The mouse acrodermatitis enteropathica gene Slc39a4 (Zip4) is essential for early development and heterozygosity causes hypersensitivity to zinc deficiency

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The human Zip4 gene (Slc39a4) is mutated in the rare recessive genetic disorder of zinc metabolism acrodermatitis enteropathica, but the physiological functions of Zip4 are not well understood. Herein we demonstrate that homozygous Zip4-knockout mouse embryos die during early morphogenesis and heterozygous offspring are significantly underrepresented. At mid-gestation, an array of developmental defects including exencephalia, anophthalmia and severe growth retardation were noted in heterozygous embryos, and at weaning, many (63/280) heterozygous offspring were hydrocephalic, growth retarded and missing one or both eyes. Maternal dietary zinc deficiency during pregnancy exacerbated these effects, whereas zinc excess ameliorated these effects and protected embryonic development of heterozygotes but failed to rescue homozygous embryos. Heterozygous Zip4 embryos were not underrepresented in litters from wild-type mothers, but were ~10 times more likely to develop abnormally than were their wild-type littermates during zinc deficiency. Thus, both embryonic and maternal Zip4 gene expressions are critical for proper zinc homeostasis. These studies suggest that heterozygous mutations in the acrodermatitis gene Zip4 may be associated with a wider range of developmental defects than was previously appreciated, particularly when dietary zinc is limiting.

INTRODUCTION

A deficiency of the essential metal zinc can cause growth retardation, immune system dysfunction, male hypogonadism, skin lesions and neurological disorders in humans (1–3). Maternal zinc deficiency impairs embryonic, fetal and postnatal development (4–6). In humans, the rare autosomal recessive disorder acrodermatitis enteropathica (AE) is thought to be caused by the inability to absorb sufficient intestinal zinc (7). Symptoms of severe nutritional zinc deficiency often develop soon after birth in bottle-fed infants or after weaning from breast milk (8). However, zinc supplementation reverses many of the symptoms of this otherwise lethal disease.

For over 30 years, little was known about the genetic cause of AE. The gene responsible for AE was mapped to human chromosomal region 8q24.3, and in 2002, it was shown to be a member of the solute carrier 39A superfamily (Slc39a) (9,10), historically named the ZIP family (11). The AE gene is Zip4 (Slc39a4) and much of what we understand about Zip4 regulation and function comes from recent studies of mice and transfected cells in culture (12–16). The mouse and human ZIP4 proteins are well conserved, and of the multiple amino acid changes due to point mutations found in

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AE patients (9,10,17–19), almost all occur at residues that are also conserved in the mouse protein (12). Other Zip4 mutations in AE patients involve premature termination codons, insertions, deletions or rearrangements of the gene that are likely to cause a complete loss of function, although this has not been directly demonstrated. Mouse ZIP4 functions as a zinc transporter in transfected cells, and although several of the amino acid substitutions found in AE patients appear to abolish its activity, others may only diminish its zinc transporter activity (12,13).

Zip4 gene expression is most active in mouse tissues involved in nutrient uptake, such as the intestine and embryonic visceral yolk sac, and the abundance of Zip4 mRNA and the cellular localization and turn-over of this protein are regulated by zinc availability in these tissues (12,14–16). During periods of zinc deficiency, Zip4 mRNA is induced and the encoded protein accumulates at the apical surfaces of enterocytes and visceral endoderm cells (12,15). In contrast, zinc repletion can cause a slow loss of this mRNA and the rapid internalization and degradation of Zip4 in enterocytes (Weaver et al., manuscript submitted) and in transfected cells (14,16). This dynamic post-transcriptional control of Zip4 in response to zinc suggests that it plays an important role in zinc homeostasis, consistent with the finding that this gene is mutated in humans with AE.

To directly test this hypothesis, we created mice in which the Zip4 gene was knocked out. This gene was shown to be essential for development of the early post-implantation mouse embryo, likely due to its function in the visceral endoderm. Remarkably, Zip4 heterozygosity was found to exert pleiotropic effects on embryogenesis when dietary zinc was adequate and to cause hypersensitivity to the teratogenic and embryotoxic effects of maternal dietary zinc deficiency during pregnancy. Both maternal and embryonic Zip4 genes were found to play critical roles in zinc homeostasis during pregnancy. These studies further suggest the possibility that loss of function of a single human Zip4 allele may be associated with dramatically increased sensitivity to dietary zinc deficiency and the risk of abnormal embryonic development.

