Two mutations, D1152H and L997F, deserve further comment. Neonate 2B (table 2) carried, on different alleles, 1717-1G→A and D1152H. His IRT value at birth was unusually high, even for CF, and remained raised at the time of the sweat test as well; also, his sweat chloride level, even though under 40 mEq/kg, was the highest among the neonates under study. D1152H has been reported in association with isolated CBAVD, but also with mild, late onset lung disease and pancreatitis in conjunction with normal sweat values.[24]

Another peculiar result of the study was the finding on four occasions of L997F, a mutation usually rare in CF, but perhaps more common in idiopathic disseminated bronchiectasis,[12] as well as in idiopathic pancreatitis. Unpublished data from our group show that L997H was found in four cases from a subset of 32 subjects suffering from idiopathic pancreatitis, but in none of 100 ΔF508 carriers.

In conclusion, standard mutation panels can detect a high prevalence of *CFTR* mutations among subjects with neonatal hypertrypsinaemia and negative sweat chloride, but even more mutations can be found by a more thorough gene search. Close clinical follow up should help in clarifying the extent of the disease, if any, in compound heterozygous newborns.

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A heterozygous endothelin 3 mutation in Waardenburg-Hirschsprung disease: is there a dosage effect of *EDN3*/*EDNRB* gene mutations on neurocristopathy phenotypes?

EDITOR—Hirschsprung disease and Waardenburg syndrome are congenital malformations involving neural crest derivatives. Several genes are involved in these diseases, defining a complex pattern of inheritance. Hirschsprung disease (HSCR) is characterised by the absence of intramural ganglia in the distal bowel. This lack of enteric innervation results in intestinal obstruction or severe constipation. The incidence of HSCR is 1 per 5000 live births and both genetic and environmental factors are thought to contribute to the phenotype. The mode of inheritance is dominant in some families and recessive or multifactorial in others.[1] In a number of cases, mutations of the *RET* proto-oncogene, a tyrosine kinase receptor, result in a dominant disease with incomplete penetrance.[2–4] Mutations in the *RET* ligand *GDNF* (glial cell line derived neurotrophic factor) may also affect the phenotype.[5–7] A few patients with HSCR were found to have heterozygous mutations in the genes encoding the endothelin B receptor (*EDNRB*)[8–10] or its ligand endothelin 3 (*EDN3*).[11 12]

Waardenburg syndrome (WS) is characterised by a combination of sensorineural deafness and abnormal pigmentation, including a white forelock and eyelashes, heterochromia irides, and areas of skin depigmentation. Four subtypes of WS have been described on the basis of clinical features.[13] Types 1 and 3 and type 2 are associated with mutations in the *PAX3* and microphthalmia associated transcription factor (*MITF*) genes,[14] respectively. Patients with type 4 WS (WS4, Waardenburg-Hirschsprung disease or Shah-Waardenburg syndrome)

have features of both WS and HSCR.[15] Several WS4 subjects have homozygous *EDNRB* or *EDN3* gene mutations,[12 16–19] whereas other patients have heterozygous mutations in the gene encoding SOX10,[20] a transcription factor expressed in emerging neural crest cells.

Endothelins are a family of three vasoactive peptides recognised by two G protein coupled heptahelical receptors. Endothelin 3 preferentially binds the endothelin B receptor. Endothelin mRNAs are first translated into preproendothelin, which undergoes two step enzymatic cleavage that generates the active endothelin peptide[21 22] (fig 1). This peptide is composed of 21 amino acids, and contains four cysteines involved in two disulphide bonds. Targeted or spontaneous homozygous mutations of the *Ednrb* or *Edn3* gene in mice generate a strikingly similar phenotype, with white coat spotting and aganglionic megacolon,[24 25] suggesting that endothelin 3 is a physiological ligand for the endothelin B receptor. The phenotype is reminiscent of WS4 in humans, in whom homozygous mutations of *EDNRB* and *EDN3* were first described.[16–19] Heterozygous mutations were later reported in patients with isolated HSCR.[8–12] Following these observations, WS4 was described as a recessive condition, and isolated HSCR as a dominant disease with incomplete penetrance when the result of *EDN3* or *EDNRB* mutations.

