MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

Mice Knocked Out for the Primary Brain Calcification—Associated Gene *Slc20a2* Show Unimpaired Prenatal Survival but Retarded Growth and Nodules in the Brain that Grow and Calcify Over Time

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Brain calcification of especially the basal ganglia characterizes primary familial brain calcification (PFBC). PFBC is a rare neurodegenerative disorder with neuropsychiatric and motor symptoms, and only symptomatic treatment is available. Four PFBC-associated genes are known; approximately 40% of patients carry mutations in the gene *SLC20A2*, which encodes the type III sodium-dependent inorganic phosphate transporter Pit2. To investigate the role of Pit2 in PFBC development, we studied *Slc20a2*-knockout (KO) mice using histology, microcomputed tomography, electron microscopy, and energy-dispersive X-ray spectroscopy. *Slc20a2*-KO mice showed histologically detectable nodules in the brain already at 8 weeks of age, which contained organic material and were weakly calcified. In 15-week-old mice, the nodules were increased in size and number and were markedly more calcified. The major minerals in overt calcifications were Ca and P, but Fe, Zn, and Al were also generally present. Electron microscopy suggested that the calcifications initiate intracellularly, mainly in pericytes and astrocytes. As the calcification grew, they incorporated organic material. Furthermore, endogenous IgG was detected around nodules, suggesting local increased blood-brain barrier permeabilities. Nodules were found in all 8-week-old *Slc20a2*-KO mice, but no prenatal or marked postnatal lethality was observed. Thus, besides allowing for the study of PFBC development, the *Slc20a2*-KO mouse is a potential solid preclinical model for evaluation of PFBC treatments. (*Am J Pathol* 2018, 188: 1865–1881; <https://doi.org/10.1016/j.ajpath.2018.04.010>)

Primary familial brain calcification (PFBC; Online Mendelian Inheritance in Man no. 213600, no. 616413, no. 615007, and no. 615483) is a neurodegenerative disease characterized by symmetric calcifications of the basal ganglia and other brain regions (eg, thalamus and cerebellum), in the absence of altered serum levels of phosphate, calcium, and parathyroid hormone.¹ The disease is also known as Fahr disease and familial idiopathic basal ganglia calcification. No association of PFBC with systemic disorders (eg, hormonal disturbances) has been identified; PFBC is usually found coincidentally on neuroimaging addressing the neuropsychiatric and motor symptoms.¹ The symptoms

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are heterogeneous and include movement disorders, such as parkinsonism, cognitive abnormalities, ranging from mild cognitive impairment to dementia, and psychiatric disorders that include mood disorders and psychotic signs.^{1–4} PFBC is usually inherited in an autosomal dominant pattern, but de novo cases have also been reported.^{5,6} Although it is a rare genetic disease, brain calcification is now more frequently detected because of extended use of brain computed tomography (CT).⁷

In PFBC patients, the calcifications are commonly found closely associated with the vasculature, and the generally accepted hypothesis is that the calcifications originate from the vasculature.^{8–10} The disease mechanism is unknown, but there are clear genetic culprits. Wang et al¹¹ found deleterious mutations in *SLC20A2*, encoding the type III sodium-dependent inorganic phosphate (P_i) transporter PiT2, in affected families from China, Brazil, and Spain. Since then, *SLC20A2* mutations have been found in PFBC families worldwide,^{12–25} and Lemos et al¹⁴ have reviewed 50 reported variants of *SLC20A2* in 55 unrelated patients. Three other genes linked to the disease are *PDGFRB*, *PDGFB*, and *XPRI*.^{26–28} They encode the platelet-derived growth factor receptor β , its specific ligand, the platelet-derived growth factor subunit B, and the multimembrane spanning protein xenotropic and polytropic retrovirus receptor 1, recently identified as a cellular P_i exporter.²⁹

Mutations in *SLC20A2* are the most common and are estimated to be responsible for at least 40% of the PFBC cases.¹² The mutation type varies: some lead to aberrant mRNAs, and others lead to PiT2 proteins that are shown or predicted to be unable to transport P_i .^{11–25,30–32} Wang and colleagues¹¹ suggested that cells carrying a PiT2 transport-knockout (KO) mutation in one allele will show reduced P_i uptake because of haploinsufficiency. However, the effect of heterozygous mutations may be more complex. Recently, an autopsied patient carrying a heterozygous mutation (p.Ser637Arg) was reported to show severely reduced to absent PiT2 protein in extracts from frontal cortex and cerebellum and from putamen, respectively.⁸ We recently obtained results that provide a potential explanation for the observations by Kimura et al.⁸ Missense variants were shown to have a dominant negative effect on PiT2-mediated P_i uptake. Heterozygous mutations in mammalian cells were mimicked by coexpressing PiT2-missense variants that are unable to transport P_i and wild-type (WT) PiT2; these missense variants reduced the P_i -uptake ability of WT PiT2.³³ Because PiT2 can oligomerize,^{31,34–36} we hypothesize that WT PiT2 protein oligomerized with certain variant PiT2 proteins is recognized by the cells as a misfolded structure and degraded, resulting in little to absent WT and variant PiT2 proteins in the cells, as reported by Kimura et al.⁸

To investigate the role of PiT2 in PFBC, *Slc20a2*-KO mice were studied. We previously showed bilateral brain calcifications in two 19-week-old *Slc20a2*-KO mice.³⁷ Herein, we present new insight into the development of brain calcifications and additional characterization of this PFBC model.

Slc20a2-KO mice had brain nodules already at 8 weeks of age, which appear weakly calcified. At 15 weeks, the nodules had calcified and grown in size and number. The nodules were almost exclusively associated with blood vessels, and IgG antibodies associated with nodules could not be detected, suggesting an affected blood-brain barrier (BBB). Extensive bilateral calcification of the hypothalamus and midbrain was revealed by micro-CT (μ CT) scans of 20-week-old KO mice. The μ CT scans also revealed that mice at the same age could show high variation in the degree of calcification, which reflects observations in PFBC. On closer inspection of the nodules with transmission electron microscopy (TEM), intracellular calcification was found in pericytes and astrocytes; degenerated astrocytes were also detected. Scanning electron microscopy/energy-dispersive X-ray spectroscopy (SEM/EDX) confirmed the histologic findings, showing large amount of P and Ca in the calcifications, but also smaller amount of Fe, Zn, Al and in some Mg and S. Both TEM and SEM/EDX confirmed the presence of organic material in the calcifications. Finally, the KO mice were smaller than WT and heterozygous (HET) mice, but increased prenatal lethality or marked postnatal lethality of the KO mice was not observed.

Materials and Methods

Animals

Transgenic C57BL/6NTac mice with an inserted KO cassette in *Slc20a2* were obtained from the European Mouse Mutant Archive (Munich, Germany). The strain is denoted C57BL/6N^{Tac}-*Slc20a2*^{tm1a-(EUCOMM)Wtsi}/leg (EM: 05549) and is HET for the KO cassette, L1L2-PGK-P, inserted in *Slc20a2* after coding exon 2 and flanking coding exon 3. It introduces splice acceptor and SV40 polyadenylation sequences in *Slc20a2* between the second and third coding exons. The mice were bred and housed in the Animal Facility at the Health Faculty, Aarhus University (Aarhus, Denmark). All were fed the same standard diet ad libitum [autoclaved Altromin 1319F, made by Altromin Spezialfütter GmbH, Lage, Germany (supplied by Brogaarden, Lyngby, Denmark)] and had free access to water. They were housed under a 12-hour light/dark schedule, with lights on at 6 AM and off at 6 PM. The pups were weaned at 3 to 4 weeks of age and then housed in groups of two to six for males and two to eight for females. Cage size depended on the size of the group (females: two to four mice: 350 cm²; and five to eight mice: 800 cm²; males: two to three mice: 350 cm²; and four to six mice: 800 cm²).

The mice were handled according to the Danish law on animal experimentation and the Animal Welfare Policy at Aarhus University. Procedures were approved by the Danish Animal Experiments Inspectorate, license number 2013-15-2934-00894, and reported in accordance with the Animal Research: Reporting *in Vivo* Experiments guidelines.

Offspring from HET breeding pairs were weighed once a week, starting between 8 and 21 days of age, and continued

until euthanasia. Weight data obtained from 131 female mice (36 WT, 62 HET, and 33 KO) and 165 male mice (36 WT, 91 HET, and 38 KO) were modeled with a logistic growth curve. Mice weighed fewer than three times were excluded from the analysis.

Genotyping was performed, as previously described.³⁷ Brains were preserved by perfusion fixation through the heart or by immersion fixation in 4% formaldehyde (formalin, 10% buffered) (catalog number 1000.5000; Hounisen, Skanderborg, Denmark). Mice were anesthetized by inhalation of 3.75% isoflurane before and during perfusion fixation. This anesthesia was used because it is highly effective at obtaining a deep surgical level of anesthesia in mice without overdosing.

