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Abstract

Brain calcification of especially the basal ganglia characterises 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; about 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, micro-computed tomography, electron microscopy (EM), and energy-dispersive X-ray spectroscopy. Slc20a2-KO mice showed histologically detectable nodules in the brain already at eight 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 were 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 pre-clinical model for evaluation of PFBC treatments.
Introduction

Primary familial brain calcification (PFBC) (OMIM: #213600, #616413, #615007, and #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. The disease is also known as Fahr’s disease and familial idiopathic basal ganglia calcification (FIBGC). No association of PFBC with systemic disorders, eg, hormonal disturbances, has been identified; PFBC is usually found coincidentally upon neuroimaging addressing the neuropsychiatric and motor symptoms. The symptoms 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. PFBC is usually inherited in an autosomal dominant pattern, but de novo cases have also been reported. Although it is a rare genetic disease, brain calcification is now more frequently detected due to extended use of brain computed tomography (CT).

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. The disease mechanism is unknown, but there are clear genetic culprits. Wang et al found deleterious mutations in SLC20A2, encoding the type III sodium-dependent inorganic phosphate (Pi) transporter PiT2, in affected families from China, Brazil, and Spain. Since then, SLC20A2 mutations have been found in PFBC families worldwide, and Lemos et al have reviewed 50 reported variants of SLC20A2 in 55 unrelated patients. Three other genes linked to the disease are PDGFRB, PDGFB, and XPR1. They encode the platelet-derived growth factor receptor β (PDGF-Rβ), its specific ligand, the platelet-derived growth factor subunit B (PDGF-B), and the multi-membrane spanning protein XPR1, recently identified as a cellular Pi exporter.

Mutations in SLC20A2 are the most common and are estimated to be responsible for at least 40% of the PFBC cases. The mutation type varies; some lead to aberrant mRNAs and others to PiT2 proteins that are shown or predicted to be unable to transport Pi. Wang and colleagues suggested that cells carrying a PiT2 transport-knockout (KO) mutation in one allele will show reduced Pi uptake due to 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. We recently obtained results that provide a potential explanation for the observations by Kimura and co-workers. Missense variants were shown to have a dominant negative effect on PiT2-mediated Pi-uptake.

Heterozygous mutations in mammalian cells were mimicked by co-expressing 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.\textsuperscript{33} Since PiT2 can oligomerize,\textsuperscript{31,34-36} we hypothesise that WT PiT2 protein oligomerized with certain variant PiT2 proteins is recognized by the cells as a misfolded structure and degraded, resulting in very little to absent WT and variant PiT2 proteins in the cells as reported by Kimura and co-workers.\textsuperscript{8}

To investigate the role of PiT2 in PFBC, \textit{Slc20a2}-KO mice were studied. We previously showed bilateral brain calcifications in two 19-week–old \textit{Slc20a2}-KO mice.\textsuperscript{37} Here we present new insight into the development of brain calcifications and additional characterisation of this PFBC model. \textit{Slc20a2}-KO mice had brain nodules already at eight 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 (\textmu CT) scans of 20-week–old KO mice. The \textmu CT scans also revealed that mice at the same age could show high variation in the degree of calcification, which reflects observations in PFBC. Upon closer inspection of the nodules with transmission electron microscopy (TEM), intracellular calcification was found in pericytes and astrocytes and degenerated astrocytes.

Scanning EM/energy-dispersive X-ray spectroscopy (SEM/EDX) confirmed the histological 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 \textit{Slc20a2} were obtained from the European Mouse Mutant Archive, Munich, Germany. The strain is denoted C57BL/6NTac-\textit{Slc20a2}^{tm1a(EUCOMM)Wtsi}/Ieg (EM: 05549) and is HET for the KO-cassette, L1L2-PGK-P, inserted in \textit{Slc20a2} after coding exon 2 and flanking coding exon 3. It introduces splice acceptor and SV40 polyadenylation sequences in \textit{Slc20a2} between the second and third coding exons. The mice were bred and housed in the Animal Facility at the Health Faculty, Aarhus University. All were fed the same standard diet \textit{ad libitum} (autoclaved Altromin 1319F made by Altromin Spezialfütter GmbH, Lage, Germany (supplied
by Brogaarden, Lynge, Denmark), and had free access to water. They were housed under a 12 h light/dark schedule, with lights on at 6.00 a.m. and off at 6.00 p.m. The pups were weaned at three to four 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 cm$^2$, and five to eight mice: 800 cm$^2$, males: two to three mice: 350 cm$^2$, and four to six mice: 800 cm$^2$).