However, contrary to this simple model, a homozygous *EDNRB* mutation was found in a patient with isolated HSCR[26] and a heterozygous mutation was identified in a patient with WS4, whose affected sibs only had features of WS.[27] *EDNRB* mutations manifest themselves in a more complex manner than previously believed. In their study of a large Mennonite family, Puffenberger *et al*[16] showed that both homozygotes and heterozygotes for a *EDNRB* mutation exhibited the intestinal phenotype, but with very different penetrance (21% in heterozygotes, 74% in homozygotes). They suggested that the *EDNRB* mutation found in this family was dosage sensitive and neither fully dominant nor fully recessive. This explanation for the variable penetrance in homozygotes and heterozygotes might also apply to features of WS. However, it would not predict whether a particular *EDNRB* mutation would be more strongly associated with HSCR or with WS, as modifier genes could also contribute to determining whether a heterozygote develops one syndrome or the other.

Similarly, heterozygous *EDN3* mutations have been identified in patients with HSCR and homozygous *EDN3* mutations in patients with WS4.[12 28] We report a novel *EDN3* mutation carried in the heterozygous state by a girl with WS4, showing that, like *EDNRB*, heterozygous *EDN3* mutations can result in either WS4 or isolated HSCR.

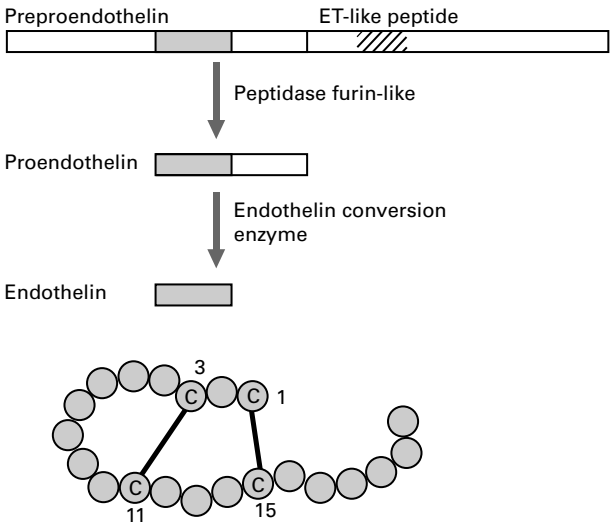

Figure 1 Enzymatic processing of preproendothelin into proendothelin (also termed "big endothelin") and into mature endothelin, according to Yanagisawa et al.[23]

The index case (III.2) was born in a well nourished state after a pregnancy marked by sonographic diagnosis of an intestinal obstruction at 33 weeks. The karyotype was normal (46,XX). Laparotomy on day 3 of life established the diagnosis of HSCR involving the colon and ileum. Multiple biopsy specimens of the distal ileum and colon showed no ganglion cells in the submucosa or intermuscular nerve plexuses and no increase in nerve fibres. An ileostomy in the dilated ileum failed to function and jejunostomy was carried out on day 15, 40 cm from the duodenum. The child required almost total parenteral nutrition. During the neonatal period the baby had a white forelock, which gradually disappeared over a period of months. Mild sensorineural hearing loss was diagnosed when she was 4 months old. Chronic intestinal infection with cholangitis and liver dysfunction occurred, together with several episodes of septicaemia requiring antibiotics (including aminoglycosides). Physical examination at 1 year of age showed areas of hypopigmentation on the hands, and an electrophysiological hearing test showed severe, bilateral, sensorineural hearing loss. Heterochromia of the irises and dystopia canthorum were absent. The child failed to thrive and liver failure necessitated liver and intestinal transplantation at the age of 5 years. She died six weeks later of septic shock.