Animals were analyzed for the presence of brain calcification. Both females and males were used, because both sexes developed similar calcifications. The following animals were analyzed for brain calcifications: 8-week-old mice: 10 WT and 7 KO mice (histology); 9-week-old mice: three WT and three KO mice (μ CT); 15-week-old mice: three WT and seven KO mice (histology); 19-week-old mice: one WT and one KO mice (TEM and SEM/EDX); 20-week-old mice: three WT and three KO mice (μ CT); 1-year-old mice: eight WT and seven HET mice (histology); and 1.5-year-old mice: one HET mouse (μ CT). Sexes are indicated in the following paragraphs, under the respectable methods, and in [Results](#).

Histology

Histologic methods were performed, as previously described.³⁷ Briefly, brains were fixed in 4% formaldehyde and embedded in paraffin. Histologic staining was performed on 4- μ m-thick sections. The sections were deparaffinized in Histo-clear (catalog number HS-200; National Diagnostics, Atlanta, GA) and rehydrated. For detection of phosphate, sections were stained with the von Kossa staining technique. They were incubated 20 minutes in 10% silver nitrate, and rinsed in sterile water, followed by 2 minutes in 5% pyrogallol. After being rinsed again in sterile water, the sections were placed in 5% sodium thiosulfate for 5 minutes. For counterstaining, eosin was used.

Periodic acid-Schiff (PAS)—hematoxylin staining was performed using a standard protocol; Mayer's hematoxylin was used as counterstain. PAS stains organic material containing carbohydrates containing 1-2 glycols or structures closely related to 1-2 glycols. The hematoxylin solution stains nuclei; however, calcium is a mordant for hematoxylin and structures rich in calcium can also stain deep purple with hematoxylin. Alizarin Red S (AR) staining was performed by deparaffinizing sections in xylene, rehydrating to 96% ethanol, and subsequent staining in 40 mmol/L AR, pH 4.3, for 6 minutes.

For immunohistochemistry, antigen retrieval was performed on deparaffinized sections by boiling in TEG (10 mmol/L Tris-HCl and 0.5 mmol/L EGTA, pH 9.0) in a microwave oven for 32 minutes. Sections were incubated with 5% goat serum (X0907; Dako, Agilent Technologies, Santa

Clara, CA) for 5 minutes or with 1:10 dilutions of FcR Blocking Reagent mouse (catalog number 130-092-575; Miltenyi Biotec, Bergisch Gladbach, Germany). Mouse IgG was detected using OptiView DAB IHC Detection kit on a Ventana Benchmark (Roche, Basel, Switzerland). Last, the sections were stained with Mayer's hematoxylin (Ampliqon, Odense, Denmark) to visualize cell nuclei.

Mice analyzed with von Kossa and PAS were as follows: 8-week-old mice: 10 WT (six females and four males) and seven KO (three females and four males); 15-week-old mice: three WT (one female and two males) and seven KO (one female and six males); and 1-year-old mice: eight WT (two females and six males) and seven HET (two females and five males). Mice analyzed with AR were as follows: 8-week-old mice: four WT (two females and two males) and four KO (one female and three males); and 15-week-old mice: three WT (two females and one male) and four KO (four males). Mice analyzed with immunohistochemistry for mouse IgG detection were as follows: 8-week-old mice: four WT (one female and three males) and four KO (one female and three males); and 15-week-old mice: three WT (two females and one male) and four KO (males).

Isolation of Primary Murine Fibroblasts

Isolation of fibroblasts from 4-week-old male and female mice was performed according to the protocol by Seluanov et al,³⁸ with small modifications. Mice were euthanized by cervical dislocation and placed on ice. The fur was soaked in 70% ethanol and shaved. A skin sample was collected from the chest area, placed in cold phosphate-buffered saline, and transferred to plates in a laminar airflow bench, where they were cut to a jelly-like consistency. The tissue was then added to a mixture of Liberase TM Research Grade (catalog number 05401119001; Roche), Dulbecco's modified Eagle's medium (DMEM; catalog number 41966-029; Gibco, Dublin, Ireland), and 1% antibiotic/antimycotic (catalog number 15240-062; Gibco) and incubated at 37°C, while stirred slowly, for at least 90 minutes. The solutions containing the tissue were transferred to DMEM supplemented with 15% fetal bovine serum (catalog number 10270-160; Gibco) and 1% antibiotic/antimycotic. The samples were centrifuged, supernatants were removed, and the pellets were resuspended in DMEM—15% fetal bovine serum—antibiotic/antimycotic; this procedure was repeated two times to remove the remaining traces of Liberase. The samples were transferred to tissue culture—treated plates and incubated at 37°C in a 5% CO₂ atmosphere. The cells were passaged, when the fibroblast outgrowths were dense (ie, after approximately 8 to 14 days), at which point antibiotic/antimycotic was replaced with 1% penicillin/streptomycin (catalog number 15140-122; Gibco).

RT-qPCR

Tail tissue was stored in RNAlater (Invitrogen, Carlsbad, CA). It was homogenized using pestles and lysed in lysis/binding

buffer containing guanidinium thiocyanate provided with the RNeasy-4PCR Kit (Applied Biosystems, Foster City, CA). RNA was purified from the lysates using the RNeasy-4PCR Kit, as described by the manufacturer. RNA purification from formalin-fixed, paraffin-embedded (FFPE) tissue was done using the PureLink FFPE RNA Isolation Kit (Invitrogen), as described by the manufacturer. Purified RNA was quantified by UV spectrometry. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time quantitative RT-PCR (RT-qPCR) analysis, the following TaqMan Gene Expression Assays (Applied Biosystems) were used: Mm00660204-mH (*Slc20a2*) and, as endogenous control, Mm00437762-m1 [β -2-microglobulin (*B2M*)]. Each RT-qPCR contained 10 μ L TaqMan Fast Universal PCR Master Mix, 1 μ L TaqMan Gene Expression Assay (*Slc20a2*, FAM), 1 μ L TaqMan Gene Expression Assay (*B2M*, VIC), and 8 μ L cDNA (approximately 10 ng). The PCR cycles used were as follows: 95°C for 10 minutes and 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The relative mRNA levels were calculated, as previously described.³⁹ RT-qPCRs were set up as triplicates. Tail tissue from 8-week-old mice [five WT (three females and two males) and four KO (four males)] and FFPE brain tissue from 8-week-old mice [10 WT (six females and four males) and 7 KO (one female and six males)] were analyzed for *Slc20a2* expression.

Transduction of Primary Murine Fibroblasts with A-MLV and 10A1 Pseudotyped Vectors

Cultures of primary murine fibroblasts isolated from 4-week-old WT and KO mice were exposed to amphotropic murine leukemia virus (A-MLV) pseudotyped vectors (derived from the packaging cell line PA317) or 10A1 pseudotyped vectors (derived from the packaging cell line PT67) carrying enhanced yellow fluorescent protein or enhanced green fluorescent protein expressing MND-X-SN transfer vectors, respectively.⁴⁰ Cells were seeded in 24-well plates, and the next day pure filtrated (0.45- μ m) vector-containing supernatants or media (mock) supplemented with 8 μ g/mL polybrene (catalog number H9268; Sigma-Aldrich, St. Louis, MO) were added. The cells were incubated for 70 to 80 minutes, after which DMEM–15% fetal bovine serum–penicillin/streptomycin was added to dilute the polybrene to 2 μ g/mL. The cells were then incubated for another 4 to 5 days. Phenol red–free DMEM was added to the wells, before examining the cells for enhanced green fluorescent protein or enhanced yellow fluorescent protein expression using an Axiovert 200M fluorescence microscope (Zeiss, Göttingen, Germany) equipped with a CoolSNAP HQ camera (Photometrics, Tucson, AZ). Images were analyzed using ImageJ software version 1.48 (NIH, Bethesda, MD; <http://rsb.info.nih.gov/ij>).⁴¹ The transduction experiment was performed twice. Each time, independent replicates of cells from two WT (one male and one female, or two females) and two KO

(males) mice were subjected to vector-containing supernatants or mock transduced.

μ CT

Brains from the following animals were analyzed by μ CT: 9-week-old mice: three WT (one female and two males) and three KO (one female and two males); 20-week-old mice: three WT (one female and two males) and three KO (one female and two males); and 1.5-year-old mice: one HET male. The brains were surface stained by immersion in Ultravist (370 mg iopromide/mL; Bayer Pharma AG, Berlin, Germany) for 5 minutes. Then, the brains were gently wiped off and scanned in a desktop μ CT scanner (Scanco μ CT 35; Scanco Medical AG, Brüttisellen, Switzerland). The scans were performed in high-resolution mode (1000 projections/180 degrees) with a spatial resolution of $6 \times 6 \times 6 \mu\text{m}^3$, an X-ray tube voltage of 45 kV_p, an X-ray current of 177 μ A, and an integration time of 800 milliseconds. After scanning, three-dimensional image sets were reconstructed using the software provided with the scanner. Three-dimensional visualization was performed using Amira version 5.6 (FEI Visualization Science Group, Mérignac, France).

TEM and SEM/EDX

For TEM, tissue from brains fixed in 4% formaldehyde was post fixed in 2% glutaraldehyde in 0.04 mol/L phosphate buffer. The tissue was cut in smaller pieces and further post fixed in osmium tetroxide for 1 hour. The specimens were dehydrated in graded alcohols and propylene oxide, and infiltrated with epoxy resin. The epoxy was polymerized at 60°C. Ultrathin sections (60 nm thick) were cut, and some of the grids were stained with uranyl acetate and lead citrate. The sections were analyzed on a JEM 2100 electron microscope (JEOL, Freising, Germany). Mice analyzed with TEM were as follows: 19-week-old mice: one WT (male) and one KO (male).