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 no. 2013-15-2934-00894 and reported in accordance with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

Offspring from HET breeding pairs were weighed once a week starting between eight to 21 days of age and continued until euthanasia. Weight data obtained from 131 female mice (36 WT, 62 HET, 33 KO) and 165 male mice (36 WT, 91 HET, 38 KO) were modelled with a logistic growth curve. Mice weighed fewer than three times were excluded from the analysis.

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

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

**Histology**

Histological methods were performed as previously described. Briefly, brains were fixed in 4% formaldehyde and embedded in paraffin. Histological staining was performed on 4-µm-thick sections. The sections were deparaffinized in Histo-clear (National Diagnostics, cat. no. HS-200) and rehydrated. For detection of phosphate, sections were stained with the von Kossa staining technique. They were incubated 20 min in 10% silver nitrate, rinsed in sterile water,
followed by 2 min in 5% pyrogallol. After being rinsed again in sterile water, the sections were placed in 5% sodium thiosulfate for 5 min. For counterstaining, eosin was used.

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

For immunohistochemistry, antigen retrieval was performed on deparaffinized sections by boiling in TEG (10 mM Tris-HCl, 0.5 mM EGTA, pH 9.0) in a microwave oven for 32 min. Sections were incubated with 5% goat serum (X0907, Dako, Agilent) for 5 min, or with 1:10 dilutions of FcR Blocking Reagent mouse (Cat. no. 130-092-575, Miltenyi Biotec). Mouse IgG was detected using OptiView DAB IHC Detection kit on a Ventana Benchmark (Roche). Lastly, the sections were stained with Mayer’s Haematoxylin (Ampliqon) to visualize cell nuclei.

Mice analyzed with von Kossa and PAS: 8-week–old: 10 WT (six females, four males) and seven KO (three females, four males), 15-week–old: three WT (one female, two males) and seven KO (one female, six males), and 1-year–old: eight WT (two females, six males) and seven HET (two females, 5 males). Mice analyzed with AR: 8-week–old: four WT (two females, two males) and four KO (one female, three males); 15-week–old: three WT (two females, one male) and four KO (four males). Mice analyzed with immunohistochemistry for mouse IgG detection: 8-week–old: four WT (one female, three males) and four KO (one female, three males), 15-week–old: three WT (two females, 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. 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 PBS, 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 (Roche, cat. no. 05401119001), DMEM (Gibco, cat.no. 41966-029), and 1% Antibiotic/Antimycotic (AA, Gibco, cat.no. 15240-062) and incubated at 37 °C, while stirred slowly, for at least 90 min. The solutions containing the tissue were transferred to DMEM supplemented with 15% FBS (Gibco, cat.no. 15240-062) and incubated at 37 °C, while stirred slowly, for at least 90 min. The solutions containing the tissue were transferred to DMEM supplemented with 15% FBS (Gibco, cat.no. 15240-062) and incubated at 37 °C, while stirred slowly, for at least 90 min.
10270-160) and 1% AA (DMEM-15% FBS-AA). The samples were centrifuged, supernatants removed, and the pellets resuspended in DMEM-15% FBS-AA; 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 about eight to 14 days, at which point AA was replaced with 1% Penicillin/Streptomycin (PS, Gibco, cat.no. 15140-122).

Quantitative reverse transcription-PCR (RT-qPCR)
Tail tissue was stored in RNA later (Invitrogen). It was homogenized using pestles and lysed in lysis/binding buffer containing guanidinium thiocyanate provided with the RNAqueous-4PCR Kit (Applied Biosystems). RNA was purified from the lysates using the RNAqueous-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 RT-qPCR analysis, the following TaqMan Gene Expression Assays (Applied Biosystems) were employed: Mm00660204-mH (Slc20a2), and, as endogenous control, Mm00437762-m1 (β-2-microglobin (B2M)). Each RT-qPCR reaction contained 10 µL TaqMan Fast Universal PCR Master Mix, 1 µL TaqMan Gene Expression Assay (Slc20a2, FAM), 1 µL TaqMan Gene Expression Assay (B2M, VIC), and 8 µL cDNA (approximately 10 ng). The PCR cycles employed were as follows: 95 °C for 10 min and 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The relative mRNA levels were calculated as previously described.39 RT-qPCR reactions were set up as triplicates. Tail tissue from 8-week–old mice (five WT (three females, two males) and four KO (four males)) and FFPE brain tissue from 8-week–old mice (10 WT (six females, four males) and seven KO (one female, six males)) were analyzed for Slc20a2 expression.