RESULTS

Generation of Slc39a4 (Zip4)-knockout mice

Mice with a targeted disruption of the Zip4 gene were generated by homologous recombination in embryonic stem (ES) cells. Two ES cell clones (C4 and D4) were selected to create chimeras and derive separate knockout lines that have yielded similar phenotypes. The targeting construct fused the initiator methionine codon of Zip4 with the open-reading frame of the enhanced green fluorescent protein (EGFP) reporter followed by several stop codons. This disrupted the protein-coding sequence of Zip4 and deleted the remaining codons in exon 1. The remainder of the gene was not altered (Fig. 1A). This allowed for EGFP expression that was driven by the Zip4 promoter in targeted cells. A loxP-flanked Neo cassette located downstream of the EGFP cDNA was used for positive selection, and flanking thymidine kinase and diphtheria toxin cassettes were used for negative selection.

Homologous recombinants were identified by Southern blot analysis of XhoI-digested genomic DNA (Fig. 1B), and the MC1-Neo cassette was then deleted by breeding heterozygous Zip4-knockout mice with commercially available transgenic mice that ubiquitously express Cre recombinase early in development. Successful Cre-mediated excision was confirmed by PCR, and a PCR screen was designed to allow rapid Zip4 genotyping of mice (Fig. 1C). Multiple heterozygous Zip4-knockout mice, in which excision of the MC1-Neo cassette was complete, were crossed to generate working colonies of wild-type and heterozygous Zip4-knockouts for experimentation.

Homozygous Slc39a4 (Zip4)-knockout mice die in utero during early development when expression of Zip4 is restricted to the visceral endoderm

Crossing Zip4+/− mice failed to yield homozygous offspring in mice raised on the zinc-adequate diet, and at weaning, the heterozygous pups were also significantly underrepresented in the population (Fig. 2). At mid-gestation (day 9), visceral yolk sacs and placentae from homozygous implantation sites could be identified (Fig. 3) and were represented in the expected (25%) frequency (Fig. 2). However, none of these embryos progressed past the egg cylinder stage, and by day 10, only a residual mass of embryonic cells remained (Fig. 3).

In the homozygous and heterozygous implantation sites, only the visceral yolk sac actively expressed EGFP under control of the Zip4 promoter (Fig. 3). EGFP expression was not detected in the placenta or in the embryo proper. This result is consistent with and extends earlier studies of expression of the endogenous Zip4 gene (12,15). Expression of EGFP was restricted to the visceral yolk sac, and examination of day 8 implantation sites using an antibody against EGFP to increase sensitivity and specificity revealed expression only in the visceral endoderm at the time when morphogenesis of the homozygous embryo fails (Fig. 4). However, EGFP expression was detectable in the inner cell mass cells as well as in the trophectoderm of day 4 blastocysts before differentiation of the visceral endoderm occurs (Fig. 4C).

Slc39a4 (Zip4) haploinsufficiency causes hypersensitivity to zinc deficiency

As mentioned earlier, the Zip4+/− offspring were underrepresented at weaning, and loss of these offspring apparently occurs after mid-gestation in mice fed the zinc-adequate diet (Fig. 2). Examination of one litter of embryos at day 10 revealed a wide range of abnormalities (Fig. 5). In most litters, there were more apparently normal embryos and fetuses than there were abnormal ones. However, compared with their wild-type littermates, the Zip4+/− embryos often varied greatly in size and morphology. Many were smaller and some were severely growth retarded and exhibited abnormal cranio-facial development. Remarkably, embryos with exencephalia were occasionally found at mid-gestation (Fig. 5). These morphological abnormalities are reminiscent of the teratology of maternal zinc deficiency (20,21) and were never seen in wild-type embryos in these litters.
After parturition, a few heterozygous offspring died before weaning and 22% (63/280) of those that survived past weaning were also morphologically abnormal. Thus, 40% of the heterozygous post-implantation embryos were ultimately abnormal. An extreme example of an abnormal but weaned heterozygous Zip4-knockout mouse is shown in Fig. 6. These mice were often much smaller and had a domed head. High-field magnetic resonance imaging (MRI) of several weaned Zip4-+/2 mice revealed striking results (Fig. 7). These three heterozygous mice had no eyes or only one eye and showed various degrees of hydrocephalus. This was verified by examination of paraffin sections from the brain from an affected Zip4-+/2 mouse after perfusion fixation (Fig. 7). In addition, abnormal thickening of the ventricular septum and wall of the left ventricle (Fig. 7) and skeletal abnormalities were present in some of these affected Zip4-+/2 mice. These abnormalities were never seen among the hundreds of wild-type offspring we examined.

