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Supplemental Data

Loss-of-Function Mutations in *UNC45A* Cause a Syndrome Associating Cholestasis, Diarrhea, Impaired Hearing, and Bone Fragility

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Supplemental Data

Supplemental Case report

Family A

The proband is a 5-year-old girl, the second child of healthy unrelated parents with no family history of digestive disease. There were no significant medical events her father's family or in her sister. The proband was born at term after an uneventful pregnancy by elective caesarean section. Birth weight was 2.980 g (-1 standard deviation (SD)), length was 46 cm (-2.5 SD) and occipital-frontal-circumference was 32 cm (-2.5 SD). Dehydration linked to severe profuse hydric diarrhea occurred from the fouth day of life leading to a weight loss of 20%. Initial explorations showed a secretory diarrhea profile with no evidence of infectious, immunologic or thyroid disease. There was no evidence of malabsorption or exudative enteropathy. The osmole gap and ionic stool concentration showed a rather secretory profile of the diarrhea. The carmine red test revealed a dramatic increase in intestinal transit time. Oral feeding with high degree cows' milk protein hydrolysate and enteral nutrition failed to improve the digestive leaks and exclusive parenteral nutrition was started. Endoscopic explorations with repeated intestinal biopsies using the periodic acid–Schiff showed focal abnormalities of the brush border which could be evocative of atypical microvillus inclusion disease. No tufting brush border was discovered during the pathology examination. Liver exploration was normal. Conjunctive biopsy did not show the presence of tufts. The hypothesis of a potential endocrine secreting tumor was ruled out by imaging and biochemical assays. In order to look for malformations associated with the digestive phenotype, cardiac ultrasound and MRI were performed but revealed no abnormalities. Immune and metabolic explorations were negative.

Nutritional management was marked by cyclization difficulties of parenteral nutrition and the impossibility to restart enteral feeding. Persistent hypokalemia was treated with supplementation, and chronic anemia with repeated iron infusions. The evolution was marked by language delay linked to severe bilateral deafness, which appeared during evolution, without psychomotor retardation. Growth retardation was only moderate thanks to regular adaptation of parenteral nutritional intake. Targeted sequencing of the *MYO5B* gene was negative. There was no history of bone frailty and DXA performed at age 4 years was normal. She has no muscle weakness and present normal Creatine Phosphokinase level.

Family B

Patient B.II.3

Patient B.II.3 is the third of four children, born at 38 weeks of gestation and is currently 23 years old. At 15 days of life she presented with neonatal jaundice with normal level of GGT associated with pale stools, dark urine and stagnation weight. Initial diagnosis was a Byler disease because of the association of a cholestasis with a persistent jaundice with normal GGT, elevated level of serum bile acids and some level of fibrosis on a liver biopsy performed at 10 months of life. On the clinical and biological levels, bilirubin levels returned to normal at 2.5 years of age. However major pruritus persisted with elevated serum bile acid, leading to partial internal biliary diversion for intractable pruritus at the age of 19. Surgery allowed for a decrease of the serum bile acid and a transient decrease of the pruritus.

The proband also presented with multiple fractures (23 during a period of 23 years) with no biological deficiency of D vitamin. The osteodensitometry test (bone density scan) at 20 years showed a whole body bone densitometry (BDM) with a T-score at -

2.9 and a Z-score at -1.5. Another at 24 years showed a vertebra BDM T-score of -3 and Z-score of -2.4 and femora BDM T-score of -0.9 and a Z-score of -0.6. Additionally, she had bilateral perception hearing loss diagnosed around the age of 5 years. The development of her language was poor and she presented with mild intellectual disability with a delay of acquisitions. She also had muscular inter ventricular communication, diagnosed at the neonatal period which resolved spontaneously, and a persistent asthma (treated). On the digestive level, she had a diverse food regimen and no diarrhea though her growth was restricted. She has a current weight of 38.5 kg and height of 147.5 cm.

Patient B.II.4

Patient B.II.4 is the fourth child, born at term and eutrophic, currently 18 years old. At 7 days, she presented with cholestasis with elevated GGT (Gamma Glutamyl Transaminases) up to 21 times normal values, resolving by the age of 3. Similar to her sister, a significant pruritus persisted, leading to a bypass surgery cholecysto-jejunocolostomy at the age of 12 years. The improvement was temporary, and itching and increased bile acid persists. On the digestive level, there is a syndrome of food intolerance with very early onset of diarrhea and failure to thrive requiring the introduction of parenteral nutrition and enteral feeding in combination with diversified regimen in small quantities. Diarrhea spontaneously resolved with time. Endoscopy was performed at the age of 1 year, because of a non-specific aspect of villous atrophy on intestine. At the age of 17, she presented a severe acute gastroenteritis due to Salmonella complicated by Clostridium infection when returning from Tunisia, with a very severe dehydration leading to hospitalization in resuscitation unit. Since then, the stools remain liquid, sometimes slimy but not bleeding, accompanied by

intermittent abdominal pain relieved by issuing stools. As her sister, she presented severe bone fragility, also with multiple fractures (Figure S6) and osteonecrosis of the femoral head at the age of 14 years, secondary to left hip dysplasia at birth (Figure S5). On the growth plan, we also reported a significant failure to thrive. Initially, hearing assessments have reported a normal hearing. Perception deafness needing hearing aid was diagnosed in adolescence. Neurologically, she presented a prenatal diagnosis of hydrocephalus related to stenosis Silvius aqueduct. She therefore received a ventricular-peritoneal shunt at the age of 4 months, removed 13 years later because of poor tolerance. She presented psychomotor retardation with delayed language and scored acquisitions, necessitating her management in Medical Educational Institute. On the urological plan vesico-ureteral reflux stage II was diagnosed at the age of 1 year, spontaneously evolving favorably. No anomaly was noted in the heart and breathing plan. The growth remains poor with at last evaluation a weight of 38.5kg (-3SD) and a height of 147.5cm (-2.5SD).