Transduction of primary murine fibroblasts with A-MLV and 10A1 pseudo-typed vectors
Cultures of primary murine fibroblasts isolated from 4-week–old WT and KO mice were exposed to amphotropic murine leukemia virus (A-MLV) pseudo-typed vectors (derived from the packaging cell line PA317) or 10A1 pseudo-typed vectors (derived from the packaging cell line PT67) carrying eYFP or eGFP expressing MND-X-SN transfer vectors, respectively.40 Cells were seeded in 24-well plates, and the next day they were added pure filtrated (0.45 µm) vector-containing supernatants or media (mock) supplemented with 8 µg/mL polybrene (PB). The cells were incubated
for 70 to 80 min, after which DMEM-15% FBS-PS was added to dilute the PB to 2 µg/mL. The cells were then incubated for another four to five days. Phenolred-free DMEM was added to the wells, before examining the cells for eGFP or eYFP expression using a Zeiss Axiovert 200M Fluorescence microscope equipped with a Photometrics CoolSNAP HQ camera. Images were analyzed using ImageJ (http://rsb.info.nih.gov/ij). The transduction experiment was performed twice. Each time independent replicates of cells from two WT (one male, one female, or two females) and two KO (male) mice were subjected to vector-containing supernatants or mock-transduced.

µCT

Brains from the following animals were analyzed by µCT: 9-week–old: three WT (one female, two males) and three KO (one female, two males), 20-week–old: three WT (one female, two males) and three KO (one female, two males), 1.5-year–old: one HET male. The brains were surface-stained by immersion in Ultravist (370 mg Iopromide/mL, Bayer Pharma AG, Berlin, Germany) for 5 min. Then the brains were gently wiped off and scanned in a desktop µCT scanner (Scanco µCT 35, Scanco Medical AG, Brüttiselen, Switzerland). The scans were performed in high-resolution mode (1000 projections/180°) with a spatial resolution of 6 × 6 × 6 µm³, an X-ray tube voltage of 45 kVp, an X-ray current of 177 µA, and an integration time of 800 ms. After scanning, 3D image sets were reconstructed using the software provided with the scanner. 3D visualization was performed using Amira 5.6 (FEI Visualization Science Group, Mérignac, France).

TEM and SEM/EDX

For TEM, tissue from brains fixed in 4% formaldehyde were post fixed in 2% glutaraldehyde in 0.04 M phosphate buffer. The tissue was cut in smaller pieces and further post fixed in osmium tetroxide for 1 h. 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), were cut and some of the grids were stained with uranyl acetate and lead citrate. The sections were analyzed on a JEOL2100 electron microscope. Mice analyzed with TEM: 19-week–old: 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) 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.
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: 19-week-old: one KO (male).

Statistical analysis

For RT-qPCR data, differences between groups were analyzed with a two-tailed Student’s *t*-test. The distribution of genotypes was tested for a 1:2:1 segregation with a Chi-square test.

Statistical analysis of weight data was performed by fitting a logistic growth curve by nonlinear mixed effects regression using maximum log-likelihood. The analysis was done in R (http://www.r-project.org) using the package *nlme* (v. 3.1.122). 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 *α* = 0.05.

Results

Throughout this study, mice were selected on the basis of age and included both females and males, as 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.

