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Syndromic congenital myelofibrosis associated with a loss-of-function variant in *RBSN*

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To the editor:

The human proteins rabenosyn-5 and VPS45 form a complex that plays a key role in early endocytosis[1-4]. Pathogenic variants in *VPS45* cause severe congenital neutropenia (SCN) with impaired neutrophil function, reticulin fibrosis of the bone marrow, and extramedullary hematopoiesis (OMIM: 615285)[5-8]. Patients with a specific *VPS45* variant (p.Glu238Lys) also have intellectual disability and bilateral optic nerve hypoplasia[5,6]. To date, the only evidence of a potential role for *RBSN* in human disease is the report of a homozygous missense variant (p.Gly425Arg) in a patient with intellectual disability, seizures, microcephaly, osteopenia, mild reticulin fibrosis of the bone marrow, and transient neutropenia[9].

We evaluated three siblings, born to consanguineous parents, for a phenotype of SCN, anemia, thrombocytopenia, reticulin fibrosis of the bone marrow, dysmorphic facial features, osteopenia, hypertriglyceridemia, hepatomegaly, microphthalmia, and optic nerve hypoplasia (Figure 1 A-E). Bone marrow examination revealed trilineage hematopoiesis without maturation arrest (Figure S1). Myelofibrosis was documented as early as 4 weeks of age and was progressive and severe (Figures S1 and S2). The proband (patient VI.2) had complete 46,XY male-to-female sex reversal and died at age 20 months after multiple infections. At autopsy, she was found to have extensive extramedullary hematopoiesis. The other two affected siblings (patients VI.3, and VI.4) are currently ages 9 and 5 years. They underwent unrelated-donor bone marrow or stem cell transplantation at 8 and 6.5 months of age, respectively. Post-transplantation bone marrow examinations have shown a reduction in myelofibrosis (Figure 1F) and their circulating blood counts have remained normal (Figure S3). Both have severe intellectual disability, are non-ambulatory and non-verbal. The clinical presentation is detailed in Table 1, Figures S1-S3, and Table S1.

The parents and a fourth sibling (patient VI.5), who is currently seven months of age, have no neurologic or ophthalmologic abnormalities. The fourth sibling had transient neonatal thrombocytopenia and anemia, and has persistent, mild neutropenia (Figure S4). The mother has had chronic moderate thrombocytopenia, an elevated immature platelet fraction, and peripheral blood smear with macrothrombocytes among variably-sized platelets. These findings have been interpreted as consistent with immune thrombocytopenia. Her CBC is otherwise normal. The father's CBC is normal.

Whole-exome sequencing on the proband (patient VI.2) revealed a variant (NM_022340.3:c.289G>C; NP_001289307.1:p.Gly97Arg) in *RBSN*, the gene encoding rabenosyn-5, which was homozygous in the three affected children, heterozygous in each parent and patient VI.5, and predicted to affect mRNA splicing and protein function (Figures S5 and S6A).

To assess splicing, we sequenced cDNA libraries from the proband (patient VI.2) and the father. Normally-spliced transcripts were not observed in the proband and represented 45% of transcripts in the father. The most common abnormal transcripts had skipping of exon 5 or an alternative exon 5a (Figure S7). High-throughput RNA sequencing confirmed these observations and revealed abnormally spliced transcripts that retain exon 5 and its neighboring introns (Figure S6B).

Immunoblotting showed an absence of intact rabenosyn-5 in the affected patients (Figure S6C). Immunofluorescence staining revealed larger, clustered EEA1-positive endosomes in patient fibroblasts compared to controls (Figure S6D). These findings suggest that the absence of intact rabenosyn-5 affects the structure and function of early endosomes.

Transferrin uptake and recycling assays revealed quantitative and qualitative differences in cells from the affected patients compared to those from a healthy control. After 30 minutes of uptake, transferrin was localized to a more clustered perinuclear region in cells from the affected patient (Figure S6E). In addition, total transferrin accumulation was higher, and the washout phase was delayed, in cells from the affected patient (Figure S6F). These findings are consistent with a decreased rate of recycling of the transferrin receptor and increased steady-state intracellular accumulation of the ligand in cells harboring the *RBSN* variant. The perinuclear clustering and the increased transferrin accumulation were reversed in cells transfected with a version of the human *RBSN* gene that does not have the variant (Figure S8).