For these 2 children, geneticists reported the plane dysmorphic blue sclera, abnormalities of the extremities, malocclusions, the watch glass nails. There was no muscular weakness with normal CPK and both have normal puberty. Diverse genetic explorations were performed with a CGH array dismissing a chromosome rearrangement and direct sequencing of *ATP8B1* [MIM: 602397] and *ABCB11* [MIM: 603201] implicated in Progressive Familial Intrahepatic Cholestasis-1 (PFIC1 [MIM: 211600]) and 2 respectively (PFIC-2 [MIM: 601847]) and of *VPS33b* [MIM: 608552] implicated in arthrogryposis, renal dysfunction, and cholestasis 1 (ARC syndrome [MIM: 208085]) was normal.

Furthermore, immunolabeling experiments on liver biopsies show mislocalization of hepatic canalicular proteins (such as ABCB11, CD13 and GGT1) (Figure S1, S2). This suggests that *UNC45A* is involved in the distribution of these proteins in the cell, although the underlying mechanisms are unknown.

Reassessment of liver pathology in Family B (Figure S1)

The patient B.II.3 a liver biopsy was taken at the age of 19 years. Histologically, the liver architecture was preserved and there was no clear sign of fibrosis. The portal tracts showed a discrete ductular reaction with inflammation. Bile duct damage was not clearly identified. In the parenchyma, there was no sign of lobular inflammation or steatosis as view by immunohistochemistry. The Aminopeptidase N, CD13 showed a canalicular expression in the hepatocytes and an apical expression in the bile ducts, ABCB11 a canalicular expression in the hepatocytes and ABCC2 (ATP-binding cassette, subfamily c, member 2, also called MRP2) a canalicular expression in the hepatocytes. However, sequencing of *ABCB4* (also called *MDR3* for Multi-Drug Resistant 3) involved in PFIC3 [MIM: 602347]) and GGT1 (gamma-glutamyltransferase1) showed no mutation (Figure S1).

For the patient B.II.4, the liver biopsy was sampled at the age 12 years. The liver architecture was preserved, however, it showed a pericellular fibrosis, periductal fibrosis in the portal tracts, and ballooned hepatocytes with occasionally Mallory denk body indicating a (fibrosing) cholestatic pathology. Immunohistochemically, MDR3 and GGT1 were negative in the hepatocytes, whereas canalicular ABCB11, ABCC2, and CD13 immunoreactivity in the hepatocytes was identified (Figure S1).

Family C

The proband is 5 years old girl. She was born to healthy unrelated parents of Turkish origin. The father is carrier of a Polyglobulia. The pregnancy was uneventful. Birth occurred at term 41 weeks, Birth weight: 2800g, Height 49.5 cm, Head circumference: 32 cm.

At 1 month, she was admitted in a tertiary center for jaundice and a failure to thrive. Investigations showed no evidence for usual causes of cholestasis. At nearly 2 months of age, because stools were partially discolored, a transvesicular cholangiography was performed which showed a normal biliary tree. A Liver biopsy performed in the same time showed: severe hepatocellular cholestasis, giant cell transformation, micro vesicular steatosis (20%), portal fibrosis and lobular fibrosis without septa. There was no ductular reaction or bile duct paucity. In addition she presented with severe watery diarrhea, vomiting (improving by fasting), spontaneous ileo-ileal invaginations twice, a tubulopathy with hyperdiurese, proteinuria, acidosis he whole leading to a failure to thrive.

Because of the severe undernutrition (4.4kg at 5 months) a parenteral nutrition was settled at the age of 6 months, which allowed the normalization of the growth although the diarrhea persisted as well as intermittent tubulopathy and cytolysis.

At six months of life she presented a bone demineralization without evidence of rickets or imperfecta osteogenesis and two severe spontaneous fractures occurred (tibia and femur) when she was 2.

There was no neurologic deficit, and no hearing deficit (hearing tests PEA and OEA positive). There was slight developmental delay but no autistic traits to date.

The probands present a small facial dimorphism with large forehead, but no hair changes nor cutaneous spots.

Intestinal biopsies found a partial and mild villous atrophy with brush border abnormalities with no argument for celiac disease. The *MYO5B* gene was normal as well as the genes of the tricho-hepato-enteric syndrome (THES1 [MIM: 222470]; THES2 [MIM: 614602]) *TTC37* [MIM: 614589] and *SKIV2L* [MIM: 600478]. Fecal elastase was normal. During the follow up, the child remains under parenteral nutrition till now (5 years) but oral alimentation has been initiated (around 3 years of age) without increasing the stool output but did not allow the complete weaning of parenteral nutrition.

During the course of the follow up the evolution was marked by episodes of increased stool output, tubulopathy with proteinuria, all contributing to dehydration, and fluctuant cytolysis without oubvious starting event . She experienced very few infectious events.