For SEM-EDX, the tissue was embedded in epon without prior osmium fixation. Sections (200 to 300 nm thick) were cut, collected on water, and placed on a single-hole copper formvar grid or an uncoated nickel mesh grid. No contrast staining with uranium or lead was performed. The sections were analyzed on a Talos F200X S/TEM from FEI Visualization Science Group. Localized chemical analyses were obtained by bombarding with a 200-kV electron beam and detecting the emitted X-ray spectrum. Mice analyzed with SEM/EDX were as follows: 19-week-old mice: one KO (male).

Statistical Analysis

For RT-qPCR data, differences between groups were analyzed with a two-tailed *t*-test. The distribution of genotypes was tested for a 1:2:1 segregation with a χ^2 test.

Statistical analysis of weight data was performed by fitting a logistic growth curve by nonlinear mixed effects regression⁴² using maximum log-likelihood. The analysis

was done in R (The R Project for Statistical Computing; <http://www.r-project.org>) using the package *nlme* version 3.1-122 (CRAN; <https://mran.revolutionanalytics.com/snapshot/2016-01-01/web/packages/nlme/index.html>). The coefficients in the growth model were fitted as a linear combination of genotype (WT/HET/KO), sex (female/male), and the interaction between genotype and sex. In addition, the model contained intercepts for each mouse as a random effect. Group-level inference was made by comparing models fitted to observed data with and without the fixed effects (genotype, sex, and the interaction between genotype and sex). First, a null model was made, consisting of only a common intercept for all groups and the random effect. Then, the fixed effects for genotype, sex, and the interaction between genotype and sex were added one at a time. Significance testing was done by comparing pairs of models with and without the fixed effects by likelihood-ratio tests.

All statistical tests were used with critical $\alpha = 0.05$.

Results

Throughout this study, mice were selected on the basis of age and included both females and males, because there was no indication of differences in calcification development between the sexes. Including both females and males reduced the number of mice bred in compliance with the 3Rs (Replacement, Refinement, and Reduction of Animals in Research).⁴³

Characterization of *Slc20a2* Expression

The effect of the presence of the KO-cassette in both *Slc20a2* alleles on expression at mRNA and protein levels was investigated (Figure 1). Specifically, mRNA was purified from tail

Figure 1 Analysis of *Slc20a2*/Pi2 expression in wild-type (WT) mice and mice homozygous for the knockout cassette (KO). **A:** mRNA was purified from tail and formalin-fixed, paraffin-embedded brain tissues from 8-week-old mice, and analyzed by real-time quantitative RT-PCR (RT-qPCR). The bars show the average *Slc20a2* mRNA expression levels relative to B2M. KO compared with WT: Brain and tail relative *Slc20a2* mRNA expression levels ($P < 0.00001$ and $P < 0.01$, respectively). **B:** Analyses of transducibility with retroviral vectors targeting PiT1 and/or PiT2. Fibroblasts were cultured from skin obtained from 4-week-old mice. Cells from WT (one female and one male, or two males) and KO (two males) mice were seeded in 24-well plates at 10,000 cells/cm² and exposed to amphotropic murine leukemia virus (A-MLV) pseudotyped transfer vectors encoding enhanced yellow fluorescent protein (eYFP) or 10A1 pseudotyped transfer vectors encoding enhanced green fluorescent protein (eGFP). Representative images of WT and KO cells exposed to medium containing A-MLV or 10A1 pseudotyped vectors are shown. $n = 10$ (6 females and 4 males) (A, brain tissue, WT); $n = 7$ (1 female and 6 males) (A, brain tissue, KO); $n = 5$ (3 females and 2 males) (A, tail tissue, WT); $n = 4$ (4 males) (A, tail tissue, KO). Original magnification, $\times 10$ (B).

tissue [WT, $n = 5$ (three females and two males); KO, $n = 4$ (males)] and from FFPE brain tissue [WT, $n = 10$ (six females and four males); KO, $n = 7$ (one female and six males)] from 8-week-old mice. The mRNA levels of *Slc20a2* were analyzed by RT-qPCR using primers spanning exons 9 to 10. Severe reduction of *Slc20a2* mRNA was found in both brain and tail tissue from KO mice (Figure 1A). To confirm the functional loss at the protein level, a retroviral vector transduction assay was used. PiT2 is a receptor for the retrovirus A-MLV; both PiT2 and PiT1, encoded by the paralog *Slc20a1*, are receptors for the A-MLV-related isolate, 10A1.^{44–46} Thus, transduction via PiT1 was used as a positive control. Fibroblasts from WT (one male and one female, or two males) and KO (two males) mice were subjected to retroviral transfer vectors encoding enhanced yellow fluorescent protein or enhanced green fluorescent protein and pseudotyped with surface proteins from A-MLV or 10A1, respectively. The WT cells were transduced by both A-MLV and 10A1 pseudotyped vectors (Figure 1B). Fibroblasts from KO mice, however, were only transduced by the 10A1 pseudotyped vector (Figure 1B). These results show that PiT1 was available for transduction in the *Slc20a2*-KO cells, but PiT2 was not (Figure 1B). In conclusion, the severe reduction at the mRNA level was reflected in a lack of PiT2 protein in the cell membrane.

μ CT Scans Reveal Variation in Calcification Pattern in *Slc20a2*-KO Mice

μ CT scans were performed on brains from 9-week-old mice [WT, $n = 3$ (one female and two males); KO, $n = 3$ (one female and two males)] and 20-week-old mice [WT, $n = 3$ (one female and two males); KO, $n = 3$ (one female and two males)]. At the voxel size used (6 μ m), the

smallest detectable objects are 12 μ m in size. Calcifications were not detected in 9-week-old mice (data not shown). But the brains from 20-week-old KO mice contained extensive and bilateral calcification of the hypothalamus and midbrain and a lower level of calcification of the thalamus and pons (Figure 2, A–C). All three 20-week-old KO mice had overall similar calcification patterns; the degree of calcification, however, varied. Figure 2A shows the most severely calcified brain, and Figure 2C shows the least calcified brain. The most and least calcified brains were from male mice, whereas the calcification degree in the female mouse was in between the two. The density of individually detected calcifications varied between 914 and 1027 mg/cm³ (determined using brains that were not stained with iopromide), which is similar to the density of human cortical bone. No calcifications were found in brains of WT mice (Figure 2D). The lack and presence of calcifications in the μ CT scans of brains from 9- and 20-week-old mice, respectively, suggest an onset of the calcification during early adulthood.

Calcifications Can Initiate Intracellularly, Incorporate Organic Material, and Develop Over Time

To narrow down the time point at which the first pathologic alterations can be identified and to confirm the localization of the calcifications identified by μ CT, histologic analysis of sections from another set of mice was performed. Moreover, it was addressed whether the calcifications developed over time. For the histologic analysis, mice were sacrificed at the ages of 8 weeks [WT, $n = 10$ (six females and four males); KO, $n = 7$ (three females and four males)] and 15 weeks

Figure 2 Micro-computed tomographic scans of 20-week-old mice. **A:** Male knockout (KO) mouse with extensive calcification. **B:** The same brain as in **A** shown in superior view at an angle showing the bilateral distribution of the calcifications. **C:** Male KO mouse with a less widespread calcification. **D:** Male wild-type (WT) mouse. Calcifications are seen in hypothalamus (H), thalamus (T), midbrain (M), and pons (P; arrows). No calcification was present in the WT mice. The contrast of soft tissue structures was increased by incubation in iopromide for 5 minutes before the scanning. Continuously white-stained areas, as apparent in **D**, are artifacts caused by the contrast fluid.

[WT, $n = 3$ (one female and two males); KO, $n = 7$ (one female and six males)]. Horizontally sectioned FFPE brains were analyzed for nodules using von Kossa and PAS-hematoxylin staining. Nodules were found in all KO mice, and no nodules were found in WT mice. The location of the nodules corresponded with the μ CT scans. The 8-week-old KO mice had small nodules in the midbrain (Figure 3) and hypothalamus and few in the thalamus and pons (data not shown). At this age, the nodules stained only weakly with von Kossa staining, which detects phosphate, and were better visualized with the PAS-hematoxylin staining (Figures 3 and 4, A and B), indicating that they were not fully calcified. In 15-week-old KO mice, the nodules were present in the same regions as in 8-week-old KO mice (ie, mainly in the midbrain and hypothalamus). They now, however, stained strongly with von Kossa staining and had grown in both number and size compared with 8-week-old mice (Figure 3).

The presence of calcium in the nodules was verified by staining brain sections from 8- and 15-week-old WT and KO mice with AR (Figure 5) [8-week-old mice: WT, $n = 4$ (two females and two males); KO, $n = 4$ (one female and three males); 15-week-old mice: WT, $n = 3$ (two females and one male); KO, $n = 4$ (four males)]. As with von Kossa staining, a few weakly stained nodules were found in 8-week-old KO mice (Figure 5, A and C), and more and strongly stained larger nodules were found in 15-week-old KO mice (Figure 5, B and D); no nodules were present in WT mice at either age (data not shown).