Our observations suggest that *RBSN* loss of function causes a severe syndromic form of congenital myelofibrosis, and they confirm that rabenosyn-5 plays a vital role in human development and hematopoiesis. The mechanisms by which *RBSN* loss of function leads to hematologic and developmental abnormalities are unknown. As previously suggested for *VPS45*, the neutropenia may result from apoptosis induced by the toxic effects of impaired endosomal trafficking[5,7]. It is important to note, however, that other congenital disorders associated with increased neutrophil apoptosis, such as pathogenic variants in *ELANE*, do not also result in congenital myelofibrosis. Thus, additional factors must be at play. One hypothesis is that the myelofibrosis results from impaired trafficking of α-granules and their release from megakaryocytes, resulting in a pro-inflammatory, profibrotic response. Also, given that *RBSN* loss of function is predicted to result in a reduction of β1 integrin expression at the cell surface, as observed for VPS45 deficiency[5], it is tempting to speculate that the optic pathway and other central nervous system effects are the result of abnormal axonal transport of integrin transmembrane proteins during development.

The phenotypic overlap between our patients and those with mutation affecting *VPS45*[5-8] suggests the existence of a distinct disorder of early endocytosis caused by pathogenic variants in the genes encoding either member of the rabenosyn-5/VPS45 complex. SCN without maturation arrest, reticulin fibrosis of the bone marrow, and extramedullary hematopoiesis appear to be the hallmarks of the disease. Anemia and thrombocytopenia in our patients were variable and exacerbated by infection and progressive hepatosplenomegaly (Table S1 and Figure S3). Long-term survival without substantial hematologic or infectious complications was possible after bone marrow or stem cell transplantation, suggesting the hematopoietic defect is intrinsic to hematopoietic stem and progenitor cells, although a transferable factor from the transplanted cells to the bone marrow cannot be excluded. The neurologic and ophthalmologic abnormalities in the surviving patients have remained stable. Other concordant features in our patients include facial dysmorphism, abnormal bone development, and hypertriglyceridemia. The only sibling homozygous for the *RBSN* variant who had a 46,XY chromosome complement also had a disorder of sex development. This appears to be part of the syndrome, and we hypothesize that it could be the result of ineffective endocytosis of sex hormones *in utero*, as has been observed in animal models[10]. Confirmation will require additional cases of 46,XY individuals with loss-of-function *RBSN* variants.

The mother's hematologic abnormality is consistent with immune thrombocytopenia. This could explain the transient neonatal thrombocytopenia in the heterozygous sibling (patient VI.5), but it would not explain his transient anemia and persistent, mild neutropenia. It is certainly possible that the hematologic manifestations in these two individuals represent a milder phenotype caused by the *RBSN* variant in heterozygous state.

The structural and phenotypic effects of the variant we report are considerably more severe than those of the previously reported *RBSN* variant (p.Gly425Arg)[9]. In that patient, intact rabenosyn-5 was detected by immunoblot, the sub-cellular localization of the protein by immunofluorescence staining was normal, and transferrin endocytosis and recycling assays showed a significantly enhanced rate of recycling suggestive of a gain-of-function effect. However, the phenotypic overlap with our patients suggests that the p.Gly425Arg variant is also pathogenic, leading to a milder phenotype within the same spectrum. Therefore, pathogenic *RBSN* variants, in homozygous or heterozygous state, should also be considered in cases of unexplained transient neutropenia, particularly when neurologic abnormalities and/or myelofibrosis are also present.

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This study was approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine. The parents gave written informed consent before evaluation, counseling, and testing.

Explanation of Author Contributions:

Designed and performed research: Shchelochkov, Bainbridge, Yatsenko, Searby, Zapata, Hernandez, Gadkari, Einhaus, Muzny, Gibbs, Bertuch, Corvera, Franco

Collected data: Magoulas, Shchelochkov, Ben-Shachar, Potocki, Lewis, Bertuch, Scott, Franco

Analyzed and interpreted data: Magoulas, Shchelochkov, Bainbridge, Yatsenko, Lewis, Searby, Marcogliese, Elghetany, Zapata, Hernandez, Gadkari, Einhaus, Muzny, Gibbs, Bertuch, Scott, Corvera, Franco

Performed statistical analysis: Corvera

Wrote the manuscript: Magoulas, Shchelochkov, Lewis, Bertuch, Scott, Corvera, Franco

Mrs. Magoulas, Dr. Shchelochkov, and Dr. Franco had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Mrs. Magoulas and Dr. Shchelochkov contributed equally to this study.