Although the bone demineralization remained unchanged, she did not experience other fractures nor complained about bone pain. The calcium- phosphorus balance, D vitamin and PTH remained normal except during the acute phases of tubulopathy.

She has no muscle weakness and all present normal Creatine Phosphokinase level.

Recently she developed a poor glycemic regulation requiring insulin therapy, but the mechanism is so far unclear. She is still under parenteral nutrition.

Supplemental figures and legends

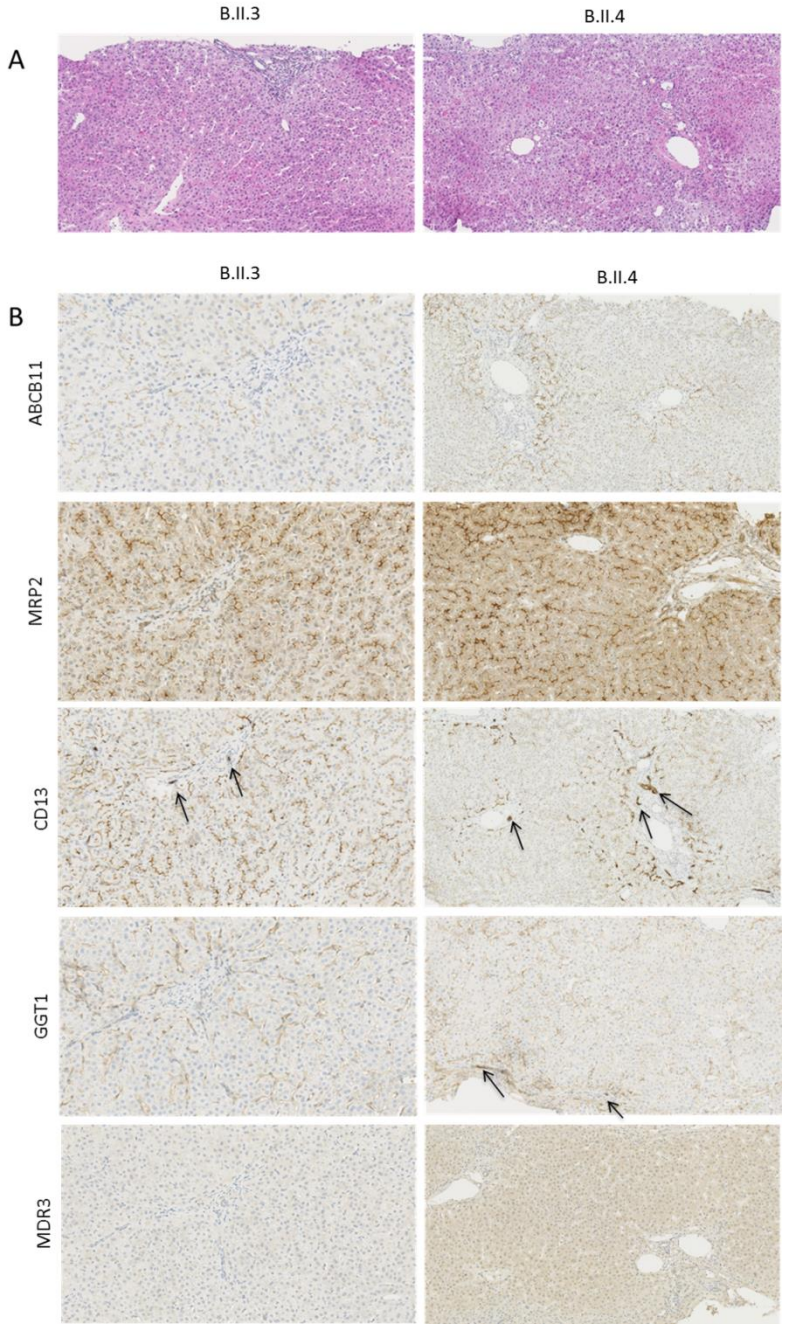

Figure S1: Photomicrographs and immunolabeling on liver biopsies of the Family B patients at 19 and 12 years respectively.

(A) Photomicrographs of patients B.II.3 and B.II.4. Individual B.II.3: Liver architecture is preserved. No clear sign of fibrosis. Portal tracts: discrete ductular reaction with inflammation. No biliary damage. Parenchyme: no steatosis. No cholestatic feature. No ballooned hepatocytes. Patient B.II.4: Liver architecture is preserved; however, it shows (fibrosing) cholestatic pathology, such as pericellular fibrosis, periductal fibrosis in the portal tracts, ballooned hepatocytes with occasionally mallory denk body. (B) Immunolabbeling. Patient B.II.3 CD13: canalicular expression in the

hepatocytes. Apical expression in the bile ducts (arrow); ABCB11: canalicular expression in the hepatocytes; GGT1: negative; MRP2: canaliculare expression in the hepatocytes. Patient B.II.4: ABCB11: deletion of the canalicular expression in the hepatocytes; CD13: deletion of the canalicular expression in the hepatocytes, however, apical expression of the bile ducts is preserved (arrow); GGT1: absence of the canalicular expression in the hepatocytes, however, apical expression of the bile ducts is preserved (arrow); MRP2 (normal pattern): canalicular expression in the hepatocytes and apical expression in the bile ducts (arrow).