The nodules were almost exclusively found in, around, or close to blood vessels, as visualized with PAS-hematoxylin staining, in both 8- and 15-week-old mice (Figures 3 and 4A). It was hypothesized that leakage/transport of plasma proteins over the BBB might contribute to the formation or growth of the nodules. To test for this, brain sections were stained for mouse IgG [8-week-old mice: WT, $n = 4$ (one

Figure 3 von Kossa and periodic acid-Schiff (PAS)—hematoxylin staining of 8- and 15-week-old wild-type (WT; **left column**) and knockout (KO; **middle and right columns**) mice. The von Kossa staining was performed simultaneously on brain slices from 8- and 15-week-old mice. A stronger staining of nodules in the 15-week-old mice is seen. The PAS-hematoxylin staining shows nodules/calcifications lying in and around blood vessels. Representative images of the midbrain are shown (8-week-old mice: male WT and female KO; 15-week-old mice: female WT and male KO). Boxed areas in **middle column** are shown in higher magnification in **right column**. **Arrows** point to nodules. Scale bars: 200 μ m (**left and middle columns**); 25 μ m (**right column**).

Figure 4 **A** and **B**: Periodic acid-Schiff (PAS)–hematoxylin staining (**A**) and von Kossa staining (**B**) on consecutive brain sections from an 8-week-old male knockout (KO) mouse. The same nodule is seen in the two images (**arrows**); it shows evident PAS staining (**A**) but only weak von Kossa staining (**B**). **C** and **D**: Immunostaining of endogenous IgG on brain sections from 8-week-old (**C**) and 15-week-old (**D**) KO mouse. IgG stain is visible around nodules (**arrows**). Scale bar = 25 μ m.

female and three males); KO, $n = 4$ (one female and three males); and 15-week-old mice: WT, $n = 3$ (two females and one male); KO, $n = 4$ (males)]. Nodules in 8-week-old mice were few and in general small, but nodules staining positive for mouse IgG could be discerned (Figure 4C); in 15-week-old KO mice, IgG-positive nodules were easily detectable (Figure 4D).

Closer inspections of the calcified areas were performed using TEM and SEM/EDX on a 19-week-old male

mouse. At sites of calcifications, TEM revealed multiple intracellular calcifications (Figure 6A), including in astrocytes (Figure 6E) and pericytes (Figure 6F), as well as degenerated astrocytes (Figure 6H). Calcifications could also be detected in what appeared to be a neuronal dendrite (Figure 6G). A pericyte with internal calcification at site of contact with an endothelial cell is shown in Figure 6F. No overt effect on the endothelium was observed.

Figure 5 Alizarin Red S staining of 8- and 15-week-old knockout (KO) mice. **A–D**: Representative images are shown: 8-week-old mice, one female (**A**) and one male (**C**); 15-week-old mice, two males (**B** and **D**). **Arrows** point to nodules. A stronger staining of nodules in the 15-week-old KO mice (**B** and **D**) compared with 8-week-old KO mice (**A** and **C**) is seen. Scale bar = 10 μ m.

The calcifications showed different morphologies (Figure 6, B and C). Calcifications with lamellar structure were present (Figure 6B), indicative of a sequential formation. Larger deposits with dense core and surrounded by a lighter corona with small needle-shaped structures were also observed (Figure 6C). Figure 6D shows a large deposit in a section not stained with uranyl acetate and lead citrate. The paleness of the deposit, compared with the uranyl acetate and lead citrate–stained sections, suggests the presence of organic material in the calcification, and cell material can be seen engulfed in its periphery.

To determine the composition of the calcified nodules, SEM/EDX was performed on sections from the same mouse as shown in Figure 6 (representative results are shown in Figure 7 and Table 1). The calcifications mainly consisted of Ca and P, with a Ca/P ratio between 1.00 and 1.28. Fe was less abundant, but also present in all analyzed areas of overt calcifications (Figure 7), independent of size. Furthermore, small amounts of Zn and Al were detected, albeit not always in the corona of larger calcifications (Figure 7A and Table 1). Small amounts of Mg and S were detected in approximately 40% to 50% of the analyzed areas of the

Figure 6 Transmission electron microscopy analyses of brain sections from a 19-week-old knockout mouse. **A:** Multiple small intracellular deposits (arrows). **B** and **C:** Larger deposits with different morphologies. **B:** The lamellar structure of one of the deposits (arrow) indicates a sequential formation. **C:** Larger deposit with dense core and a lighter corona. Small needle-shaped crystals can be seen in the corona (inset). **D:** Deposition in preparation where contrasting with uranyl acetate and lead citrate has been omitted, revealing the needle-shaped structures also to be present, thus indicating that uranium and lead stain additional material. Cell material can be seen engulfed in the periphery of the deposit. **E:** Deposits in close relation to filaments of intermediate size (arrow), indicating deposition in an astrocyte. **F:** Part of capillary with a red blood cell (R), endothelial cell (E), and pericyte (P). The pericyte is separated from the endothelial cell by a basement lamina (arrows), except between the arrowheads, where a deposit is also found. **G:** Deposit in a smaller structure in the neuropil in direct apposition to a bouton with dense core vesicles (arrow). **H:** Degenerated astrocyte with intermediate filaments (arrow). Scale bars: 500 nm (A and D); 2 μ m (B); 1 μ m (C and F–H); 500 nm (C, inset); 200 nm (E).

Figure 7 Scanning electron microscopy/energy-dispersive X-ray spectroscopy (SEM/EDX) analysis of the same brain region, as shown in Figure 6. Representative areas of sections with calcifications are shown. Unstained 200- to 300-nm-thick brain sections were collected either on a formvar-coated single-hole grid (A) or on an uncoated mesh grid (B), and analyzed using SEM/EDX. Individual figures show high-angle annular dark field (HAADF) images of the area and mapping of elements, as indicated. **Green boxed areas** in HAADF images were subjected to element analysis. The analysis of areas 2 to 9 in A is shown in Table 1. There is an apparent higher oxygen (O) content in the tissue in A compared with B, and this may reflect the difference in use of formvar coating. Scale bars: 5 μ m (A); 2 μ m (B). Al, aluminum; C, carbon; Ca, calcium; Cl, chlorine; Fe, iron; Mg, magnesium; N, nitrogen; P, phosphorus; S, sulfur; Zn, zinc.

Table 1 Element Analysis of Indicated Areas in Figure 7A

Element	Area							
	2	3	4	5	6	7	8	9
Phosphorus (P)	ND	TA	44.74	44.77	44.96	45.17	40.26	45.04
Calcium (Ca)	ND	ND	47.60	46.90	46.78	47.48	51.65	50.30
Iron (Fe)	ND	ND	4.08	3.21	4.83	3.26	7.19	4.66
Zinc (Zn)	ND	ND	2.01	1.82	1.74	2.22	0.9	ND
Aluminum (Al)	ND	ND	1.57	3.30	1.69	1.87	ND	ND

Data are given as the relative percentage of each element in the indicated area.

ND, not detected; TA, trace amount.

calcifications, although not in those analyzed in Figure 7A. Concerning the potential presence of Cu, the results were inconclusive. O, N, and C were not included in the analysis of the relative distribution of elements present, because of imprecision in their determination. The element mapping, however, showed that the calcifications were clearly enriched with O (Figure 7). Compared with the surrounding tissue, N was slightly enriched in the calcifications, or at the same level (Figure 7). For all areas analyzed, smaller amounts of C and Cl were detected in the calcifications compared with the surrounding tissue (Figure 7B). Interestingly, some areas close to calcifications and originally identified as areas of different structure in the high-angle annular dark field and with denser N mapping as areas 3 and 4 in Figure 7B showed detectable P but no Ca signals. No P or Ca signal was detectable in area 2 in the same section. Moreover, two other areas were identified in the same manner in the sections (data not shown); in these areas, Ca could be detected, but the Ca/P ratios (0.11 and 0.24, respectively) were lower than those observed in any part of overt calcifications (including the corona), which had Ca/P ratios closer to 1. These areas might represent the initiation of new areas of calcification, which suggests that calcifications initiate with a surplus of P compared with Ca.

In conclusion, the size and calcification of nodules increased markedly from 8 to 15 weeks of age. The calcifications consisted largely of P and Ca and small amounts of Fe, Zn, and Al and in some also Mg and S. Moreover, O and to some extent also N were enriched in the calcifications compared with the surrounding tissue. Intracellular calcifications were found in pericytes and astrocytes, and degenerated astrocytes were found at sites of calcification. IgGs could be detected already at 8 weeks and were restricted to areas around nodules.

Slc20a2-HET Mice Rarely Present with Nodules

It was also addressed whether mice heterozygous for the KO-cassette showed brain calcification at 1 year of age. FFPE brains from eight WT (two females and six males) and seven HET (two females and five males) mice were sectioned horizontally and analyzed for nodules by von Kossa staining. One HET mouse had few nodules that were

similar in appearance to those found in 8-week-old KO mice. No nodules were detected in the remaining six HET mice or in the WT mice (data not shown). A μ CT scan was also performed on a 1.5-year-old male HET mouse without finding calcifications (data not shown). Given the 100% penetrance of the brain pathology in KO mice at 8 weeks of age and the progression to extensive calcifications at 15 and 20 weeks of age (Figures 2, 3, 4, and 5), it was concluded that the development of nodules in the *Slc20a2*-HET mice is a rare event.