These results were all obtained with mice fed with a zinc-adequate diet. Also, including excess zinc (250 p.p.m. ZnCl2) in the drinking water and feeding the zinc-adequate diet from day 1 of pregnancy until weaning protected development of heterozygous Zip4 embryos, but failed to rescue the homozygous Zip4-knockout embryos (Fig. 2). At weaning, wild-type and heterozygous pups were present at the expected Mendelian frequency, and only one heterozygous pup (3%) displayed the abnormal eye phenotype.

To further test the hypothesis that the pleiotropic teratogenic and embryotoxic effects of Zip4 heterozygosity reflect impaired zinc homeostasis during embryogenesis, Zip4-+/2 mice were mated and pregnant females were fed a zinc-deficient diet beginning on day 8 of pregnancy. Embryos were examined on day 14 and genotyped by PCR (Fig. 8). Zip4-+/2 embryos were significantly underrepresented in the population (21 wild-type and 23 heterozygous), and 50% were lost by day 14. About two-thirds of the remaining Zip4-+/2 embryos showed severe abnormalities, with the majority (61%) being classified as severely abnormal. In contrast, 71% of the wild-type embryos in these litters appeared morphologically normal and only a small percentage (4.7%) was severely abnormal (Fig. 8). Thus, maternal zinc status modulates the detrimental effects of embryonic Zip4 heterozygosity on development, and heterozygous embryos are hypersensitive to zinc deficiency.

The contributions of maternal Zip4 gene expression to the teratogenic and embryotoxic effects of Zip4 heterozygosity...
were examined. Wild-type females were mated with Zip4<sup>+/−</sup> males and the relationship between genotype and phenotype of the day 14 embryos in response to maternal dietary zinc deficiency was determined. Remarkably, Zip4<sup>+/−</sup> embryos in these litters were not underrepresented at day 14 (Fig. 8), nor were Zip4<sup>+/−</sup> offspring underrepresented (44 Zip4<sup>+/−</sup> and 42 wild-type) at weaning when dietary zinc is adequate. However, 18% (eight pups) of those weaned pups also display the same types of abnormalities and had failed to thrive. Maternal zinc deficiency also profoundly affected the development of Zip4<sup>+/−</sup> embryos in wild-type females. About two-thirds of the heterozygous embryos were morphologically abnormal, and 41% were severely abnormal in dams fed a zinc-deficient diet. In contrast, 94% of the wild-type embryos were normal and none were severely abnormal in these litters (Fig. 8). Among the abnormal Zip4<sup>+/−</sup>
embryos, seven of the eight embryos had one or no eyes, and four had exencephalia at day 14.

These results establish that maternal Zip4 heterozygosity sensitizes the developing embryo (wild-type and Zip4\(^{+/2}\)) to the deleterious effects of zinc deficiency during pregnancy. Furthermore, in combination with embryonic Zip4 heterozygosity, maternal Zip4 heterozygosity dramatically increases the chances of abnormal development and diminishes the chances of survival of the embryo to parturition, and these effects are exacerbated by dietary zinc deficiency during pregnancy. Finally, these results suggest that the abnormal phenotypes noted at weaning in Zip4 heterozygous offspring reflect the diminished function of the embryonic Zip4 gene.

