampus\_7d | 2.75 | 3.36E-09 |         |
|        |               | Striatum\_7d | 1.66 | 8.88E-04 |         |
| Ms4a4b | GW AS hit     | Hippocampus\_7d | 2.11 | 8.51E-06 |         |
|        |               | Striatum\_7d | 1.72 | 4.70E-04 |         |
| Ms4a6b | GW AS hit     | Hippocampus\_7d | 1.8 | 3.57E-07 |         |
|        |               | Striatum\_7d | 1.48 | 4.41E-05 |         |
| Ms4a7  | GW AS hit     | Hippocampus\_7d | 2.65 | 1.50E-09 |         |
|        |               | Striatum\_7d | 1.89 | 3.89E-05 |         |
| CD33   | GW AS hit     | Hippocampus\_7d | 2.18 | 1.52E-22 |         |
| Cd68   | Microglia marker | Hippocampus\_2d | 1.46 | 6.68E-04 |         |
|        |               | Hippocampus\_7d | 2.68 | 3.16E-13 |         |
|        |               | Striatum\_7d | 2.33 | 5.05E-10 |         |
| Aif1   | Microglia marker | Hippocampus\_7d | 2.25 | 5.64E-17 |         |
|        |               | Striatum\_7d | 1.57 | 1.16E-08 |         |

The authors hypothesized that A\textsubscript{B} may have been the result of APP overexpression during ischemic stress\textsuperscript{65}. Importantly, we show that
acutely-subacutely hypoerfused brain areas and particularly reactive astrocytes and hippocampal neurons are positive for Aβ oligomers rather than β amyloid, that it is likely to represent a late and chronic event in the APP misfolding cascade. Moreover, we detected Aβ oligomers in the hypoperfused brain regions, displaying gliosis but...
not necessarily gray or white matter hyperintensities detectable on T2-weighted MRI (Fig. 5, Fig. 3A,B). Thus suggesting that a marked degree of hyperperfusion-ischemia that may remain however below the T2-weighted MRI detectability and does not lead to strokes, may trigger APP misfolding and may explain the link between brain microstructural changes detected on diffusion tensor imaging (DTI) and likely hypoxic-ischemic hyperintensities in white matter, detected decades before the onset of symptoms and autosomal dominant AD cases66,67 as well as common late-onset sporadic cases and elderly people68.

Therefore, our data may unveil at least some of the pathogenic mechanisms by which ischemic stroke may precipitate the progression of AD in experimental models and patients and why cerebrovascular accidents may accelerate AD onset particularly in asymptomatic elderly patients with AD pathology69. Indeed, the enrichment for genetic variability in APP-Aβ degradation genes has been reported playing a key role in sporadic late-onset AD whereas increases in Aβ production currently explain a minority of AD cases37,70.

In summary, we provide genetic, gene-expression and histological data supporting a shared pathogenic ground between LOAD and SVID-acute ischemia. Our genetic data in SVID patients, together with expression data in a vascular dementia mouse model show that 1) APOE transcriptional regulation but not e4 allele may play a role in brain hyperperfusion and small vessel ischemic disease; 2) APP-Aβ degradation plays a prevalent role upon APP-Aβ production; 3) APP, PSEN1 and PSEN2 are not common pathogenic factors in SVID; 4) CD33, CR1, EP1A1 and the MS4A cluster may be involved in SVID and brain subacute hypoperfusion-ischemia and 4) acute and mainly subacute ischemia may trigger Aβ toxic oligomer formation. Thus suggesting that the vascular hypothesis73 and the amyloid cascade hypothesis72 in AD may complement each other, rather than being mutually exclusive. Our findings warrant further genetic screening in a larger cohort and functional studies.

Data availability
All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author contributions
S.B., M.F., C.S. and U.D. planned the experiments. S.B., M.F., C.S., D.B., S.M. performed the experiments. S.B., M.F., S.M., M.H., K.B., D.B., D.B., U.D. and C.S. analysed the data and drafted the manuscript.

Competing interests
The authors declare no competing interests.

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