Segregation of Genotypes and Viability of *Slc20a2*-KO Mice

Despite the absence of PiT2, the KO mice are viable in contrast to PiT1 KO mice.^{47,48} The distribution of genotypes was analyzed in 721 mice from HET breeders (Table 2), and a 1:2:1 segregation was found (χ^2 test: $P > 0.1$). Thus, no prenatal lethality of KO mice was observed. Of 37 KO mice, bred from HET parents and meant to reach at least 17 weeks of age, only one died of unknown cause at 16 weeks of age. Four mice were euthanized prematurely, three because of insufficient growth at 3, 5, and 7 weeks of age, respectively, and one because of seizures involving uncontrolled movements of forelimbs, which were observed in a total of two KO mice at 20 to 21 week of age. In addition, one KO mouse was found dead at 17 weeks of age because of fighting between males. In summary, 2.7% of the KO mice died prematurely of unknown cause, whereas 10.8% were euthanized because of animal welfare considerations. The remaining mice lived to an average age of 23.7 (range, 17 to 31) weeks before planned euthanasia. Higher mortality was observed in KO mice bred from KO parents, where 3 of 11 KO mice, that were bred simultaneously, died of unknown causes at 2 weeks, 3 weeks, and 7 months of age, respectively. This, however, together with poor health of the female KO breeders after their second litter, led us to abandon the use of KO mice as breeders. Thus, we do not have a large data set on the mortality of KO mice from KO breeders. It was recently reported by Wallingford et al⁴⁹ that pregnant *Slc20a2*-KO mice had placental abnormalities, including calcifications and abnormal vascular structure, which are likely to contribute to their poor health

Table 2 Distribution of Genotypes from HET Breeders

Genotype	Number of mice*	Percentage of mice
WT	179	24.8
HET	379	52.6
KO	163	22.6
Total	721	100

Distribution of genotypes between 721 mice.

*WT, HET, and KO are distributed 1:2:1.

HET, heterozygous; KO, knockout; WT, wild type.

when used as breeders and to affect the nutrition of the fetuses.

Growth Differences between Genotypes

Differences in weight and growth were analyzed by weighing the mice from the general breeding scheme once a week, starting at 8 to 21 days of age and until euthanized. The mice were weighed 3 to 14 times (median, 9 times). The weight data from mice of 8 to 100 days of age were modeled with a logistic growth curve (Figure 8). Comparison of models with and without the relevant factors (genotype, sex, and interaction between genotype and sex) showed significant different growth curves as an effect of genotype [$\chi^2(6) = 194.73$, $P < 0.0001$]. The KO mice differed from both WT and HET by having a lower upper bound asymptotic weight (ie, expected weight when fully grown), and the KO mice obtained half their predicted final weight approximately 6 to 10 days later than WT and HET mice, depending on the sex of the mice. There was a significant difference between male and female mice [$\chi^2(3) = 223.63$, $P < 0.0001$], as is expected, and a significant interaction between genotype and sex [$\chi^2(6) = 16.92$, $P = 0.0096$]. The interaction was driven by a minor difference in the steepness of the growth curves between male and female mice. The interaction does not alter the overall pattern in the differences between KO mice and WT and HET mice on the growth curves between male and female mice: For both male and female mice, KO mice reached a lower final weight and at a slower growth rate (Figure 8).

Discussion

We have herein shown that knockout of Pit2 in mice results in localized brain calcification associated with blood vessels in 8- and 15-week-old mice, which is in agreement with our original observation³⁷ showing that Pit2 KO led to brain calcification in two 19-week-old mice (one male and one female). Using histology and μ CT, we found that

the calcification progressed significantly from the age of 8 to 15 weeks.

Histologic analysis revealed small nodules (by PAS-hematoxylin staining) already in 8-week-old mice, which were only weakly stained for phosphate (von Kossa) and calcium (AR). In 9-week-old mice, the nodules were undetectable by μ CT (ie, the calcification of the nodules was $<12 \mu\text{m}$, the resolution of the scanner). However, analysis of 15-week-old mice by von Kossa, PAS-hematoxylin, and AR stains, and of 20-week-old mice by μ CT revealed extensive, localized, and bilateral brain calcification in the KO mice. Thus, compared with the nodules in 15- and 20-week-old mice, the nodules in 8- and 9-week-old mice are not fully calcified. Closer inspection of tissue sections with calcifications and determination of the element composition of calcifications were performed using TEM and SEM/EDX, respectively, on sections from a 19-week-old mouse. SEM/EDX revealed that the calcified nodules largely consisted of P and Ca, in approximately a 1:1 ratio, and of O, as well as small amounts of Fe, Zn, and Al. In addition, Mg and S could be detected in 40% to 50% of the analyzed areas of the calcifications. The presence of N and small amounts of C in the calcifications supports the findings by TEM and histology of presence of organic material in the calcifications. Interestingly, with SEM/EDX, tissue areas with elevated N and P, but no Ca content, were detected. Areas with elevated N and substantially lower Ca/P ratios than in overt calcifications were also found. This observation could indicate that a surplus of P initiates the calcification process.

The multiple calcifications in the cytosol observed by TEM suggest an intracellular cytosolic origin of the calcifications. In the histologic staining, nuclei entangled in/associated with the calcifications were also observed (Figures 3 and 4, A, C, and D). Indeed, in some stained sections, each calcified nodule appeared to be associated with a nucleus (data not shown), in agreement with an intracellular origin of the calcifications. TEM also revealed the presence of degenerated astrocytes at sites of calcification. No overt effect was observed on the endothelium. Pericytes and astrocytes are, however, involved in maintaining the BBB,^{50,51} and recent

Figure 8 Results of analysis of weight difference between genotypes for male and female mice over a period of 100 days. Mice from heterozygous (HET) breeders were weighed once a week from 8 to 21 days of age until euthanasia at different time points. The curves show the predicted weight for each genotype as a function of time from days 0 to 100. Both male and female knockout (KO) mice reached a lower asymptotic weight than wild-type (WT) and HET mice ($P < 0.0001$) and grew at a slower rate. Each point represents the weight of one mouse at the given time point. The data were obtained from 131 female mice (36 WT, 62 HET, and 33 KO) and 165 male mice (36 WT, 91 HET, and 38 KO).

studies in mice have shown that reduction in pericytes alone causes increased transport of endogenous IgG across an intact BBB.⁵⁰ In agreement with an impairment of pericyte function, endogenous IgG was detected in areas around nodules, in agreement with an increased IgG transport over the BBB. The restriction of IgG to the areas of calcifications suggests that IgG transport is secondary to intracellular calcifications/development of nodules.

The mice, mentioned above, with reduction in pericytes and which show increased IgG transport over an intact BBB⁵⁰ also develop brain calcifications and represent a mouse model for the form of PFBC associated with defects in *PDGFB* and *PDGFRB*.²⁶

Presently, there are few published histologic analyses of calcifications in autopsied PFBC patients with confirmed *SLC20A2* causality.^{8,52} There are, however, analyses published on autopsied PFBC patients with a large genomic deletion on chromosome 8, which results in complete deletion of *SLC20A2* and complete or partial deletion of seven other genes.^{9,10,52} Furthermore, analyses on autopsied PFBC patients have been published, where, to our knowledge, the causative genes are not known.^{53,54} A characteristic of calcifications in the autopsied PFBC patients is that they are found in close proximity to blood vessels or within vessel walls,^{8–10,52,53} as we find them in the *Slc20a2*-KO mouse. The involvement of pericytes and astrocytes in the calcification was also reported,⁵³ in a deceased 53-year-old PFBC patient. Specifically, intracellular calcifications were found in the cytoplasm of pericytes and glia cells, and deposits connected with processes of fibrous astrocytes were found. As in the *Slc20a2*-KO mouse, this patient did not present with calcifications or overt changes in endothelial cells. Histologic examination of a deceased 71-year-old PFBC patient with seemingly more severe calcifications, however, showed signs of endothelial death in heavily calcified areas, suggesting that endothelial cells can become damaged as the disease progresses.¹⁰

Similar structures of calcifications, as found in the *Slc20a2*-KO mouse, have been reported in PFBC patients.^{53,54} With regard to the composition of the calcifications in the *Slc20a2*-KO mouse, the principle minerals were Ca and P, which is in agreement with two previously reported extensive studies^{10,54} on two PFBC patients. In addition to Ca and P, both studies found C and O and small amounts of Mg, S, and Na in calcifications in PFBC patients. In the *Slc20a2*-KO mouse, O was found; and in 40% to 50% of measurements, small amounts of Mg and S were also found. In addition, smaller amounts of Fe, Zn, and Al were found, as reported in a PFBC patient.⁵⁴ Smeyers-Verbeke et al⁵⁴ compared the amounts of trace elements with those in bone and teeth, and found relatively large amounts of Mn. This was not reported by Miklossy et al,¹⁰ and Mn was not detected in the mouse. Finally, N and small amounts of C were found in the calcifications, which is in agreement with the inclusion of organic material, as reported in PFBC patients.^{10,54}