**DISCUSSION**

The human recessive genetic disorder AE is caused by mutations in the Slc39a4 (Zip4) gene, and the studies of Zip4-knockout mice reported herein confirm that this zinc transporter plays a pivotal role in zinc homeostasis in the mammalian embryo and newborn. Some patients with AE are homozygous for mutations in the Zip4 gene that likely lead to a complete loss of function of this protein. For example, patients homozygous for a point mutation, a 5 bp deletion or 53 bp insertion that each introduces a premature stop codon within the coding region of Zip4 mRNA have been described (10,17,18). These mutations would cause the synthesis of severely truncated peptides, although that has not been proven to occur. A complete loss of function was mimicked in these Zip4-knockout mice. In mice, a complete loss of function of ZIP4 was embryonic lethal. Similarly, in humans, the apparently complete loss of ZIP4 function is lethal but at a later stage (postnatal) unless zinc is supplemented in the diet. Therefore, the early post-implantation death of homozygous Zip4-knockout mouse

Figure 6. Gross morphology of a wild-type and an Slc39a4 (Zip4\(^{+/+}\)) heterozygous-knockout mouse just after weaning. A wild-type (+/+ ) and a severely abnormal Zip4 heterozygous (+/−) mouse just after weaning were photographed and then analyzed by high-field MRI at the Hoglund Brain Imaging Center at our institution (see first two columns in the top set of panels in Fig. 7).

![Figure 6](https://academic.oup.com/hmg/article-abstract/16/12/1391/2355926)

**Figure 7.** Pleiotropic phenotypes associated with Slc39a4 (Zip4) haploinsufficiency in weaned mice. Wild-type mice and Zip4\(^{+/−}\) knockout mice (Zip4\(^{+/−}\)) that were obviously abnormal were examined using high-field MRI (top three rows). Top row: axial sections through the face at the plane of the eyes; second row: axial sections through the midbrain region; third row: sagittal sections through the midbrain region; white areas in the brain indicate water accumulation. Images are not displayed proportional to the size of the mice. Middle two panels (left): histology of the normal mouse brain at bregma 1.18–0.86 mm. SMC, sensory motor cortex; CC, corpus callosum; LV, lateral ventricle; CPu, caudate putamen; ICJ\(M\), island of Calleja (major); SNu, septal nuclei; (right): paraffin section of an affected Zip4\(^{+/−}\) brain stained with toluidine blue-confirmed hydrocephalus. Bottom set of four panels: sagittal section from near the center of the mouse heart stained with H&E. Histology of wild-type (WT) and three Zip4\(^{+/−}\) hearts from affected weaned mice (not those shown in MRI panels). RV, right ventricle; LV, left ventricle; VS, ventricular septum; TC, trabeculae carneae. Arrows indicate the relative thickness of the ventricular septum and the outer wall of the left ventricle. The heart on the bottom right was smaller than the others.
embryos was an unexpected finding. Human AE patients generally present symptoms after birth, and they often benefit greatly by zinc therapy (22). This suggests the existence of secondary zinc uptake systems that can compensate for the loss of ZIP4 function in the newborn human. However, we were unable to rescue the embryonic lethality caused by the complete loss of ZIP4 function in mice by providing excess zinc orally and/or by intra-peritoneal injection to the mother, suggesting the lack of an adequate backup system for zinc uptake through the mother and into the embryonic environment at this critical stage of development. In addition to the essential function of Zip4, the copper transporter Ctr1 (23) and the zinc efflux transporter ZnT1 (24) are also essential genes at this stage of mouse development. Taken together, these studies suggest that morphogenesis of the mouse embryo requires the development of functional systems to maintain homeostasis of essential metals at the egg cylinder stage. It is interesting to note that the ‘fear-of-intimacy’ gene in Drosophila encodes an LIV-1 family zinc transporter that is essential during early development of the fly (25). Similarly, in zebrafish, a member of the ZIP superfamily (zLIV-1) is essential during early morphogenesis (26). Zip4 is the first example of a mammalian Zip gene shown to serve an essential function during embryogenesis, and the most active expression of the Zip4 gene is restricted to the visceral endoderm, although this gene was detectably expressed in all cells in the blastocyst. The visceral endoderm cells are the third cell type to differentiate from the inner cell mass, and on day 7.5, they surround the extra-embryonic region of the developing conceptus.

The pleiotropic effects of Zip4 heterozygosity on embryonic development in mice were profound, and both embryonic and maternal Zip4 genes were implicated in controlling sensitivity to zinc deficiency. Some AE patients are homozygous or compound-heterozygous for Zip4 mutations that may result in hypomorphic alleles. For example, patients homozygous for the P200L mutation have been reported (10,19), and this mutation diminishes but does not eliminate the zinc transport activity of ZIP4 in transfected cell assays (13). Although, to our knowledge there are no published studies showing that Zip4 heterozygosity in humans is associated with effects on embryonic development, our studies suggest that dietary zinc availability as well as gene dosage may modulate the severity and type of symptoms associated with AE mutations. It is interesting to note that gene dosage for Atp7a, the Menke’s disease gene, determines the sensitivity of the developing zebrafish notochord to copper deficiency (27). Similarly, transient neonatal zinc deficiency during breast-feeding has been associated with heterozygosity in the maternal ZnT2 gene in women (28). Zinc nutritional status, interacting with mutations in human Zip4 may be associated with a wide range of developmental abnormalities.