Pregnancy III.3 (fig 2) was terminated at 29 weeks, in accordance with French law, after an intestinal obstruction was identified sonographically. Necropsy showed the same pattern of HSCR affecting the ileum and colon. There were no other discernible morphogenic defects. The mother (II.4) and father (II.5) are non-consanguineous. I.1, I.2, II.4, and III.1 (9 years old) are healthy. Their physical examination showed no malformations or dysmorphism. Their audiograms were normal. I.1 and I.2 are of Yugoslavian origin. II.2 was born at term in a well nourished state, but died in Yugoslavia in the neonatal period from congenital intestinal obstruction (no medical records are available). The father (II.5) and his family (of French origin) had no relevant history.

The coding sequences of the three genes involved in WS4 (*EDN3*, *EDNRB*, and *SOX10*) were screened by means of single strand conformation polymorphism (SSCP) analysis in the index case, as previously described[12 20] (the sequences of the *EDNRB* primers were kindly provided by J Amiel). A band shift was observed in a fragment corresponding to exon 3 of the *EDN3* gene. Sequencing of the variant fragment showed a heterozygous C→A transition, which introduces a stop codon at position 169 (fig 2B). This mutation, C169X, was inherited from the healthy mother (fig 2A) and was not found in 100 control chromosomes. As WS4 is classically considered to result from homozygous *EDN3* mutations, we investigated this family further.

The coding sequence of the *RET* proto-oncogene was screened for mutations on III.3 fetal DNA by SSCP analysis (exons 1 and 2) and denaturing gradient gel electrophoresis (DGGE) (all other exons) as previously described.[29] No causative substitutions or neutral variants were detected. Haplotypes inherited at the *RET* locus were reconstructed by genotyping the parents' DNA for six known intragenic polymorphisms in exons 2, 7, 11, 13, 14, and 15.[30 31] A single nucleotide polymorphism (SNP) of intron 19 and a microsatellite marker located 80 kb upstream of the *RET* gene (MS, unpublished results) were also analysed. The presence of an interstitial microdeletion

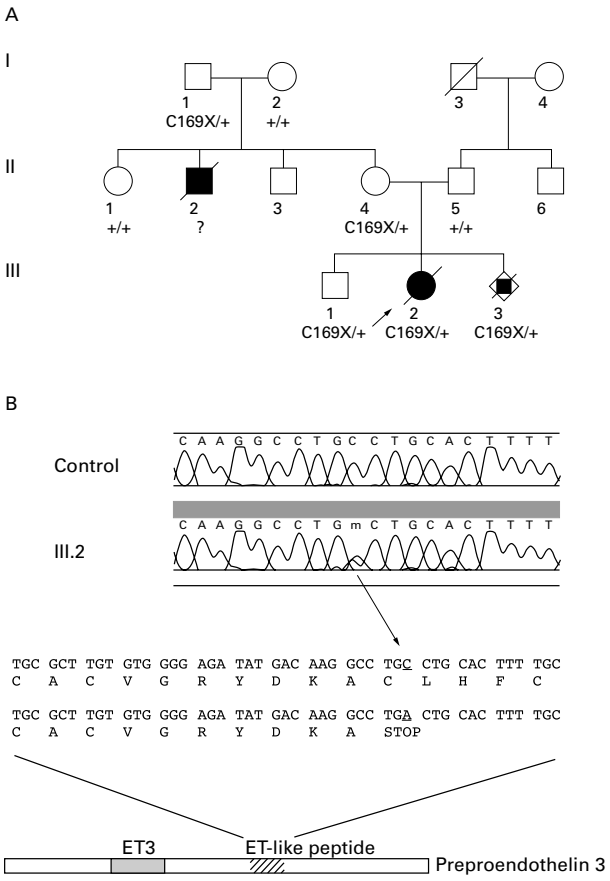

Figure 2 (A) Pedigree of the family. Subjects for whom a DNA sample was available are represented, along with the results of EDN3 genotyping. II.2 is thought to have been affected, as he died from neonatal intestinal obstruction, but no medical records are available. An intestinal obstruction was detected prenatally in the male fetus III.3 and the pregnancy was terminated. (B) The C169X mutation. Results of direct sequencing of EDN3 exon 3 from genomic DNA of a control (above) and the index case III2 (below); consequences of the mutation for preproendothelin 3 structure. The mature endothelin 3 peptide (ET3) is in grey and the ET-like peptide is striped.

was ruled out in the most proximal portion, but loss of heterozygosity (LOH) analysis was not fully informative for the rest of the gene.