In an extensively investigated *SLC20A2*-associated PFBC case, the calcification stained positive with both von Kossa and PAS,⁸ as we find in the *Slc20a2*-KO mouse. In the mouse, the presence of organic material in the calcification was also supported by the SEM/EDX and TEM analyses. Other studies also show the incorporation of organic material in the calcified nodules in PFBC patients,^{8,10,53,54} and it has been suggested that the calcification in PFBC patients may be a mineralization process (ie, a deposition of calcium-phosphate in association with an organic extracellular matrix rather than a primary precipitation of calcium-phosphate products).⁸ The presence of PAS-positive nodules with only weak phosphate staining in young KO mice suggests a similar etiology in *Slc20a2*-KO mice. A similar calcification process is also observed in mice with hypomorphic *Pdgfrb* alleles, which model the *PDGFB*-associated PFBC. These mice present with brain nodules, which initially stain positive with PAS (2-month-old mice), but not for the presence of calcium, whereas the nodules at a later time point (4-month-old mice) have grown in size and number and stain strongly for calcium.²⁶ Nevertheless, TEM of calcified tissue sections from the *Slc20a2*-KO mouse and a PFBC patient⁵³ suggests an intracellular cytosolic initiation of the calcifications. On the basis of our results, the calcification may initiate as precipitation of phosphate on an organic structure in the cell, followed by inclusion of calcium, and further build-in of organic material resulting in the calcified nodules observed with PAS, von Kossa, and AR staining.

As common for dominant hereditary diseases, the penetrance of PFBC within the same family carrying the same *SLC20A2* mutation can be highly variable. This was described by Geschwind et al⁵⁵ for a family that was shown to carry an *SLC20A2* mutation by Hsu et al¹² (family F₁). Typically, the variable penetrance is ascribed to the variable genetic background within the family. Although the genetic background almost certainly has an influence on the expression of possible modifier genes involved in regulating phosphate transport, the *Slc20a2*-KO mice also exhibit a variance in the degree of calcification, as shown by the μ CT scans of 20-week-old mice. The fact that they are on an inbred genetic background would indicate that other, more individual, stochastic effects might have an influence on the disease progression. This could, for example, be the microlocalization of the onset of nodule formation, perhaps in combination with a local influence on nodule growth and calcification.

It is unknown how the lack of functional PiT2 results in brain calcification. However, peripheral vascular calcification—referred to as medial calcification or Mönckeberg sclerosis⁵⁶—is strongly associated with hyperphosphatemia.^{57–59} And mural cells are probably involved in causing this type of calcification as a response to elevated extracellular [P_i].^{60,61} As in human PFBC patients,^{8–10,52–54} the nodules/calcifications in the *Slc20a2*-KO mice were

closely associated with the vasculature. Therefore, exposure of brain mural cells to elevated extracellular $[P_i]$ may be a factor in the development of brain calcification. This explanation would predict that the phosphate homeostasis in the brain is affected by impaired expression of PiT2 and/or lack of functional PiT2. Young *Slc20a2*-KO mice have indeed an increased cerebrospinal fluid (CSF) $[P_i]$,⁶² which was also recently reported for a 54-year-old PBC patient.⁶³

Guerreiro and colleagues⁶⁴ originally suggested that PiT2 is important for the phosphate homeostasis in the CSF. They showed the presence of PiT2 in the apical membrane of the choroid plexus epithelial cells in rat and spiny dogfish. Their *in vitro* studies, moreover, suggested that PiT2 probably was involved in transporting P_i out of the CSF, thus maintaining the characteristic lower P_i concentration in the CSF than in the blood found in, for example, mammals. In line with this, the data show that at 3 weeks of age, *Slc20a2*-KO mice had, on average, a 2.4-fold increased $[P_i]$ in the CSF compared with WT mice.⁶² Later, Wallingford et al⁶⁵ reported on comparably elevated levels of P_i in the CSF of 1-year-old *Slc20a2*-KO mice. On the basis of the low levels of small nodules at 8 weeks of age (Figures 3 and 4) (ie, at least 5 weeks after the onset of CSF hyperphosphate),⁶² we hypothesize that CSF hyperphosphate is present before the appearance of nodules, and that elevated CSF $[P_i]$ is a potential factor in early disease development. It was recently suggested that the CSF follows a para-arterial pathway into the brain parenchyma,⁶⁶ a process that mixes the CSF and the interstitial fluid. This process is likely to increase the interstitial fluid $[P_i]$ in the KO mice and lead to brain cells, including vascular cells, being exposed to an increased concentration of P_i . This could lead to brain vascular smooth muscle cells and/or pericytes reacting in a similar manner as peripheral vascular smooth muscle cells/pericytes when exposed to hyperphosphatemia, leading to their involvement in the calcification process. We found calcifications inside pericytes, which support that they are involved in initiating the formation of calcifications. The involvement of astrocytes, however, separates the calcification observed in PFBC patients and the *Slc20a2*-KO mouse from Mönckeberg sclerosis. Astrocytes are the cell type in the mouse brain neurovascular unit with the highest expression of both *Slc20a2* and its paralog *Slc20a1*, which encodes PiT1.⁶⁷ Thus, astrocytes may be especially sensitive to alterations in phosphate homeostasis, resulting in the observed intracellular calcifications and degeneration of astrocytes.

If our hypothesis is correct and elevated CSF $[P_i]$ is involved in the development of nodules and their calcification, it will likely have implications for several cerebrovascular diseases, including the small-vessel disease and intracranial artery calcification seen in patients with chronic kidney disease.^{68–70} This patient group has been shown in a study from 1932 not only to be hyperphosphatemic, but also to have increased $[P_i]$ in the CSF.⁷¹ With the *Slc20a2*-KO mouse, we have a model in which the effect of altered phosphate homeostasis in the brain can be

studied in isolation, because the mice are not hyperphosphatemic.⁶²

The mice described in the present study are from HET breeders. Knockout of PiT2 leads to mice that are smaller, compared with the HET and WT mice. The size difference can be seen already before weaning. It is, therefore, not likely to be caused by the brain nodules, which are expected to be few and small, if present in the pups. Phosphorus is important for growth at several levels, including generation of ATP. Thus, the decrease in size could be caused by an altered metabolism in the KO mice. Despite the weight difference, no prenatal or marked postnatal lethality of the KO mice was observed.

Wallingford et al⁶⁵ recently reported on the presence of calcifications in the thalamus of *Slc20a2*-HET mice and WT mice. In the *Slc20a2*-HET mice, they could detect a few small calcifications at 6 months of age, and slightly more and larger calcifications in 1- and 1.5-year-old animals. They moreover reported on weak calcification in 1-year-old WT mice. This was not observed in the present study. Thus, although 100% penetrance of development of nodules/calcifications was found in *Slc20a2*-KO mice ($n = 18$) at all ages, with the youngest mice analyzed being 8 weeks old, only few small nodules could be detected in one of seven 1-year-old *Slc20a2*-HET mice and none in 1-year-old WT mice. Wallingford et al⁶⁵ also reported the premature death of 33% of their *Slc20a2*-KO mice between 4 and 21 weeks of age, an observation not found to the same extent in our colony when HET mice were used as breeders. Rather, the *Slc20a2*-KO mice from HET breeders are viable and only few show impaired survival. We hypothesize the presence of environmental differences as the cause of these divergences. Thus, although patients are typically heterozygous for the mutations, the *Slc20a2*-KO mouse is here recommended as a model for PFBC. With the 100% penetrance of nodules/calcifications in young animals, the number of mice necessary for experiments as well as time and costs can be reduced.

In conclusion, an early brain pathology associated with KO of *Slc20a2* is the histologic presence of nodules, which are weakly calcified at 8 weeks of age. The nodules increase with age in number, size, and degree of calcification. Calcifications were found inside cells, including pericytes and astrocytes. Degenerated astrocytes were present in areas with calcification. Furthermore, a local increased BBB transport of IgG was detected, which may be caused by calcified pericytes and calcified and degenerated astrocytes. Increased BBB permeability for IgG is a trait that is common with a mouse model for *PDGFB*- and *PDGFRB*-associated PFBC. A 100% penetrance of brain calcification was observed in the *Slc20a2*-KO mice, but to varying degree as in PFBC patients. Finally, although the *Slc20a2*-KO mice were smaller than WT and HET mice, there was no marked lethality. Thus, the *Slc20a2*-KO mouse presents as a reliable model for studying the development of brain calcification. This model will likely be crucial for discovering the mechanism behind *SLC20A2*-associated brain calcification in humans. The presence of

evident nodules at 8 weeks of age, as well as their successive development and calcification, offers this mouse model as a suitable model for screening of drugs targeting PFBC. In addition, it provides a new opportunity for the study of small-vessel disease in the brain in general.