Conflict of Interest Disclosure: The authors report no conflicts of interest related to this work.

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Figure Legend

Figure 1. Family pedigree and phenotypic features of the patients.

A, Family pedigree indicating consanguinity. Filled symbols represent affected individuals (patients VI.2, VI.3, VI.4).

B, Photographs of patient VI.2 at 11 months, patient VI.3 at 9 years 2 months, and patient VI.4 at 4 years 11 months. Note microphthalmia, beaked nose, and prominent columella. Consent for the publication of images was provided by the patients' parents.

C, Absolute neutrophil count over time for the three patients. Shaded blue represents the normal reference range based on age. The upper limit of normal in the newborn period in our laboratory is 23.5x100/uL, which is above the range depicted (*). HSCT: Hematopoietic stem cell transplantation. BMT: Bone marrow transplantation. DOD: Date of death.

D, Brain MRI of patient VI.3 at 9 months of age. Note supratentorial volume loss involving the white greater than the gray matter, ex vacuo ventricular enlargement, and optic nerve hypoplasia.

E, Bone marrow biopsy for patient VI.3 at 33 days of age. Reticulin stain, 200x magnification. Note moderate to severe reticulin fibrosis. Trichrome staining for collagen fibers was not performed.

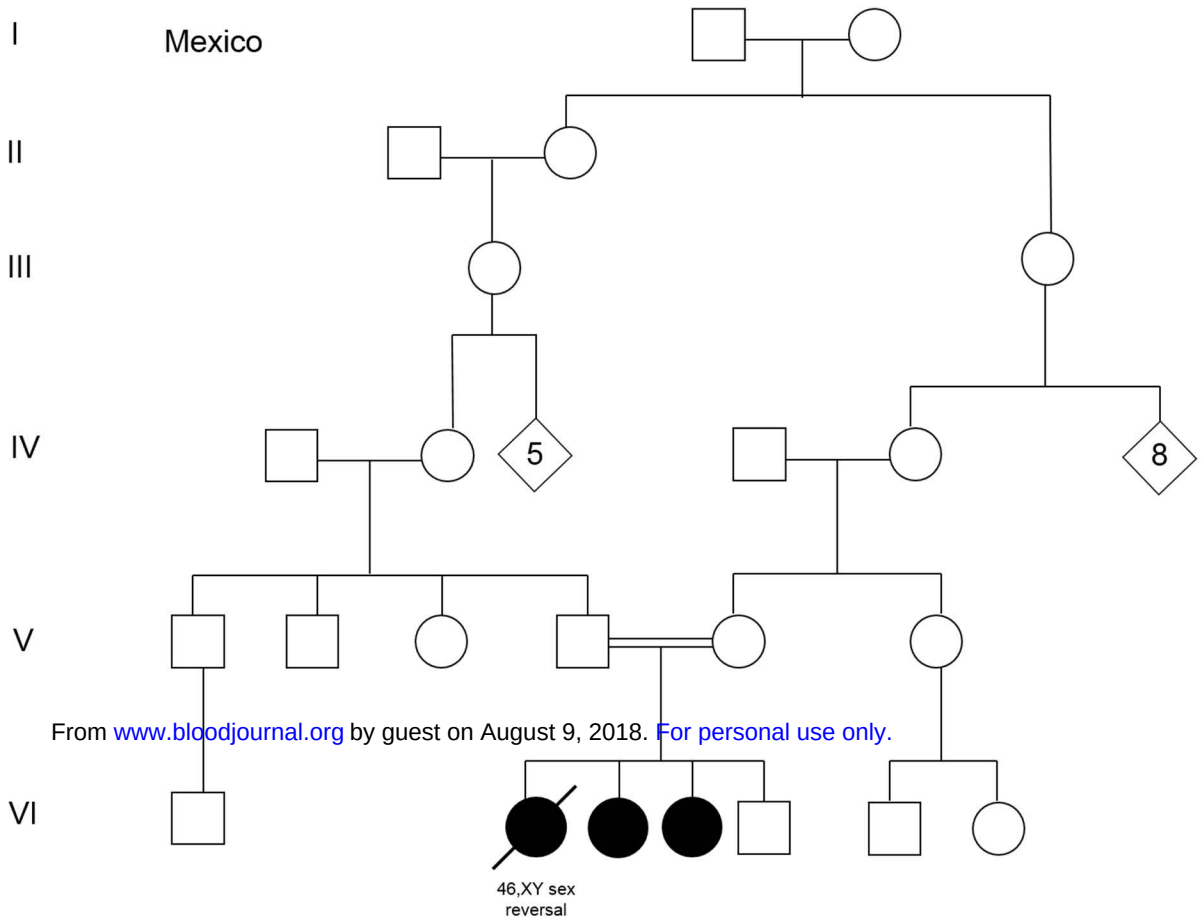
F, Bone marrow biopsy for patient VI.3 at 20 months of age, one year after bone marrow transplantation. Reticulin stain, 200x magnification. Note the reduction in reticulin fibrosis to mild and focal. Trichrome staining for collagen fibers was negative (data not shown).