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 (Fig. 1). Specifically, mRNA was purified from tail tissue (WT *n*=5 (three females and two males), KO *n*=4 (male)) 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 9-10. Severe reduction of *Slc20a2* mRNA was found in both brain and tail tissue from KO mice (Fig. 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.\textsuperscript{45-47}

Thus, transduction via PiT1 was used as a positive control. Fibroblasts from WT (one male, one female, or two males) and KO (two males) mice were subjected to retroviral transfer vectors encoding eYFP or eGFP and pseudo-typed with surface proteins from A-MLV or 10A1, respectively. The WT cells were transduced by both A-MLV and 10A1 pseudo-typed vectors (Fig. 1B). Fibroblasts from KO mice, however, were only transduced by the 10A1 pseudo-typed vector (Fig. 1B). These results show that PiT1 was available for transduction in the *Slc20a2*-KO cells, but PiT2 was not (Fig. 1B). In conclusion, the severe reduction at the mRNA level was reflected in a lack of PiT2 protein in the cell membrane.

µCT scans reveal variation in calcification pattern in *Slc20a2*-KO mice

µCT 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 µm) the smallest detectable objects are 12 µm in size. Calcifications were no detected in 9-week–old mice (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 (Figs. 2A-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 the least calcified. The most and least calcified brains were from male mice, while the calcification degree in the female mouse was in between the two. The density of individually detected calcifications varied between 914 to 1,027 mg/cm\textsuperscript{3} (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 (Fig. 2D). The lack and presence of calcifications in the µCT scans of brains from 9-week–old 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 pathological alterations can be identified and to confirm the localization of the calcifications identified by µCT, histological analysis of sections was performed from another set of mice. Moreover, it was addressed whether the calcifications developed over time. For the histological analysis, mice
were sacrificed at the ages of eight weeks (WT n=10 (six females and four males), KO n=7 (three females and four males)) and 15 weeks (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-haematoxylin staining. Nodules were found in all KO mice and none in WT-mice. The location of the nodules corresponded with the µCT scans. The 8-week–old KO mice had small nodules in the midbrain (Fig. 3) and hypothalamus and few in thalamus and pons (not shown). At this age, the nodules stained only weakly with von Kossa staining, which detects phosphate, and were better visualized with the PAS-haematoxylin staining (Fig. 3 and Figs. 4A 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 to 8-week–old mice (Fig.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 Alizarin Red S (Fig. 5) (8-week–old: WT n=4 (two females, two males), KO n=4 (one female, three males; 15-week–old: WT n=3 (two females, 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 (Figs. 5A and C), and more and strongly stained larger nodules in 15-week–old KO mice (Figs. 5B and D); no nodules were present in WT mice at either age (not shown).

The nodules were almost exclusively found in, around, or close to blood vessels, visualized with PAS-haematoxylin staining, in both 8- and 15-week–old mice (Figs. 3 and 4A). We hypothesised 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: WT n=4 (one female, three males), KO n=4 (one female, three males); 15-week–old: WT n=3 (two females, 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 (Fig. 4C); in 15-week–old KO mice IgG positive nodules were easily detectable (Fig. 4 D).

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 (Fig. 6A) including in astrocytes (Fig. 6E) and pericytes (Fig. 6F), as well as degenerated astrocytes (Fig. 6H). Calcifications could also be detected in what appeared to be a neuronal dendrite (Fig. 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.

The calcifications showed different morphologies (Figs. 6B-C). Calcifications with lamellar structure were present (Fig. 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 (Fig. 6C). Figure 6D shows a large deposit in a section not stained with uranyl acetate and lead citrate. The paleness of the deposit compared to 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 Fig. 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 (Fig. 7) independent of size. Furthermore, small amounts of Zn and Al were detected (albeit not always in the corona of larger calcifications, 7A and Table 1). Small amounts of Mg and S were detected in about 40% to 50% of the analyzed areas of the calcifications, though 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, due to imprecision in their determination. The element mapping, however, show that the calcifications were clearly enriched with O (Fig. 7). Compared to the surrounding tissue, N was slightly enriched in the calcifications, or at the same level (Fig. 7). For all areas analyzed, smaller amounts of C and Cl were detected in the calcifications compared to the surrounding tissue (Fig. 7B). Interestingly, some areas close to calcifications and originally identified as areas of different structure in the HAADF and with denser N mapping (Fig. 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 (not shown), in these 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 to Ca.