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References

- Ramos EM, Oliveira J, Sobrido MJ, Coppola G: Primary familial brain calcification. In GeneReviews [Internet]. Copyright University of Washington, Seattle. 2004. Available at <https://www.ncbi.nlm.nih.gov/books/NBK1421>. (last revised August 24, 2017)
- Nicolas G, Pottier C, Charbonnier C, Guyant-Marechal L, Le Ber I, Pariente J, Labauge P, Aygnac X, Defebvre L, Maltete D, Martinaud O, Lefacheur R, Guillin O, Wallon D, Chaumette B, Rondepierre P, Derache N, Fromager G, Schaeffer S, Krystkowiak P, Verny C, Jurici S, Sauvee M, Verin M, Lebouvier T, Rouaud O, Thauvin-Robinet C, Rousseau S, Rovelet-Lecrux A, Frebourg T, Campion D, Hannequin D, French ISG: Phenotypic spectrum of probable and genetically-confirmed idiopathic basal ganglia calcification. *Brain* 2013, 136:3395–3407
- Nicolas G, Charbonnier C, de Lemos RR, Richard AC, Guillin O, Wallon D, Legati A, Geschwind D, Coppola G, Frebourg T, Campion D, de Oliveira JR, Hannequin D; collaborators from the French IBGC Study Group: Brain calcification process and phenotypes according to age and sex: lessons from SLC20A2, PDGFB, and PDGFRB mutation carriers. *Am J Med Genet B Neuropsychiatr Genet* 2015, 168:586–594
- Batla A, Tai XY, Schottlaender L, Erro R, Balint B, Bhatia KP: Deconstructing Fahr's disease/syndrome of brain calcification in the era of new genes. *Parkinsonism Relat Disord* 2017, 37:1–10
- Nicolas G, Jacquin A, Thauvin-Robinet C, Rovelet-Lecrux A, Rouaud O, Pottier C, Aubriot-Lorton MH, Rousseau S, Wallon D, Duvillard C, Bejot Y, Frebourg T, Giroud M, Campion D, Hannequin D: A de novo nonsense PDGFB mutation causing idiopathic basal ganglia calcification with laryngeal dystonia. *Eur J Hum Genet* 2014, 22:1236–1238
- Ferreira JB, Pimentel L, Keasey MP, Lemos RR, Santos LM, Oliveira MF, Santos S, Jensen N, Teixeira K, Pedersen L, Rocha CR, Dias da Silva MR, Oliveira JR: First report of a de novo mutation at SLC20A2 in a patient with brain calcification. *J Mol Neurosci* 2014, 54:748–751
- Kiroglu Y, Calli C, Karabulut N, Oncel C: Intracranial calcifications on CT. *Diagn Interv Radiol* 2010, 16:263–269
- Kimura T, Miura T, Aoki K, Saito S, Hondo H, Konno T, Uchiyama A, Ikeuchi T, Takahashi H, Kakita A: Familial idiopathic basal ganglia calcification: histopathologic features of an autopsied patient with an SLC20A2 mutation. *Neuropathology* 2016, 36:365–371
- Wider C, Dickson DW, Schweitzer KJ, Broderick DF, Wszolek ZK: Familial idiopathic basal ganglia calcification: a challenging clinical-pathological correlation. *J Neurol* 2009, 256:839–842
- Miklossy J, Mackenzie IR, Dorovini-Zis K, Calne DB, Wszolek ZK, Klegeris A, McGeer PL: Severe vascular disturbance in a case of familial brain calcinosis. *Acta Neuropathol* 2005, 109:643–653
- Wang C, Li Y, Shi L, Ren J, Patti M, Wang T, de Oliveira JR, Sobrido MJ, Quintans B, Baquero M, Cui X, Zhang XY, Wang L, Xu H, Wang J, Yao J, Dai X, Liu J, Zhang L, Ma H, Gao Y, Ma X, Feng S, Liu M, Wang QK, Forster IC, Zhang X, Liu JY: Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. *Nat Genet* 2012, 44:254–256
- Hsu SC, Sears RL, Lemos RR, Quintans B, Huang A, Spiteri E, et al: Mutations in SLC20A2 are a major cause of familial idiopathic basal ganglia calcification. *Neurogenetics* 2013, 14:11–22
- Yamada M, Tanaka M, Takagi M, Kobayashi S, Taguchi Y, Takashima S, Tanaka K, Touge T, Hatsuta H, Murayama S, Hayashi Y, Kaneko M, Ishiura H, Mitsui J, Atsuta N, Sobue G, Shimozaawa N, Inuzuka T, Tsuji S, Hozumi I: Evaluation of SLC20A2 mutations that cause idiopathic basal ganglia calcification in Japan. *Neurology* 2014, 82:705–712
- Lemos RR, Ramos EM, Legati A, Nicolas G, Jenkinson EM, Livingston JH, Crow YJ, Campion D, Coppola G, Oliveira JR: Update and mutational analysis of SLC20A2: a major cause of primary familial brain calcification. *Hum Mutat* 2015, 36:489–495
- Taglia I, Bonifati V, Mignarri A, Dotti MT, Federico A: Primary familial brain calcification: update on molecular genetics. *Neurol Sci* 2015, 36:787–794
- David S, Ferreira J, Quenez O, Rovelet-Lecrux A, Richard AC, Verin M, Jurici S, Le Ber I, Boland A, Deleuze JF, Frebourg T, Mendes de Oliveira JR, Hannequin D, Campion D, Nicolas G: Identification of partial SLC20A2 deletions in primary brain calcification using whole-exome sequencing. *Eur J Hum Genet* 2016, 24:1630–1634
- Grütz K, Volpato CB, Domingo A, Alvarez-Fischer D, Gebert U, Schifferle G, Buffone E, Wszolek ZK, Rademakers R, Ferbert A, Hicks AA, Klein C, Pramstaller PP, Westenberger A: Primary familial brain calcification in the "IBGC2" kindred: all linkage roads lead to SLC20A2. *Mov Disord* 2016, 31:1901–1904
- Liu X, Ma G, Zhao Z, Mao F, Tang J, Li X, Zhu M: Novel mutation of SLC20A2 in a Chinese family with primary familial brain calcification. *J Neurol Sci* 2016, 360:1–3
- Pasanen P, Mäkinen J, Myllykangas L, Guerreiro R, Bras J, Valori M, Viitanen M, Baumann M, Tienari PJ, Poyhonen M, Baumann P: Primary familial brain calcification linked to deletion of 5' noncoding region of SLC20A2. *Acta Neurol Scand* 2017, 136:59–63
- Røsbj O, Legati A, Coppola G: Primary familial brain calcification in a Norwegian family, caused by a novel SLC20A2 gene mutation. *J Neurol* 2016, 263:594–596
- Takeuchi T, Muraoka K, Yamada M, Nishio Y, Hozumi I: Living with idiopathic basal ganglia calcification 3: a qualitative study describing the lives and illness of people diagnosed with a rare neurological disease. *SpringerPlus* 2016, 5:1713
- Gagliardi M, Morelli M, Iannello G, Colica C, Annesi G, Quattrone A: A SLC20A2 mutation identified in an asymptomatic patient with brain calcification. *J Neurol Sci* 2017, 372:70–72
- Kumar N, Jog M: Fahr's disease presenting as late-onset levodopa-responsive parkinsonism. *Can J Neurol Sci* 2017, 44:322–323
- Mi TM, Mao W, Cai YN, Yang CX, Wang CD, Xu EH, Zhang H, Chan P: Primary familial brain calcifications linked with a novel SLC20A2 gene mutation in a Chinese family. *J Neurogenet* 2017, 31:1–4
- Rubino E, Giorgio E, Godani M, Grosso E, Zibetti M, Lopiano L, Ferrero P, Duca S, Moretti L, Gallone S, Rainero I, Brusco A: Three