Other mutations in the *EDN3*, *EDNRB*, and *SOX10* genes were sought by directly sequencing genomic DNA extracted from the girl's blood cells (III.2) and from a lymphoblastoid cell line established from III.3 fetal cells. No other mutation was found. The absence of partial deletion or rearrangement of the *EDN3* gene was shown by Southern blotting. The girl's (III.2) and control DNA was digested with *BamH*I or *Bcl*I, and human *EDN3* cDNA[32] was used as a probe (ATCC). Finally, to detect possible extinction of the normal *EDN3* allele in the girl, we amplified exon 3 and the junction with exon 4 by reverse transcription from the lymphoblastoid cell line RNA and nested PCR using the following conditions: 20 pmol of each primer, 1.5 mmol/l Mg, annealing temperature 55°C, 35 cycles. First round PCR primers: F 5'CGAACAGACGGTGCCCTATGGAC3', R 5'ATGAGCTTTGGATGGTGGAGGTC3'. Nested primers: F 5'GACTGTCCAACTACAGAGGAAGC3', R 5'CCTGCTTGCTTTGTTGGTCCTTG3'. The PCR products were analysed on 2% agarose gel before sequencing. Several amplification products corresponding to some of the previously described alternatively spliced mRNAs were observed.[32 33] The normal and mutated *EDN3* alleles could be amplified from both the mother and the girl (not shown).

The C169X mutation of the *EDN3* gene lies in a region of the distal preproendothelin called the ET-like peptide (fig 2B). This 15 amino acid peptide shows a very high degree of homology with the mature endothelin peptide, and also with the three preproendothelins from various species. In particular, the four cysteines are conserved. The ET-like peptide might play a role in the first enzymatic cleavage step. The absence of this first cleavage step impairs the final clipping step by endothelin conversion enzyme (ECE-1).[34] As a result, the C169X mutation, by substituting the third cysteine of the ET-like peptide, prevents the disulphide bonds and probably generates an inappropriately cleaved, inactive proendothelin. It is noteworthy that another of the three *EDN3* mutations described to date in WS4 disrupts the disulphide bonds of the ET-like peptide. This defect, C159F, described in the homozygous state,[18] modifies the first cysteine. A functional in vitro assay has been used to show that this mutation results in a virtual absence of the mature endothelin 3 product, supporting the hypothesis of impaired cleavage (Yanagisawa, cited in Hofstra *et al*[28]).

To date, three heterozygous *EDN3* gene mutations have been described in isolated HSCR, and three homozygous mutations have been observed in WS4. Interestingly, in one of the WS4 families, certain members who are heterozygous for the *EDN3* gene C159F mutation have one or more WS features but are free of megacolon.[18] This is incompatible with a recessive mode of WS inheritance and with a dominant mode of HSCR transmission. Another patient, with a congenital central hypoventilation syndrome (CCHS) but free of HSCR and pigmentation defects, carries a heterozygous *EDN3* frameshift mutation involving the carboxy-terminal region of the prepropeptide.[35] However, a functional in vitro test failed to show any effect of this mutation on preproendothelin processing, raising questions as to the deleterious nature of the mutation.