In conclusion, the size and calcification of nodules increased markedly from eight 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 to the surrounding tissue. Intracellular calcifications were found in pericytes and astrocytes as well as degenerated astrocytes at sites of calcification. IgGs could be detected already at eight 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 one year of age. FFPE brains from eight WT (two females and six males) and seven HET mice (two females and five males) 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 (not shown). A µCT scan was also performed on a 1.5-year–old male HET mouse without finding calcifications (not shown). Given the 100% penetrance of the brain pathology in KO mice at eight weeks of age and the progression to extensive calcifications at 15 and 20 weeks of age (Figs. 2 to 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.48,49 The distribution of genotypes was analyzed in 721 mice from HET breeders (Table 2) and a 1:2:1 segregation was found (Chi-square test: \( P > 0.1 \)). Thus, any prenatal lethality of KO mice was not observed. Out 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 due to insufficient growth at 3, 5, and 7 weeks of age, respectively, and one due to seizures involving uncontrolled movements of forelimbs, which we observed in a total of two KO mice at 20 to 21 weeks of age. In addition, one KO mouse was found dead at 17 weeks of age due to fighting between males. In summary, 2.7% of the KO mice died prematurely of unknown cause, whereas 10.8% were euthanized due to animal welfare considerations. The remaining mice lived to an average age of 23.7 (17 to 31) weeks before planned euthanasia. It should be noted, that we observed a higher mortality in KO mice bred from KO parents, where three out 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 dataset on the mortality of KO mice from KO breeders. It was recently reported by Wallingford and co-workers that pregnant Slc20a2-KO mice had placental abnormalities including calcifications and abnormal vascular structure,50 which is likely to contribute to their poor health when used as breeders and to affect the nutrition of the foetuses.
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 three to 14 times (median = 9). The weight data were modelled from mice of eight to 100 days of age with a logistic growth curve (Fig. 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 about six 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 (Fig. 8).

Discussion

We have here 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 observation showing that Pit2 KO led to brain calcification in two 19-week–old mice (one male and one female). Using histology and µCT, we found that the calcification progressed significantly from the age of eight to 15 weeks.

Histological analysis revealed small nodules (by PAS-haematoxylin 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 µCT, ie, the calcification of the nodules was smaller than 12 μm (the resolution of the scanner). However, analysis of 15-week–old mice by von Kossa, PAS-haematoxylin, and AR stains, and of 20-week–old mice by µCT, revealed extensive, localized, and bilateral brain calcification in the KO mice. Thus, compared to 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 roughly 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% or 50% of the analyzed areas of the calcifications. The presence of N and small amounts of C in the calcifications support 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 a substantial lower Ca:P ratios than in overt calcifications was 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 histological staining, nuclei entangled in/associated with the calcifications were also observed (Figs. 3, 4A, 4C, and 4D). Indeed, in some staining each calcified nodule appeared to be associated with a nucleus (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, \textsuperscript{51, 52} and recent studies in mice, have shown that reduction in pericytes alone cause increased transport of endogenous IgG across an intact BBB. \textsuperscript{53} In agreement with an impairment of pericyte function, endogenous IgG were 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.

It is noteworthy, that the mice, mentioned above, with reduction in pericytes, and which show increased IgG transport over an intact BBB, \textsuperscript{51} also develop brain calcifications and is a mouse model for the form of PFBC associated with defects in \textit{PDGFB} and \textit{PDGFRB}. \textsuperscript{56}

Presently, there are few published histological analyses of calcifications in autopsied PFBC patients with confirmed \textit{SLC20A2} causality. \textsuperscript{8, 53} There are, however, analyses published on autopsied PFBC patients with a large genomic deletion on chromosome 8, which results in complete deletion of \textit{SLC20A2} and complete or partial deletion of seven other genes. \textsuperscript{9, 10, 53} Furthermore, analyses on autopsied PFBC patients have been published, where, to our knowledge, the causative genes are not known. \textsuperscript{54, 55} A characteristic of calcifications in the autopsied PFBC patients is that they are found in close proximity to blood vessels or within vessel walls, \textsuperscript{8, 10, 53, 54} as we find them in the \textit{Slc20a2-KO} mouse. The involvement of pericytes and astrocytes in the calcification was also reported \textsuperscript{54}, 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. As in the \textit{Slc20a2-KO} mouse, this patient did not present with calcifications or overt changes in endothelial cells. Histological 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.\textsuperscript{10}

Similar structures of calcifications, as found in the \textit{Slc}20\textit{a}2-KO mouse, have been reported in PFBC patients.\textsuperscript{54, 55}