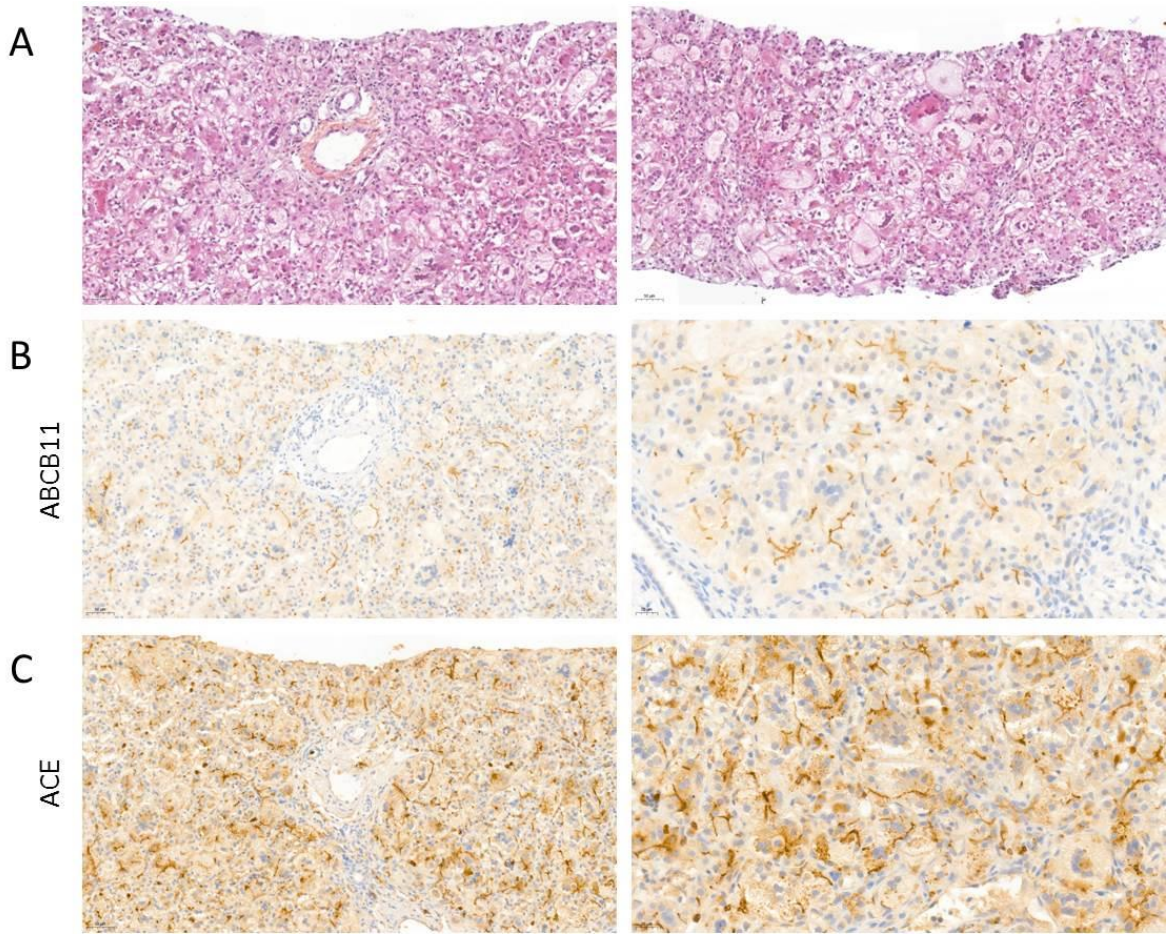

Figure S2: Photomicrographs and immunolabeling on liver biopsies of the patient C.II.1

(A) Photomicrographs of patients C.II.1 Hematoxylin and eosin staining showing hepatocellular, canalicular cholestasis, giant cell transformation of hepatocytes, microvesicular steatosis (20%), slight portal and lobular fibrosis.(B) Immunolabelling. Patient C.II.1 BSEP: canalicular expression is conserved, however a thickened canalicular staining is observed.

(C) Immunolabelling. Patient C.II.1 ECA: canalicular expression is conserved, however, a thickened canalicular staining and a granular and patchy pattern in the subcanalicular area is observed

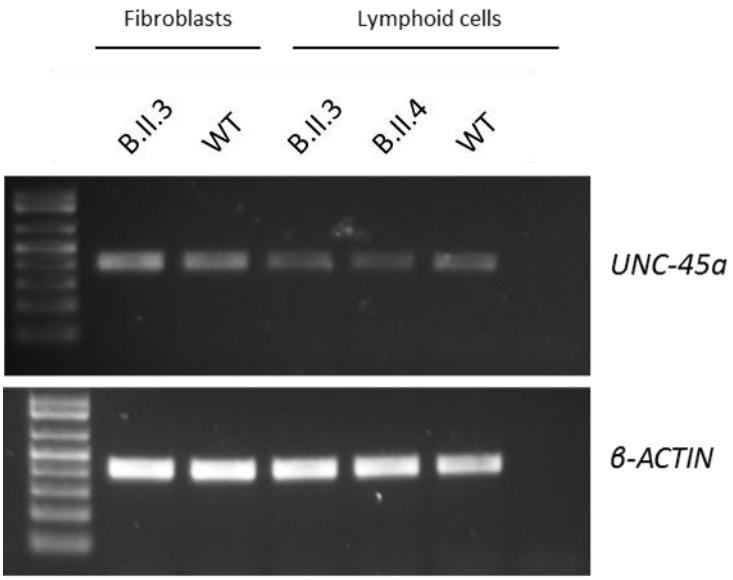

Figure S3: Detection of UNC45A transcripts by reverse transcription-PCR using mRNA of patients (Family B) and WT fibroblasts and lymphoid cells