- novel missense mutations in SLC20A2 associated with idiopathic basal ganglia calcification. *J Neurol Sci* 2017, 377:62–64
26. Keller A, Westenberger A, Sobrido MJ, García-Murias M, Domingo A, Sears RL, et al: Mutations in the gene encoding PDGFR- β cause brain calcifications in humans and mice. *Nat Genet* 2013, 45:1077–1087
 27. Legati A, Giovannini D, Nicolas G, López-Sánchez U, Quintáns B, Oliveira JR, et al: Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. *Nat Genet* 2015, 47:579–581
 28. Nicolas G, Pottier C, Maltête D, Coutant S, Rovelet-Lecrux A, Legallic S, Rousseau S, Vaschalde Y, Guyant-Maréchal L, Augustin J, Martinaud O, Defebvre L, Krystkowiak P, Pariente J, Clanet M, Labauge P, Aygnac X, Lefaucheur R, Le Ber I, Frébourg T, Hannequin D, Campion D: Mutation of the PDGFRB gene as a cause of idiopathic basal ganglia calcification. *Neurology* 2012, 80:181–187
 29. Giovannini D, Touhami J, Charnet P, Sitbon M, Battini JL: Inorganic phosphate export by the retrovirus receptor XPR1 in metazoans. *Cell Rep* 2013, 3:1866–1873
 30. Zhang Y, Guo X, Wu A: Association between a novel mutation in SLC20A2 and familial idiopathic basal ganglia calcification. *PLoS One* 2013, 8:e57060
 31. Böttger P, Pedersen L: Two highly conserved glutamate residues critical for type III sodium-dependent phosphate transport revealed by uncoupling transport function from retroviral receptor function. *J Biol Chem* 2002, 277:42741–42747
 32. Böttger P, Pedersen L: Mapping of the minimal inorganic phosphate transporting unit of human PiT2 suggests a structure universal to PiT-related proteins from all kingdoms of life. *BMC Biochem* 2011, 12:21
 33. Larsen FT, Jensen N, Autzen JK, Kongsfelt IB, Pedersen L: Primary brain calcification causal PiT2 transport-knockout variants can exert dominant negative effects on wild-type PiT2 transport function in mammalian cells. *J Mol Neurosci* 2017, 61:215–220
 34. Böttger P, Pedersen L: Evolutionary and experimental analyses of inorganic phosphate transporter PiT family reveals two related signature sequences harboring highly conserved aspartic acids critical for sodium-dependent phosphate transport function of human PiT2. *FEBS J* 2005, 272:3060–3074
 35. Salaün C, Gyan E, Rodrigues P, Heard JM: Pit2 assemblies at the cell surface are modulated by extracellular inorganic phosphate concentration. *J Virol* 2002, 76:4304–4311
 36. Salaün C, Marechal V, Heard JM: Transport-deficient Pit2 phosphate transporters still modify cell surface oligomers structure in response to inorganic phosphate. *J Mol Biol* 2004, 340:39–47
 37. Jensen N, Schröder HD, Hejblø EK, Füchtbauer EM, de Oliveira JR, Pedersen L: Loss of function of SLC20A2 associated with familial idiopathic basal ganglia calcification in humans causes brain calcifications in mice. *J Mol Neurosci* 2013, 51:994–999
 38. Seluanov A, Vaidya A, Gorbunova V: Establishing primary adult fibroblast cultures from rodents. *J Vis Exp* 2010, 44:e2033
 39. Hellemans J, Mortier G, De Paep A, Speleman F, Vandesompele J: qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 2007, 8:R19
 40. Søndergaard CS, Haldrup C, Beer C, Andersen B, Kohn DB, Pedersen L: Preloading potential of retroviral vectors is packaging cell clone dependent and centrifugation onto CH-296 ensures highest transduction efficiency. *Hum Gene Ther* 2009, 20:337–349
 41. Schneider CA, Rasband WS, Eliceiri KW: NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012, 9:671–675
 42. Pinheiro J, Bates D: *Mixed-Effects Models in S and S-PLUS*. New York, Springer, 2000
 43. Burden N, Chapman K, Sewell F, Robinson V: Pioneering better science through the 3Rs: an introduction to the national centre for the replacement, refinement, and reduction of animals in research (NC3Rs). *J Am Assoc Lab Anim Sci* 2015, 54:198–208
 44. van Zeijl M, Johann SV, Closs E, Cunningham J, Eddy R, Shows TB, O'Hara B: A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. *Proc Natl Acad Sci U S A* 1994, 91:1168–1172
 45. Miller DG, Edwards RH, Miller AD: Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. *Proc Natl Acad Sci U S A* 1994, 91:78–82
 46. Lundorf MD, Pedersen FS, O'Hara B, Pedersen L: Single amino acid insertion in loop 4 confers amphotropic murine leukemia virus receptor function upon murine Pit1. *J Virol* 1998, 72:4524–4527
 47. Festing MH, Speer MY, Yang H-Y, Giachelli CM: Generation of mouse conditional and null alleles of the type III sodium-dependent phosphate cotransporter PiT-1. *Genesis* 2009, 47:858–863
 48. Beck L, Leroy C, Beck-Cormier S, Forand A, Salaun C, Paris N, Bernier A, Urena-Torres P, Prie D, Ollero M, Coulombel L, Friedlander G: The phosphate transporter PiT1 (Slc20a1) revealed as a new essential gene for mouse liver development. *PLoS One* 2010, 5:e9148
 49. Wallingford MC, Gammill HS, Giachelli CM: Slc20a2 deficiency results in fetal growth restriction and placental calcification associated with thickened basement membranes and novel CD13 and laminin $\alpha 1$ expressing cells. *Reprod Biol* 2016, 16:13–26
 50. Villasenör R, Ozmen L, Messaddeq N, Gruninger F, Loetscher H, Keller A, Betsholtz C, Freskgard PO, Collin L: Trafficking of endogenous immunoglobulins by endothelial cells at the blood-brain barrier. *Sci Rep* 2016, 6:25658
 51. Alvarez JI, Katayama T, Prat A: Glial influence on the blood brain barrier. *Glia* 2013, 61:1939–1958
 52. Baker M, Strongosky AJ, Sanchez-Contreras MY, Yang S, Ferguson W, Calne DB, Calne S, Stoessl AJ, Allanson JE, Broderick DF, Hutton ML, Dickson DW, Ross OA, Wszolek ZK, Rademakers R: SLC20A2 and THAP1 deletion in familial basal ganglia calcification with dystonia. *Neurogenetics* 2014, 15:23–30
 53. Kobayashi S, Yamadori I, Miki H, Ohmori M: Idiopathic non-arteriosclerotic cerebral calcification (Fahr's disease): an electron-microscopic study. *Acta Neuropathol* 1987, 73:62–66
 54. Smeyers-Verbeke J, Michotte Y, Pelsmaeckers J, Lowenthal A, Massart DL, Dekegel D, Karcher D: The chemical composition of idiopathic nonarteriosclerotic cerebral calcifications. *Neurology* 1975, 25:48–57
 55. Geschwind DH, Loginov M, Stern JM: Identification of a locus on chromosome 14q for idiopathic basal ganglia calcification (Fahr disease). *Am J Hum Gen* 1999, 65:764–772
 56. Mönckeberg JG: Über die reine Mediaverkalkung der Extremitätenarterien und ihr Verhalten zur Arteriosklerose. *Virchows Arch Pathol Anat Physiol Klin Med* 1903, 171:141–167
 57. Block GA, Hulbert-Shearon TE, Levin NW, Port FK: Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998, 31:607–617
 58. Ganesh SK, Stack AG, Levin NW, Hulbert-Shearon T, Port FK: Association of elevated serum PO₄, Ca x PO₄ product, and parathyroid hormone with cardiac mortality risk in chronic hemodialysis patients. *J Am Soc Nephrol* 2001, 12:2131–2138
 59. Covic A, Kothawala P, Bernal M, Robbins S, Chalian A, Goldsmith D: Systematic review of the evidence underlying the association between mineral metabolism disturbances and risk of all-cause mortality, cardiovascular mortality and cardiovascular events in chronic kidney disease. *Nephrol Dial Transplant* 2009, 24:1506–1523
 60. Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM: Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. *Circ Res* 2011, 109:697–711

61. Collett GD, Canfield AE: Angiogenesis and pericytes in the initiation of ectopic calcification. *Circ Res* 2005, 96:930–938
62. Jensen N, Autzen JK, Pedersen L: SLC20a2 is critical for maintaining a physiologic inorganic phosphate level in cerebrospinal fluid. *Neurogenetics* 2016, 17:125–130
63. Paucar M, Almqvist H, Jelic V, Hagman G, Jörneskog G, Holmin S, Björkhem I, Svenningsson P: A SLC20A2 gene mutation carrier displaying ataxia and increased levels of cerebrospinal fluid phosphate. *J Neurol Sci* 2017, 375:245–247
64. Guerreiro PM, Bataille AM, Parker SL, Renfro JL: Active removal of inorganic phosphate from cerebrospinal fluid by the choroid plexus. *Am J Physiol Renal Physiol* 2014, 306:F1275–F1284
65. Wallingford MC, Chia J, Leaf EM, Borgeia S, Chavkin NW, Sawangmake C, Marro K, Cox TC, Speer MY, Giachelli CM: SLC20A2 deficiency in mice leads to elevated phosphate levels in cerebrospinal fluid and lymphatic pathway-associated arteriolar calcification, and recapitulates human idiopathic basal ganglia calcification. *Brain Pathol* 2016, 27:64–76
66. Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, Benveniste H, Vates GE, Deane R, Goldman SA, Nagelhus EA, Nedergaard M: A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Sci Transl Med* 2012, 4:147ra111
67. Vanlandewijck M, He L, Mae MA, Andrae J, Ando K, Del Gaudio F, Nahar K, Lebouvier T, Lavina B, Gouveia L, Sun Y, Raschperger E, Rasanen M, Zarb Y, Mochizuki N, Keller A, Lendahl U, Betsholtz C: A molecular atlas of cell types and zonation in the brain vasculature. *Nature* 2018, 554:475–480
68. Bugnicourt J-M, Chillon J-M, Massy ZA, Canaple S, Lamy C, Deramond H, Godefroy O: High prevalence of intracranial artery calcification in stroke patients with CKD: a retrospective study. *Clin J Am Soc Nephrol* 2009, 4:284–290
69. Bugnicourt JM, Godefroy O, Chillon JM, Choukroun G, Massy ZA: Cognitive disorders and dementia in CKD: the neglected kidney-brain axis. *J Am Soc Nephrol* 2013, 24:353–363
70. Toyoda K: Cerebral small vessel disease and chronic kidney disease. *J Stroke* 2015, 17:31–37
71. Cantarow AA: Calcium studies, VII: the calcium and inorganic phosphorus content of cerebrospinal fluid and blood serum in chronic glomerulonephritis with uremia. *Arch Intern Med* 1932, 49:981–993