With regard to the composition of the calcifications in the \textit{Slc}20\textit{a}2-KO mouse, the principle minerals were Ca and P, which is in agreement with two previously reported extensive studies\textsuperscript{55, 10} 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 \textit{Slc}20\textit{a}2-KO mouse, O was found and in 40\% to 50\% of measurements also small amounts of Mg and S. In addition, smaller amounts of Fe, Zn, and Al were found as reported.\textsuperscript{55} Smeyers-Verbeke and co-workers compared the amounts of trace elements to those in bone and teeth, and found relatively large amounts of Mn. This was not reported by Miklossy et al, 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\textsuperscript{55, 10}.

In an extensively investigated \textit{SLC20A2}-associated PFBC case, the calcification stained positive with both von Kossa and PAS,\textsuperscript{8} as we find in the \textit{Slc}20\textit{a}2-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,\textsuperscript{8, 10, 54, 55} 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.\textsuperscript{8} The presence of PAS-positive nodules with only weak phosphate staining in young KO-mice suggests a similar aetiology in \textit{Slc}20\textit{a}2-KO mice. A similar calcification process is also observed in mice with hypomorphic \textit{Pdgfb}-alleles, which model the \textit{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, while the nodules at a later time point (4-month–old mice) have grown in size and number and stain strongly for calcium.\textsuperscript{26} Nevertheless, TEM of calcified tissue sections from the \textit{Slc}20\textit{a}2-KO mouse and a PFBC patient\textsuperscript{54} suggest an intracellular cytosolic initiation of the calcifications. Based on 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 \textit{SLC20A2} mutation can be highly variable. This was described by Geschwind et al\textsuperscript{26} for a family that was shown to carry a \textit{SLC20A2} mutation by Hsu et al (family F1).\textsuperscript{12} 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, it is interesting to note that the
Slc20a2-KO mice also exhibit a variance in the degree of calcification as shown by the µCT 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, eg, be the micro-localization 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’s sclerosis
— is strongly associated with hyperphosphataemia.

And mural cells are probably involved in causing this type of calcification as a response to elevated extracellular [P_i]. As in human PFBC patients, 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 an increased CSF [P_i], which was also recently reported for a 54-year-old PBC patient.

Guerreiro and colleagues originally suggested that PiT2 is important for the phosphate homeostasis in the cerebrospinal fluid (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, eg, mammals. In line with this, the data show that at three weeks of age, Slc20a2-KO mice had on average a 2.4-fold increased [P_i] in the CSF compared to WT mice. Later, Wallingford and co-workers reported on comparably elevated levels of P_i in the CSF of 1-year-old Slc20a2-KO mice. Based on the low levels of small nodules at eight weeks of age (Figs. 3 and 4), ie, at least five weeks after the onset of CSF hyperphosphate, we hypothesise 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, a process that mixes the CSF and the interstitial fluid (ISF). This process is likely to increase the ISF [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 (VSMCs) and/or pericytes reacting in a similar manner as peripheral VSMCs/pericytes when exposed to hyperphosphataemia, 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’s
sclerosis. Astrocytes, are the cell type in the mouse brain neurovascular unit with the highest expression of both \textit{Slc20a2} and its paralog \textit{Slc20a1}, which encodes PiT1. 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. This patient group has been shown in a study from 1932 not only to be hyperphosphataemic, but also to have increased [P$_i$] in the CSF. With the \textit{Slc20a2}-KO mouse, we have a model where the effect of altered phosphate homeostasis in the brain can be studied in isolation, as the mice are not hyperphosphataemic.

The mice described in the present study are from HET breeders. Knockout of PiT2 leads to mice that are smaller, compared to 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 and co-workers recently reported on the presence of calcifications in the thalamus of \textit{Slc20a2}-HET mice and WT mice. In the \textit{Slc20a2}-HET mice, they could detect a few small calcifications at six months of age, and slightly more and larger calcifications in 1-year- and 1.5-year-old animals. They moreover reported on weak calcification in 1-year-old WT mice. Although 100% penetrance of development of nodules/calcifications was found in \textit{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 out of eight 1-year-old \textit{Slc20a2}-HET mice and none in 1-year-old WT mice. Wallingford and co-workers also reported the premature death of 33% of their \textit{Slc20a2}-KO mice between four and 21 weeks of age, an observation not found to the same extent when HET mice were used as breeders. In our colony, the \textit{Slc20a2}-KO mice from HET breeders are viable and only few show impaired survival. We hypothesise the presence of environmental differences as the cause of these divergences. Thus, although patients are typically heterozygous for the mutations, the \textit{Slc20a2}-KO mouse is 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 histological presence of nodules, which are weakly calcified at eight 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 eight weeks of age, as well as their successive development and calcification, offer 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