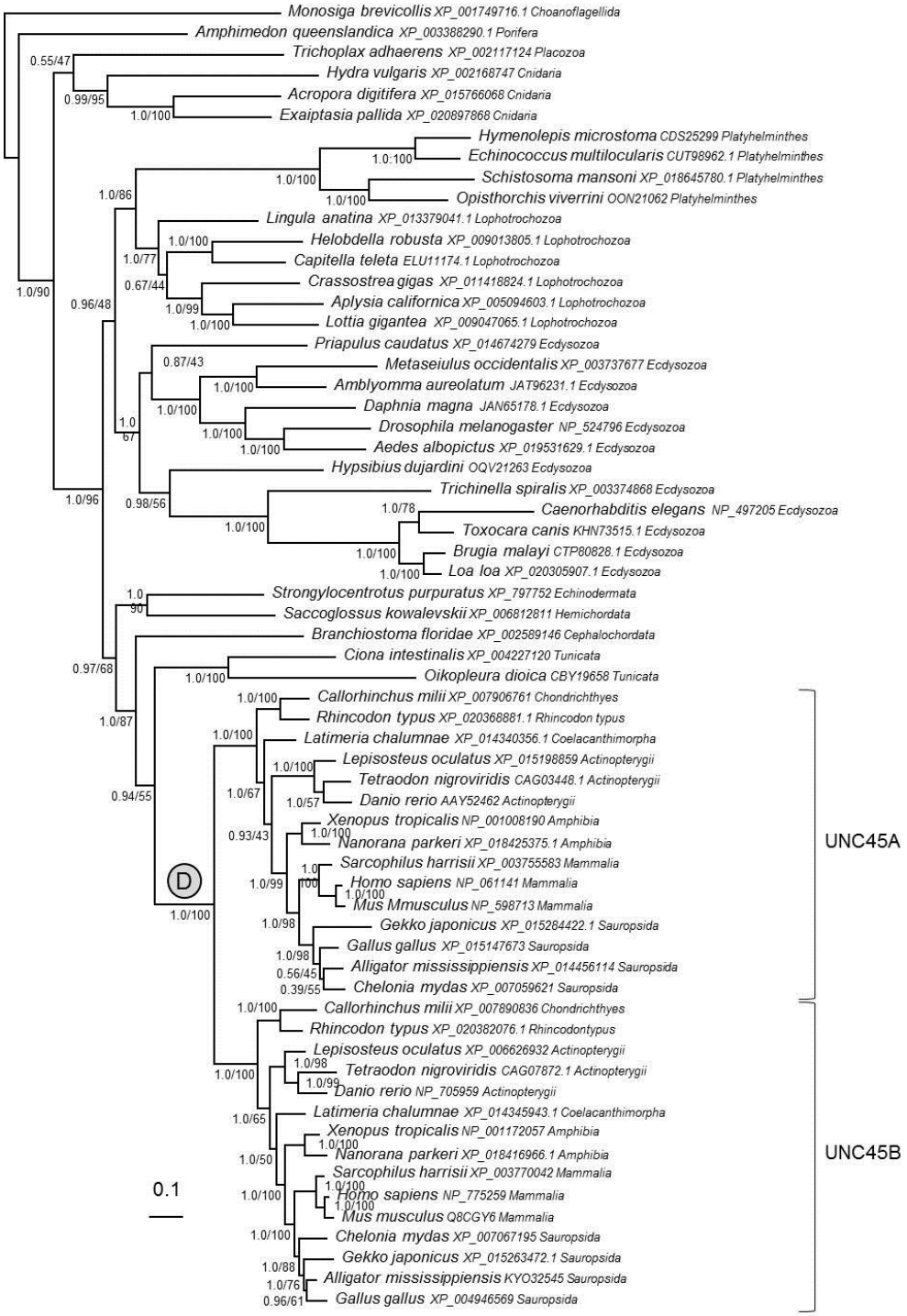

Figure S4: Bayesian tree of metazoan UNC45/UNC45A/UNC45B proteins (63 sequences, 704 amino acid positions).

The tree is rooted with the sequence of *Monosiga brevicollis* (Choanoflagellida), a close relative of animals. Numbers at branches correspond to posterior probabilities / bootstrap values. Values greater than 0.95/ 90% correspond to strongly supported branches. The scale bar indicates the average number of substitutions per site. A duplication event, indicated by a circle, occurred in branch leading to the last common ancestor of Vertebrata.