The aborted fetus III.3, which was heterozygous, also had severe intestinal disease, but the presence of WS features could not be assessed. The maternal grandfather and a healthy brother were also heterozygous. It is likely that a maternal uncle, who died at birth from intestinal obstruction, also carried the mutation. As in most other cases described, penetrance was incomplete. Two of the three heterozygous *EDN3* mutations so far identified in isolated HSCR were inherited from an asymptomatic mother,[12] while one was inherited from a mother with a mild intestinal phenotype.[11] Incomplete penetrance and phenotypic variability are frequent in neurocristopathies, particularly in HSCR. This could be explained by environmental factors, multigenic inheritance (see for example Bolk *et al*[36]), or modifier genes, or by stochastic events acting on cell fate or cell differentiation in early embryogenesis. The EDN3/EDNRB ligand/receptor interaction is essential for the development of two different cell lineages, melanocytes and enteric neurones, derived from the neural crest. It is unclear whether this interaction is required by early progenitors of both lineages or only after the lineages diverge. Differences in the chronological order and sites of emergence of distinct subsets of cells derived from the neural crest could partly account for the variable manifestations associated with *EDN3* and *EDNRB* mutations.

This characterisation of the *EDN3* C169X mutation shows that features of both WS and HSCR can result from a heterozygous *EDN3* mutation. One possible explanation for this observation includes multigenic inheritance. Indeed, involvement of an unidentified gene cannot be ruled out in this family. A mutation of the endothelin conversion enzyme gene (*ECE1*) has been described in a patient with a very particular phenotype, including cardiac defects, craniofacial abnormalities, other dysmorphic

features, and autonomic dysfunction,[37] which were not found in the family investigated here. Another possibility in keeping with our findings is a mode of inheritance which is not fully recessive and not fully dominant, with different penetrance in homozygotes and heterozygotes, as suggested for *EDNRB* mutations. Alternatively, the ET-like peptide mutations could have a particular mode of transmission and phenotypic expression.

This description of a heterozygous *EDN3* mutation in a severe case of Waardenburg-Hirschsprung disease underlines the difficulty in predicting the phenotypic manifestations of *EDN3* mutations. This situation complicates genetic counselling and requires care when assessing the recurrence risk in a family.

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The small patella syndrome: description of five cases from three families and examination of possible allelism with familial patella aplasia-hypoplasia and nail-patella syndrome

EDITOR—The small patella syndrome (SPS, *MIM 14789), also known as ischiopatellar dysplasia, coxopodo patellar syndrome, or Scott-Taor syndrome, is a rare autosomal dominant disorder, characterised by a/hypoplasia of the patellae and various anomalies of the pelvis and feet. This syndrome was first described by Scott and Taor[1] in 1979 in a large family with bilateral small or absent patellae accompanied by anomalies of the pelvic girdle and upper femora in most of the affected subjects. To our knowledge, 42 patients have been reported with this disorder,[1–9] comprising 35 cases from five families and seven sporadic cases. This bone dysplasia is characterised by patellar a/hypoplasia and pelvic anomalies, including bilateral absent or delayed ossification of the ischiopubic junction and infra-acetabular axe cut notches. Other major signs are a wide gap between the first and second toes, short fourth and fifth rays of the feet, and pes planus. Various other skeletal anomalies have been reported, such as elongated femoral necks, flattened and widened proximal femoral epiphyses, hypoplasia of the lesser trochanter, and tarsal anomalies. SPS should be clinically differentiated from disorders with a/hypoplastic patellae, in particular the autosomal dominant disorders isolated familial patella aplasia-hypoplasia (PTLAH) syndrome[10] and the more severe nail-patella syndrome (NPS).[11] The latter is caused by mutations of the *LMX1B* gene on chromosome 9q34. Recently, a locus for PTLAH has been identified on chromosome 17q21-22. As yet, it is unknown whether SPS and PTLAH are allelic disorders. Here we report on five cases from three families with SPS, compare their clinical and radiological anomalies with those of previously reported cases, and propose minimal diagnostic criteria for SPS. Given the clinical overlap between SPS, PTLAH, and NPS, we have studied the possible involvement of candidate regions for these syndromes on chromosome 17q21-22 and 9q34, respectively, by linkage analysis.