Table 1. Clinical, imaging, and histopathologic findings in three patients with a pathogenic *RBSN* variant

	Patient VI.2	Patient VI.3	Patient VI.4
Phenotype	Female	Female	Female
Chromosomes	46,XY	46,XX	46,XX
CMA	Normal (BG v.5 and 400,000 oligonucleotide research array)	Normal (BG v.6.4)	Normal (BG v.8.3)
Growth	11 m: Ht: 25%; Wt: 75%; OFC: 25-50%	9 y 2 m: Ht: <1%, Wt: 76%, OFC: 18%	4 y 11 m: Ht: <1%, Wt: 57%, OFC: 5%
Facial features	Narrow bi-temporal diameter, metopic prominence, sloping forehead, upslanting palpebral fissures, epicanthal folds, bulbous nose, depressed nasal tip	Prominent nasal bridge, low set, posteriorly rotated ears, retromicrognathia, high-arched palate	Bitemporal narrowing, tubular nose, prominent columella
Hematologic	SCN transiently responsive to intermediate-dose filgrastim, PB NRBCs, progressive anemia and thrombocytopenia, hypercellular bone marrow with severe reticulin fibrosis and myeloid hyperplasia, extramedullary hematopoiesis (liver, spleen, kidneys, lymph nodes) on PME	SCN without maturation arrest, refractory to filgrastim, transfusion-dependent anemia, variable thrombocytopenia including at birth and with infection, PB NRBCs, normocellular bone marrow with moderate to severe reticulin fibrosis and maturing trilineage hematopoiesis	SCN refractory to filgrastim, moderate anemia at birth (variably present subsequently), PB NRBCs, hypercellular bone marrow with moderate to severe reticulin fibrosis and maturing trilineage hematopoiesis
Ophthalmologic	Poor visual response, microphthalmia, hypoplastic irides, microcornea, blepharophimosis, absent retinal vessels, aplastic optic nerves	Poor visual response, microphthalmia, microcornea, blepharophimosis, aplastic optic nerves	Poor visual response, microphthalmia, aniridia with iris coloboma, blepharophimosis, dysplastic optic nerves, absent retinal vessels
Neurologic	Increased muscle tone in the lower extremities, DTRs 2+, poor head control	Increased muscle tone in the four extremities, DTRs 3+, normal EEG	Joint restriction, hypotonia, diminished strength, muscle atrophy, normal DTRs
Brain MRI	Prominent supratentorial ventricles, sulci, and cisterns, widened sylvian fissures, thin corpus callosum, cerebral atrophy, severe optic nerve and chiasm hypoplasia, diffuse expansion of diploic calvarial marrow space	Perinatal intraparenchymal and subarachnoid hemorrhages. Supratentorial volume loss, thin corpus callosum, mild myelin maturation delay. Hypoplasia of the optic nerve, chiasm, and optic tract. Bone enhancement likely related to myelofibrosis	N/A
Respiratory	Tracheomalacia	Tracheomalacia	Normal
Cardiovascular	Mild cardiomegaly on PME	Small PDA, redundant atrial septum	Small PFO, small secundum ASD
Gastrointestinal	Gastrostomy feeding only, GE reflux, hepatosplenomegaly with extramedullary hematopoiesis on PME. Accessory spleen	Gastrostomy feeding only, mild esophageal dysmotility, hepatomegaly likely related to extramedullary hematopoiesis	Gastrostomy feeding only, hepatomegaly likely related to extramedullary hematopoiesis
Orthopedic	Osteopenia, congenital hip dysplasia. Tapered 5th finger	Osteopenia, femur fracture, bilateral hip dysplasia, clinodactyly, delayed bone age (2-3 y), advanced bone age (6-7 y)	Delayed bone age (-3 SD below mean), bilateral hip dysplasia
Genitourinary	Hypoplastic labia minora and majora, underdeveloped genital tubercle, anteriorly placed anus. Streak gonads, uterine didelphys, and double vagina on PME. Nephromegaly with extramedullary hematopoiesis on PME	Nephromegaly, likely related to extramedullary hematopoiesis	Normal
Endocrine	Mild adrenal cortical lipid depletion and pituitary hypoplasia on PME, elevated FSH and LH for age, normal testosterone	Elevated FSH for age.	Normal
Audiological	Bilateral mild to moderate high-frequency sensorineural hearing impairment	Normal	N/A
Immunologic	Frequent infections (*Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Candida albicans*, influenza A virus, respiratory syncytial virus)	Normal Ig G, A, M, and E. Normal CH_{50} activity. Normal numbers and ratios of CD4+ and CD8+ T cells and CD19+ B cells. NK cells and CD4+/CD45RA+ naïve T cells increased in absolute number, normal ratio. CD4+/CD45RO+ memory cells low in absolute number and ratio	Frequent urinary tract infections
Metabolic	Hypertriglyceridemia (249-335 mg/dL)	Hypertriglyceridemia (155-1086 mg/dL)	Hypertriglyceridemia (176-296 mg/dL)
Development/ Cognition	Global developmental delay	9 y: Severe intellectual disability, non-verbal, non-ambulatory	5 y: Severe intellectual disability, non-verbal, non-ambulatory