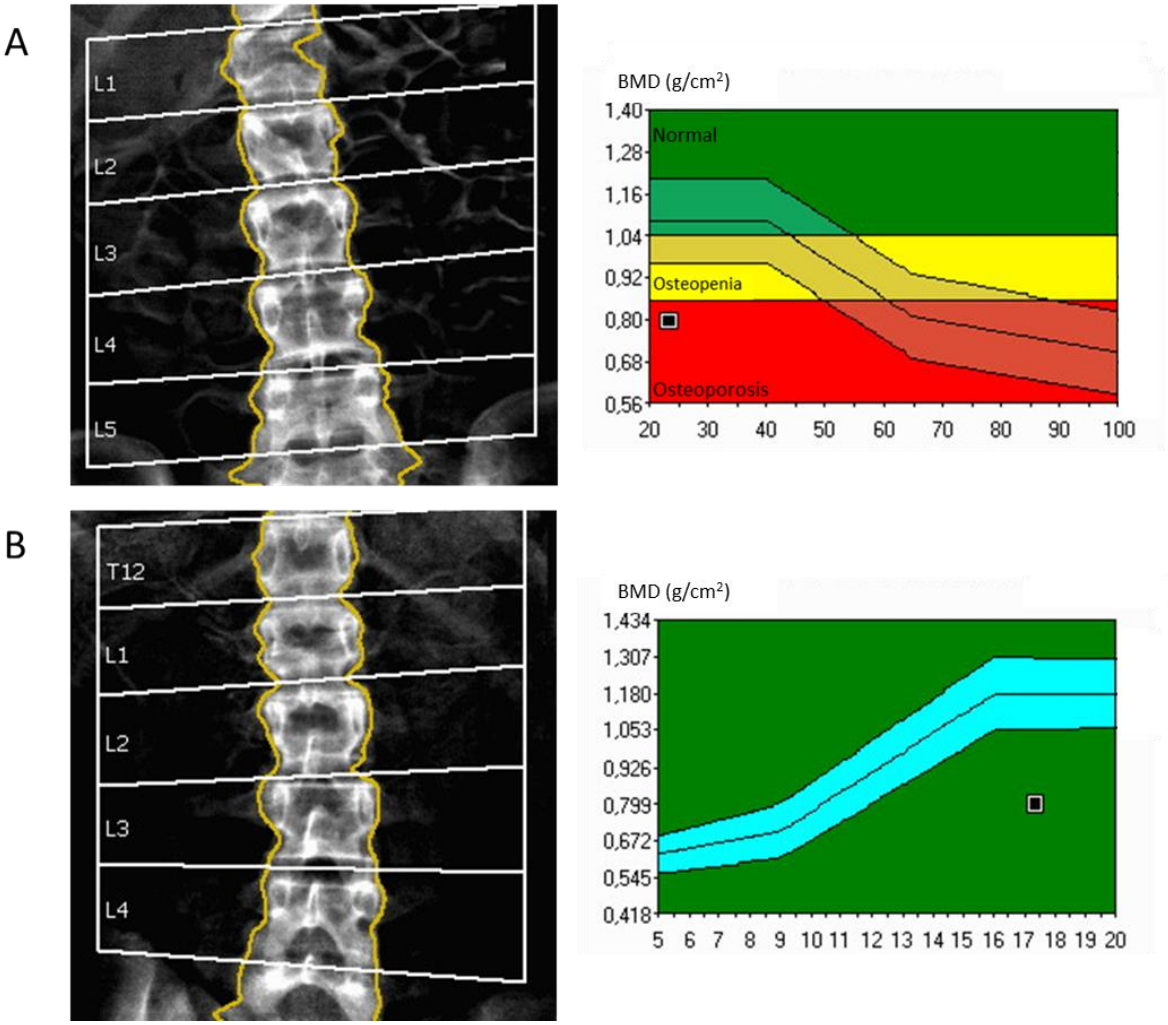

Figure S5 : Osteodensitometry analysis showing low bone densities of the two patients of Family B

(A) Patient B.II.3 at 23 years old and (B) Patient B.II.4 at 17 years old.