Figure legends

**Figure 1** Analysis of Slc20a2/PiT2 expression in WT mice and mice homozygous for the KO-cassette (KO). A: mRNA was purified from tail and FFPE brain tissues from 8-week–old mice, and analyzed by RT-qPCR. Brain tissue: WT, n=10 (six females and four males); KO, n=7 (one female and six males). Tail tissue: WT, n=5 (three females and two males); KO, n=4 (four males). The bars show the average Slc20a2 mRNA expression levels relative to B2M. KO compared to WT: Brain and tail relative Slc20a2 mRNA expression levels ($P < 0.00001$ and $P < 0.01$, respectively). B: Analyses of transducability 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/cm$^2$ and exposed to A-MLV pseudo-typed transfer vectors encoding eYFP or 10A1 pseudo-typed transfer vectors encoding eGFP. Representative photos of WT and KO cells exposed to medium containing A-MLV or 10A1 pseudo-typed vectors are shown at 10x magnification.

**Figure 2** Micro-CT scans of 20-week–old mice. A: Male 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 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 min prior to the scanning. Continuously white-stained areas, as apparent in D, are artefacts caused by the contrast fluid.

**Figure 3** von Kossa and PAS-haematoxylin staining of 8- and 15-week–old WT and KO 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-haematoxylin staining shows nodules/calcifications lying in and around blood vessels. Representative images of the midbrain are shown (8-week–old: male WT and female KO; 15-week–old: female WT and male KO). Arrows point to nodules. Scale bars for the left-most two columns (WT and KO) = 200 µm. Scale bars for right-most column (KO Higher magnification) = 25 µm.

**Figure 4 A and B:** PAS-haematoxylin staining and von Kossa staining on consecutive brain sections from an 8-week–old male 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) mouse. IgG stain is visible around nodules in C and D (arrows). Scale bars = 25 µm.

**Figure 5** AR staining of 8- and 15-week–old KO mice. Representative images are shown (8-week–old: one female (A) and one male (C); 15-week–old: 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 to 8-week–old KO mice (A and C) is seen. Scale bars = 10 µm.

**Figure 6** Transmission electron microscopy analyses of brain sections from 19-week–old KO mouse. A: Multiple small intracellular deposits (arrows). B-C: Larger deposits with different morphologies. B: Note the lamellar structure of one of the deposits (arrow) indicating a sequential formation. C: Larger deposit with dense core and a lighter corona. Small needle shaped crystals can be seen in the corona (insert). 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 stains 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 also a deposit is 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 A, D: 500 nm, B: 2 µm, C, F, G, H: 1 µm, E: 200 nm, C insert: 500 nm.

**Figure 7** 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. Al: aluminium, Ca: calcium, C: carbon, Cl: chlorine, Fe: iron, Mg: magnesium, N: nitrogen, O: oxygen, P: phosphorus, S: sulphur, Zn: zinc. Areas boxed in with green 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 O content in the tissue in A compared to B, this may reflect the difference in use of formvar coating. Scale bars A: 5 µm, B: 2 µm.
**Figure 8** Results of analysis of weight difference between genotypes for male and female mice over a period of 100 days. Mice from HET breeders were weighed once a week from eight 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 KO mice reached a lower asymptotic weight than 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, 33 KO) and 165 male mice (36 WT, 91 HET, 38 KO).
Table 1. Element analysis of indicated areas in Figure 7A

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*The relative percentage of the shown elements in the indicated area.
† ND: Not detected.
‡ TA: Trace amount.
Table 2. Distribution of genotypes from HET breeders.*

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*Distribution of genotypes between 721 mice.

†WT, HET, and KO are distributed 1:2:1.
A  

**qRT-PCR analysis**

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B

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