CMA: Chromosomal microarray analysis. BG: Baylor Genetics Laboratory. SCN: Severe congenital neutropenia. PB NRBCs: Peripheral blood nucleated red blood cells. N/A: Not assessed. m: Months of age. y: Years of age. Ht: Height. Wt: Weight. OFC: Occipital frontal circumference. DTRs: Deep tendon reflexes. EEG: Electroencephalogram. PME: Postmortem examination. PDA: Patent ductus arteriosus. PFO: Patent foramen ovale. ASD: Atrial septal defect; GE: Gastroesophageal. SD: Standard deviations.

A



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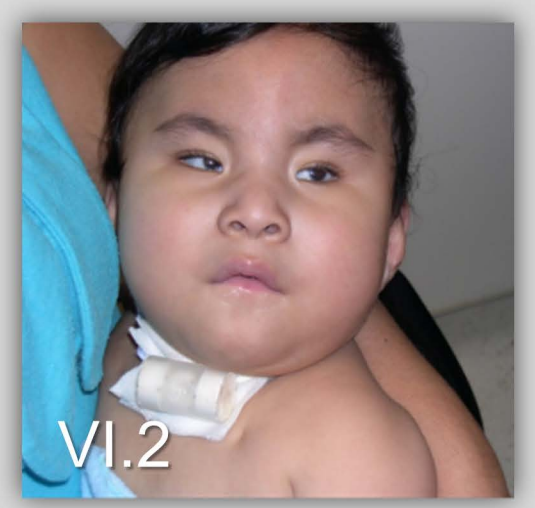