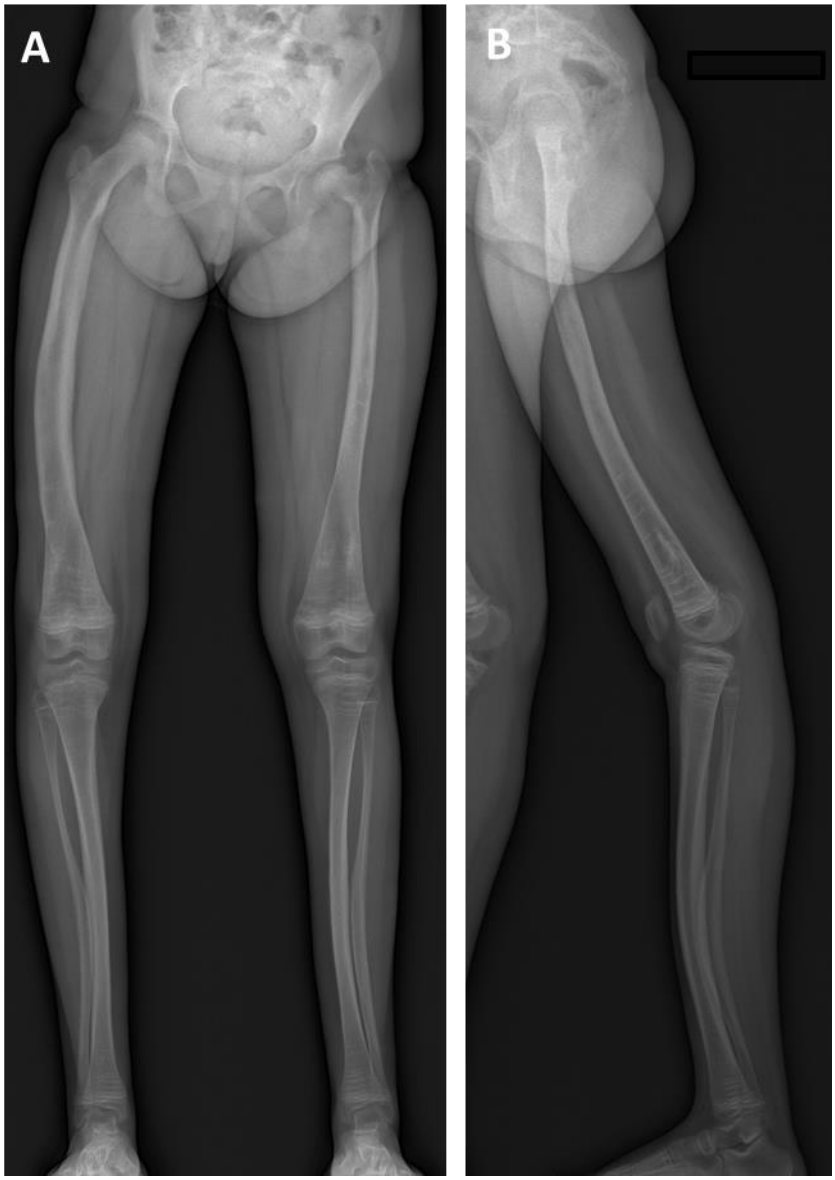

Figure S6 : Lower limb X-ray of Patient B.II.4 at 15 years old showing abnormalities of the bone remodeling with femoral deformation, bone demineralization and Left Coxa vara.

(A) front view. (B) lateral view.

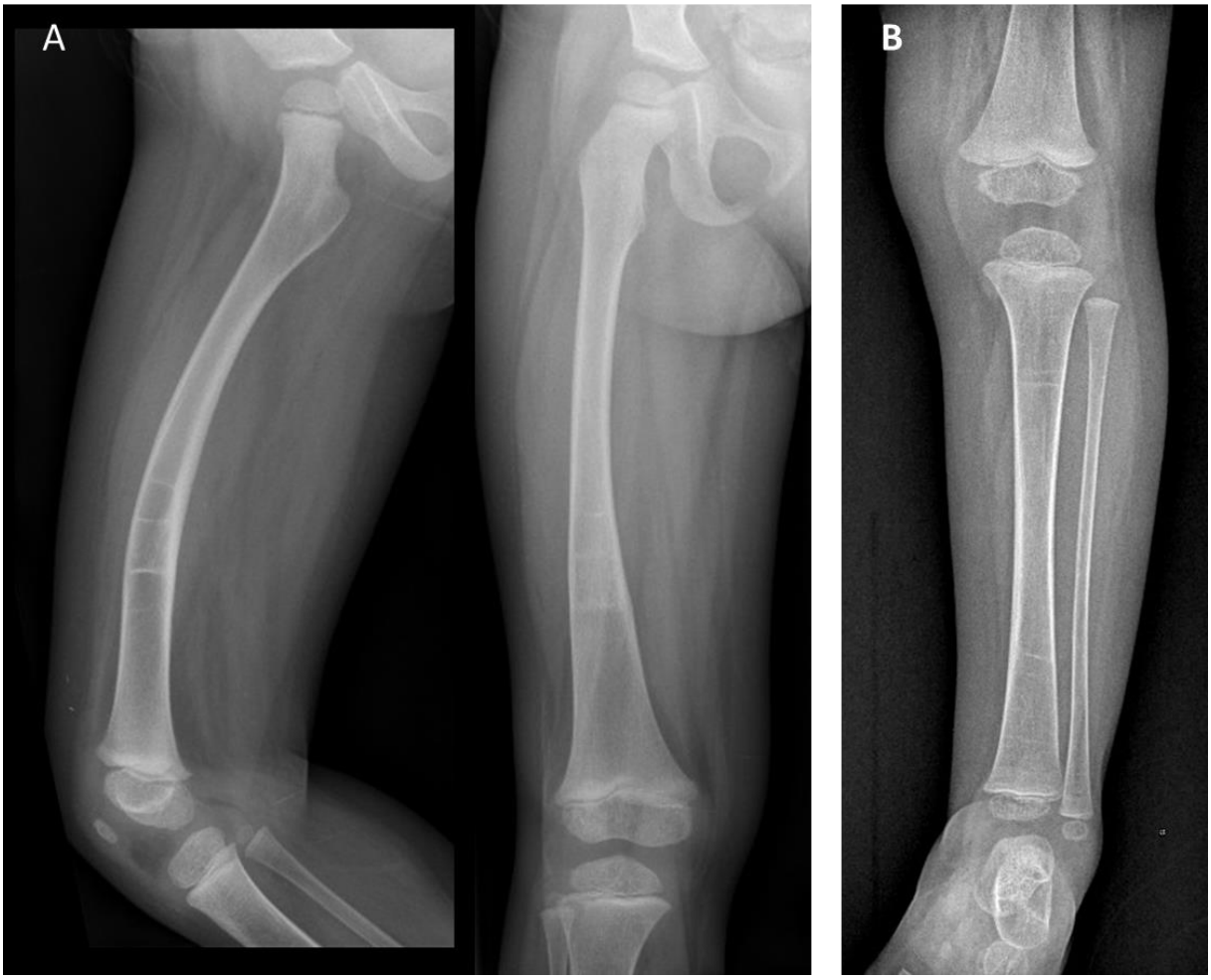

Figure S7: X-ray of Patient C.II.1 femur (A) and tibia (B) showing bone demineralization (2 years old)

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tr|Q567I0|Q567I0_DANRE             MSQDSSALREEGNNHFKAGDVQQALTCYTKALKISDCPSESAVLYRNRSACYLKLEDYTK
                                    ************************************************************

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                                    ********************************

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Figure S8: Protein sequence alignment of the 3 UNC45A isoforms from Danio Rerio :

ENSDART00000159409.2 (F1QU23), ENSDART00000171709.1 (A0A0R4IU52) and ENSDART00000163426.1 (Q567I0) CLUSTAL O(1.2.4) multiple sequence alignment.