Family A, case 1. This male patient, aged 9 years 10 months at the time of examination, was referred because of bilateral absence of the patellae. He was the third child of non-consanguineous Dutch parents. He was born at 37 weeks' gestation after an uneventful pregnancy. Birth weight was 2750 g (10th-25th centile). At birth, talipes equinovarus was noted. Motor milestones were delayed; he sat at 13 months and walked at 24 months. Mental development was normal. At the age of 6 and 8 years, surgery for flat feet was performed, but without success. At the time of examination he complained of unstable knees, muscle weakness of the lower extremities, fatigue on moderate exertion, and inability to run or to stand up from sitting without support. At the age of 9 years 10 months, weight was 30.2 kg (10th-25th centile), height 146.5 (50th-90th centile), and head circumference 51.5 cm (10th-50th centile). The ears were low set and posteriorly angulated. He had a wide gap between the first and second toes bilaterally, short fourth and fifth rays of the feet, and pes planus (fig 1A). The patellae were not palpable. Normal

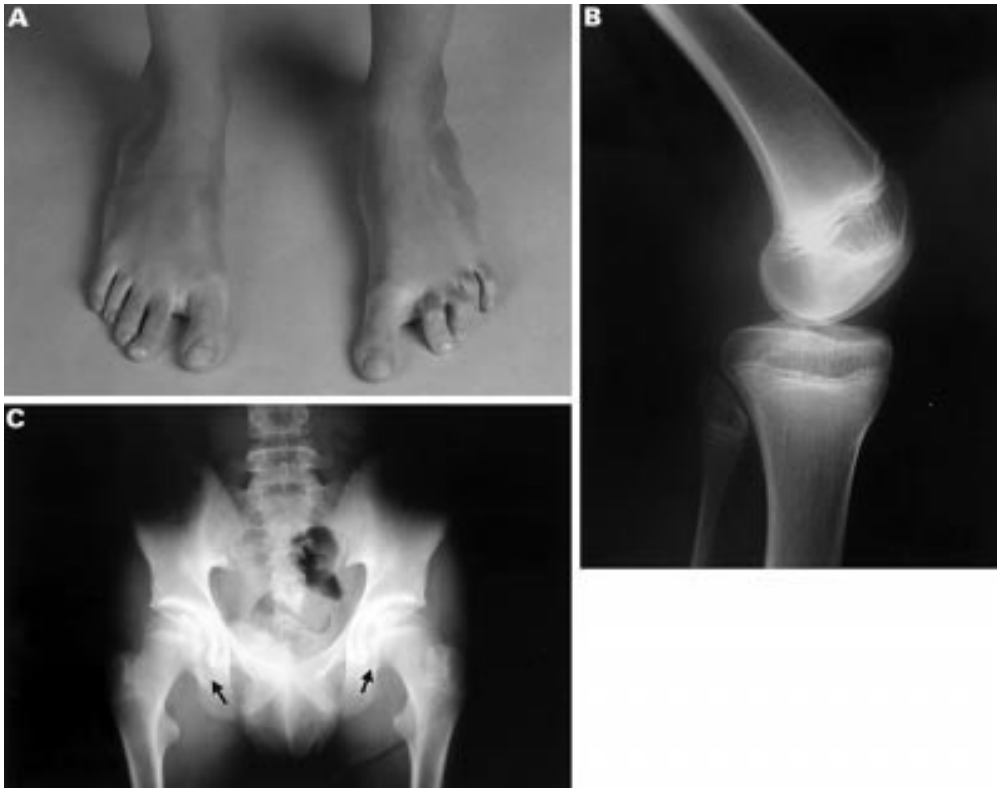

Figure 1 (A) Anterior view of the feet of the proband of family A (case 1) showing an increased space between the first and second toes and short fourth and fifth rays. (B) Radiograph of the knee at the age of 12 years 11 months. Note the absence of the patellae and dysplasia of the epiphyses of the proximal fibula. (C) Radiograph of the pelvis at the age of 12 years 11 months showing the absent ossification of the ischiopubic junction, infra-acetabular axe cut notches (arrows), and elongated femoral necks.