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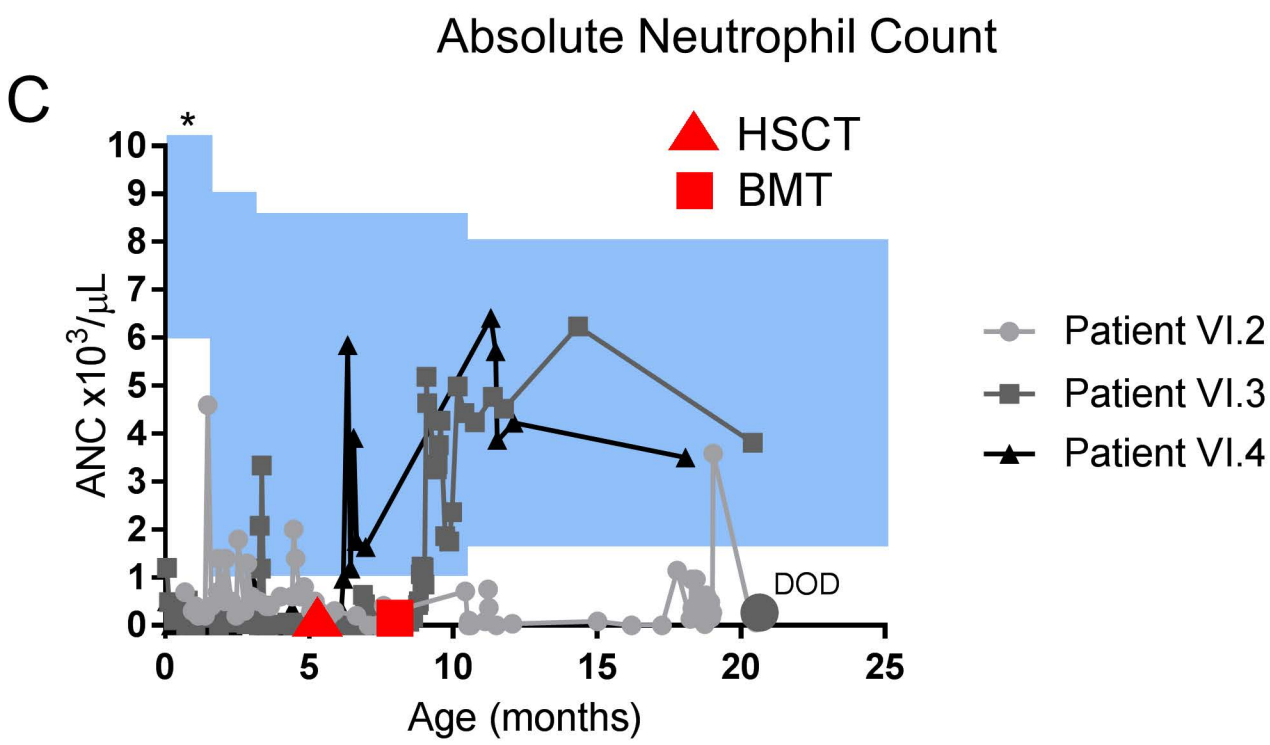

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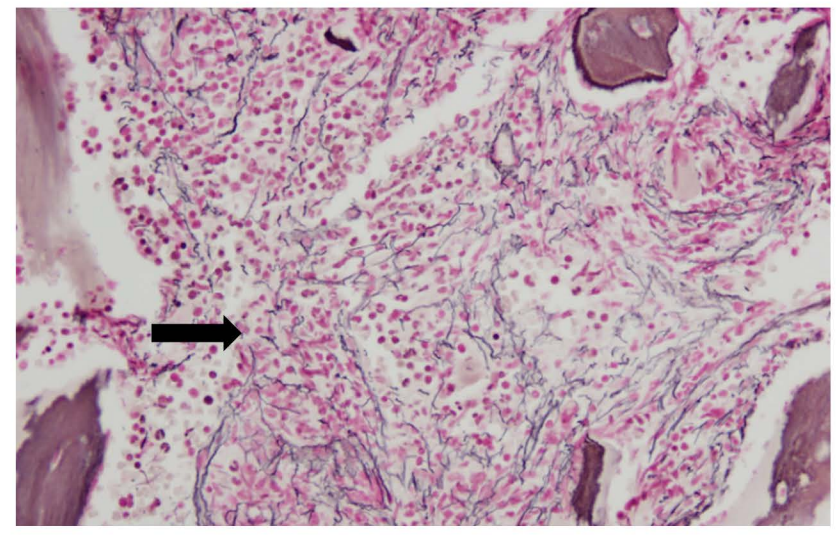