Figure S9 : Protein sequence alignment of UNC45A (Q9H3U1.1) from homo sapiens and UNC45A from Danio Rerio (F1QU23) with CLUSTAL O(1.2.4) multiple sequence alignment

The mutations found in the Family A, B and C are indicated in blue, red and green respectively

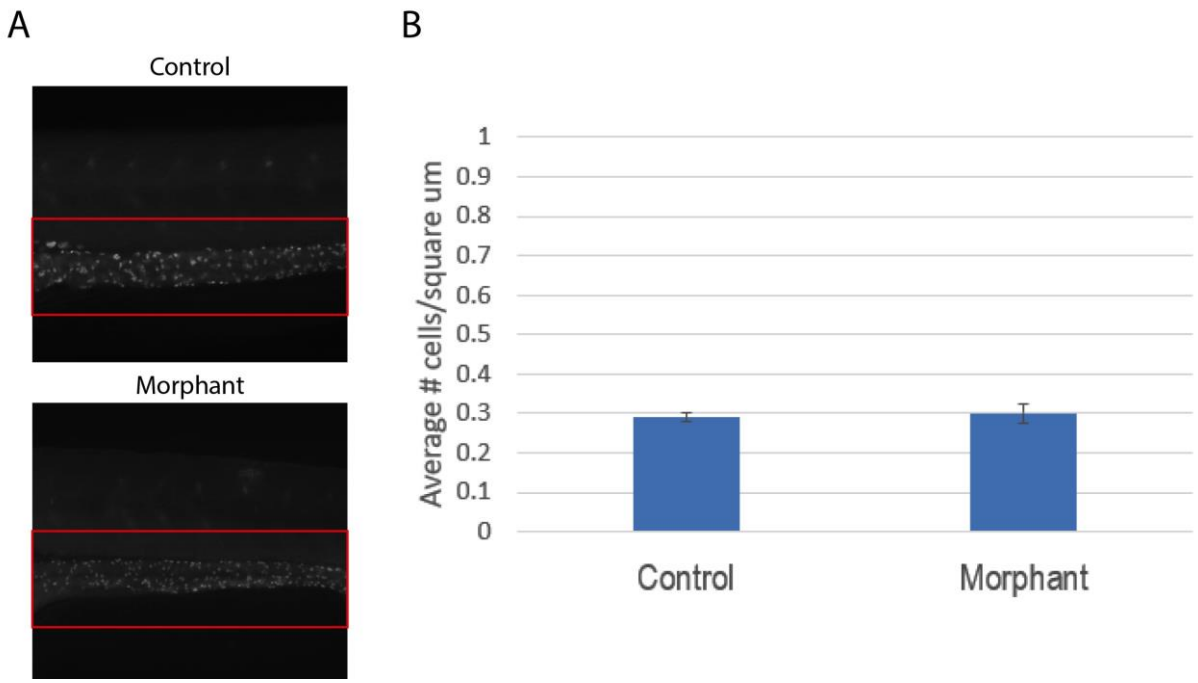

Figure S10: Enteric neuron analysis suggests that unc45a suppression does not impact neurodevelopment in the gut.

A. Antibody staining against HuC/D in whole mount zebrafish and B. the quantified the region of the number of neurons/square um in the gut (T-test p-value=0.418). Supplementary

Supplemental table

Individual	Exon	Mutation	gnomeAD frequency	UMD Predictor (score)	Polyphen (score)	SIFT
A.II.2	11	c.784C>T:p.Arg262*	3/277220	Pathogenic (100)		
	14	c.1268T>A:p.Val423Asp	NS	Pathogenic (99)	Probably damaging (0.996)	Affected Protein Function
B.II.3 ; B.II.4	23	c.2581C>T:pGln861*	NS	Pathogenic (100)		
	23	c.2633C>T:p.Ser878Leu	7/277162	Pathogenic (99)	Probably damaging (0.996)	Affected Protein Function
	23	c.2734T>G:p.Cys912Gly	NS			
C.II.1	8	c.247C>T:p.Arg83Trp	4/276948	Pathogenic (100)	Probably damaging (1)	Affected Protein Function
	13	c.983G>T:p.Gly328Val	NS	Pathogenic (100)	Probably damaging (1)	Affected Protein Function

Table S1 : *UNC45A* mutations and pathogenicity prediction

Mutations in *UNC45A*, gnomAD (genome Aggregation Database) frequency and prediction of the pathogenicity. (NS stand for Never Seen)

Supplemental References

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3. Criscuolo, A., and Gribaldo, S. (2010). BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. BMC evolutionary biology 10, 210.
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