Neurogenesis appears to be remarkably sensitive to zinc deficiency in heterozygous Zip4-knockout embryos. Exencephalia, hydrocephalus, anophthalmia and anopia all occurred in these animals under normal dietary zinc conditions. Remarkably, many of these abnormalities have been previously associated with zinc deficiency in the rat (4,6,29,30), and zinc is essential for brain development and function in monkeys as well (31). It is thought that diminished DNA and RNA synthesis in neurons during zinc deficiency may cause abnormal neurogenesis (32,33). The retina is particularly rich in zinc, which is concentrated in photoreceptors and retinal pigment epithelial cells (34), where it may function as a neuromodulator (35,36). Depletion of intracellular zinc induces caspase-dependent apoptosis in retinal cells (37).

The severity and penetrance of these abnormalities in these mice may reflect interactions of Zip4 with genetic modifiers. Inbred C57BL/6 mice have a high rate (up to 12%) of spontaneous microphthalmia and ocular infections (www.jaxmice.jax.org/library/notes/463aHTML), which is exacerbated by alcohol (38). Ocular phenotypes such as abnormal retina and optic nerve have been reported in p53-knockout mice bred onto the C57BL/6 background but not onto the 129/SvJ background (39). However, true anophthalmia and anopia as seen in these Zip4 heterozygous mice are unusual in C57BL strains of mice (38). Furthermore, these mice are on a mixed genetic background (129/SvJ:C57BL/6:B6-FVB), and none of these neuro- or ocular developmental abnormalities were seen during examination of several hundred wild-type mice from the same litters. Thus, the pleiotropic phenotypes observed here reflect the loss of function of a Zip4 allele, and there is no evidence that this exacerbates an inherent susceptibility to malformations in this genetic background. It is intriguing to note that AE has been associated with LDL deficiency (40), and many of the same phenotypes reported here in Zip4-knockout mice were also noted in apo-B-knockout mice (41). Homozygous apo-B-knockout embryos die before day 9 of gestation, and heterozygous embryos showed increased intrauterine deaths and abnormal neurogenesis. Incomplete neurotube closure and hydrocephalus were noted in some apo-B-knockout mice, but no eye phenotypes were reported, and many of the male mice were infertile (41). Whether ZIP4 influences LDL metabolism in mice remains to be examined.
MATERIALS AND METHODS

Animal care and dietary zinc manipulation

Experiments involving mice were performed in accordance with the guidelines from the National Institutes of Health for the care and use of animals and were approved by the Institutional Animal Care and Use Committee. Mouse diets were purchased from Dyets Inc. (www.dyets.com) and were identical except for zinc levels, which were as follows: zinc-deficient, 1 p.p.m. zinc; zinc-adequate, 30 p.p.m. zinc.

The effect of zinc deficiency during pregnancy on morphogenesis and growth of the embryo was monitored as described in detail previously (12,20,21).

Targeting vector construction

A bacterial artificial chromosome that contained the Zip4 gene from the 129S1/Sv mouse strain was obtained from Research Genetics (www.invitrogen.com) and sequenced by Bruce Roe (University of Oklahoma).

The 7.3 kb upstream arm of the targeting vector spanned from a HindIII site located ~7.1 kb upstream of exon 1 and extended downstream to the Zip4 start codon. A BamHI site that had been introduced immediately downstream of the start codon by PCR was fused in-frame with the coding region of the EGFP cDNA from the vector pEGFPKTIloxneo (gift from Mario Capecchi, University of Utah). A loxP-flanked MC1-Neo cassette was immediately downstream of the EGFP cDNA in this vector. The 3.7 kb downstream arm was subcloned downstream of this MC1-Neo cassette and extended from a SbfI site located within intron 1 to an AfeI site within exon 11. This targeting construct was inserted into the HpaI and Pmel sites in 4317G9 (gift from Richard Palmiter, University of Washington) which are flanked by the negative selection markers, herpes simplex virus thymidine kinase and diphtheria toxin.