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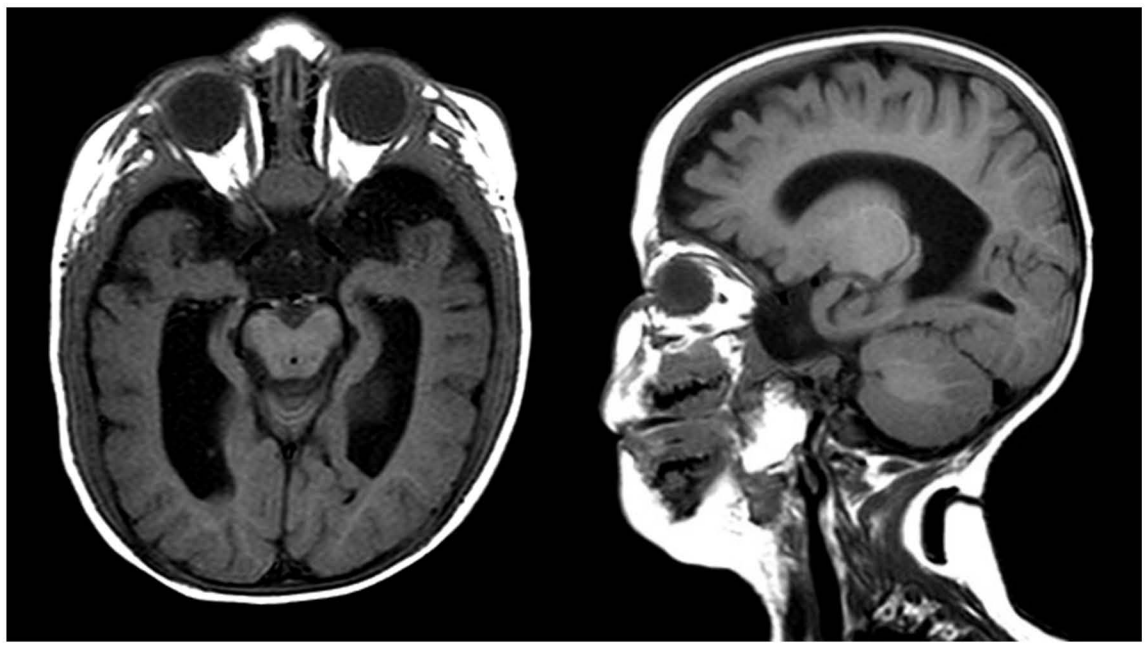

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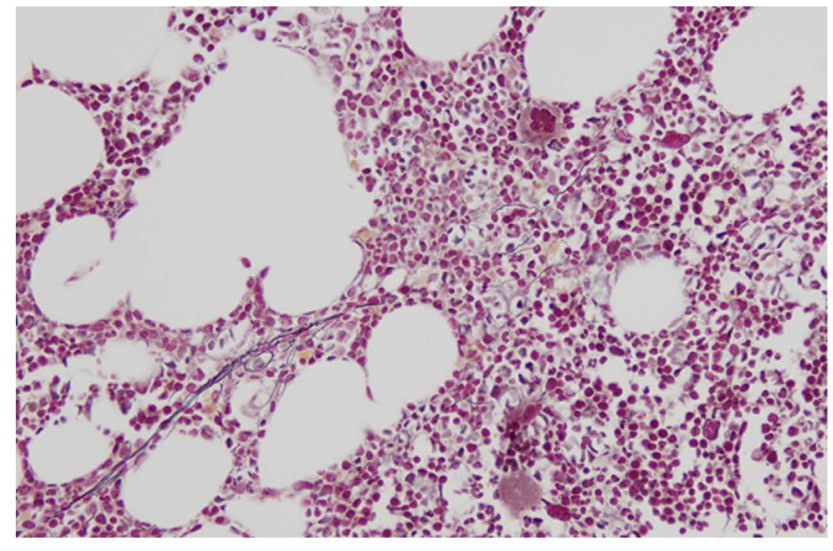

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