Targeted disruption of the Slc39a4 (Zip4) gene in ES cells

The targeting vector was linearized within the vector backbone with AscI and electroporated into C57 ES cells. Selected colonies were screened by Southern blot hybridization and karyotyped as described in detail previously (20,21). Homologous recombination of the targeting vector into the endogenous locus results in the insertion of an XhoI site, allowing the targeted and wild-type alleles to be distinguished by Southern blot hybridization using both 5’ and 3’ flanking probes.

Generation of Slc39a4 (Zip4)-knockout mice

Chimeric mice were generated by microinjection of two independent Zip4+/− ES cell clones into day 4 C57BL/6 blastocysts, followed by transfer to pseudopregnant CD-1 foster mothers. Resulting chimeric mice were crossed with C57BL/6 females (Harlan, Indianapolis, IN). Germline transmission was confirmed by PCR from tail DNA of agouti offspring, and results from the PCR screen were initially confirmed with Southern blot analysis. Agouti offspring heterozygous for the Zip4-knockout allele were mated with commercially available (B6-FVB) mice that express Cre recombinase driven by the adenovirus Elia promoter (www.jax.org) to remove the loxP-flanked MC1-Neo cassette. PCR was used to verify complete excision of the MC1-Neo cassette in the Zip4+/− x Elia-Cre F1 progeny. These mice were then genotyped using a set of three primers (primer sequences are available upon request). One primer was common to both the wild-type and targeted alleles; a second primer was specific to the wild-type allele; the third primer annuls downstream of the EGFP open-reading frame.

On the basis of this breeding scheme, the genetic background of these mice is mixed, 129S1/SV.C57BL/6.FVB. Multiple heterozygous Zip4-knockout mice in which the MC1-Neo cassette had been completely removed were used to create the working colonies of wild-type and heterozygous knockout mice. Two separate lines of Zip4-knockout mice were generated from separate ES clones. Both displayed the same homozygous lethal phenotype. Therefore, the studies reported herein focused on the C4 cell line-derived Zip4-knockout mice.

Blastocyst collection

Zip4+/− females between the age of 3 and 4 weeks were superovulated by intraperitoneal injection of 5 IU PMSG (www.sigma.com) followed 47 h later by the injection of 5 IU hCG (www.sigma.com). Females were then paired with wild-type males for mating overnight. On day 4 of pregnancy, females were euthanized and their uteri were excised and placed in 3 ml of HEPES-buffered M2 medium (www.specialtymedia.com). Uteri were flushed with this medium, and blastocysts and morulae were collected and transferred to a glass depression slide in sterile PBS for imaging. Those embryos that had not yet developed to the blastocyst stage were cultured in KSOM medium (www.specialtymedia.com) in a humidified atmosphere of 5% CO2 until the blastocyst stage was reached before imaging by microscopy, as described subsequently.

Microscopy

Fluorescence imaging and immunodetection of EGFP were performed as described in detail previously (15,20,21). EGFP fluorescence was detected using a Leica MZFLIII dissecting microscope. Antibody binding was detected using a Texas Red cube in a DM4000B Leica fluorescence microscope coupled with a DC500 digital camera (www.leica.com). Red fluorescence indicated antibody binding, whereas blue fluorescence revealed nuclei. Phase contrast microscopy was also done using the same microscope. Images were captured with Adobe Photoshop.

Scanning by high-field MRI

High-field MRI in axial and sagittal planes was performed on one wild-type and three weaned Zip4+/− mice post-mortem using a 9.4T horizontal Varian scanner (www.varian.com) with 31 cm bore, 400 mT/m gradient coil set and 38 mm inner diameter radio frequency volume coil, as described previously (42,43). The imaging parameters for the axial
scans were TR = 4000 ms, TE = 12 ms, image matrix = 128 × 128, field-of-view = 20 mm × 20 mm, slice thickness = 1 mm, number of averages = 2 and number of slices = 30. The corresponding parameters for the sagittal scans were the same except image matrix = 256 × 128, field-of-view = 55 mm × 20 mm and slice thickness = 0.5 mm.

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Conflict of Interest statement. None declared.

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