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"Genetics of gastrointestinal disease: dismotility disorders, intestinal polyposis and gene therapy"

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CONTENTS

CHAPTER 2.
LIST OF ORIGINAL PUBBLICATION

1. **Auricchio R,** De Rosa M, Quaglietta L, Miele E, Boccia G, Staiano A, Izzo P. A dramatic case of early-onset familial adenomatous polyposis. *Clin Genet.* 2005 Jan;67(1):104-6.

2. Gargiulo A, **Auricchio R,** Barone MV, Cotugno G, Reardon W, Milla PJ, Ballabio A, Ciccodicola A, Auricchio A. Filamin A is mutated in X-linked chronic idiopathic intestinal pseudo-obstruction with central nervous system involvement. *Am J Hum Genet.* 2007 Apr;80(4):751-8.

3. **Auricchio R,** Quaglietta L, Melis D, Miele E, Migliore C, Maresca I, Giannetti E, Tramontano A, Sebastio G, StaianoA. Clinical and genetic characterization of syndromic HSCR. *(paper in submittion)*

CHAPTER 2.
INTRODUCTION

Genetics in gastroenterology and clinical application.

Throughout clinical medicine, recent progress in molecular genetics has provided critical insight into disease pathogenesis, guiding basic research into previously unforeseen areas.

To better understand the relevance of genetic study in gastroenterology, first of all, we have to clarify the difference between monogenic disorders and complex diseases. The monogenic disorders are disease with mendelian inheritance,

Complex diseases are those with polygenic factors involved in their pathogenesis, such as Chron and celiac disease.

The analysis of monogenic disorders are most successful than complex disease. Possible strategies to identify candidate gene for monogenic gastrointestinal disorders could be based on functional or positional cloning. The first strategy starts from protein defect, the second one is based on the mapping of the gene in the genome and then in the identification of gene's function (Figure 1).

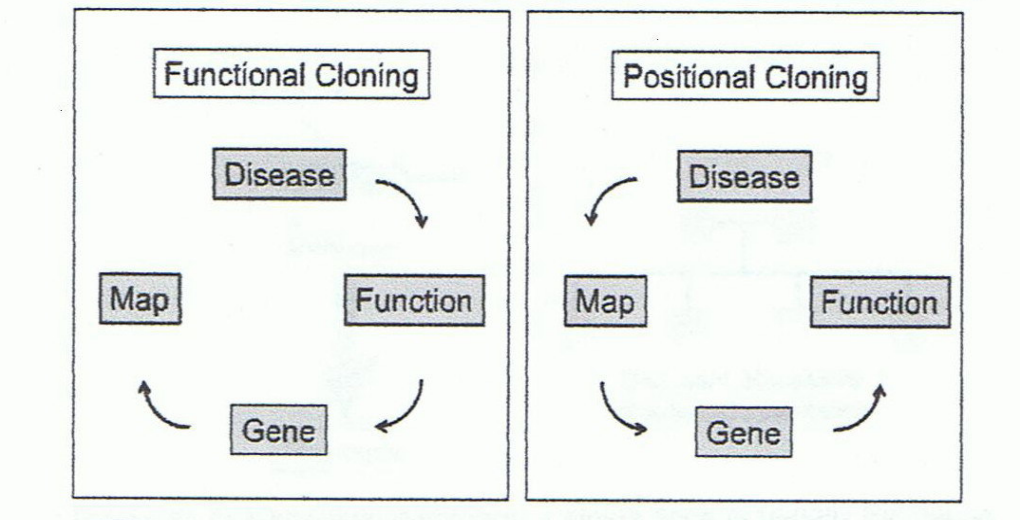

Figure 1. Possible strategies to identify gastrointestinal disease genes.

The identification of genes causing gastrointestinal disorders contribute to the understanding of disease's pathogenesis, the rule and the interaction between proteins, and to better classify diseases. Otherwise, such discoveries have raised physicians' and patients'expectations, particularly with respect to molecular genetic testing, but translating scientific progress to patient care needs careful evaluation. In specialist clinical genetics, protocols have been developed to ensure responsible molecular testing and detailed genetic counseling in fully penetrant single-gene disorders, however specialists in other disciplines, such as gastroenterology, perhaps remain less familiar with all of the complex issues surrounding genetic testing (Figure 2).

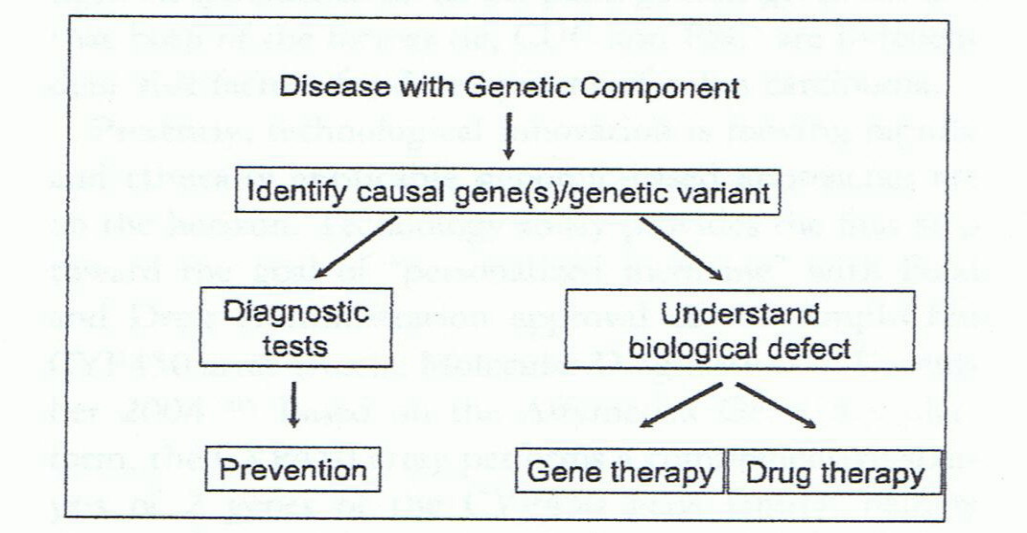

Figure 2. Impact of genomics in medical practice

Several criteria must be satisfied for any genetic test before its application in clinical medicine. The test should ideally address a reversible risk, and thereby lead to clinically useful outcome; be socially and ethically acceptable to the individuals undergoing testing; have sufficient sensitivity and specificity; and be implementable and cost effective.

In HNPCC and familial adenomatous polyposis (FAP), genetic testing not only carries the potential for identifying individuals or family members at risk, but also for directing the appropriate application of further intervention.

Finally, genetic discoveries could be relevant to address future treatment, such as gene therapy or drug therapy.

Aim of this thesis was to explore the genetic component of some gastrointestinal disorders, such as dismotility disorders (Hirschsprung disease, esophageal achalasia and congenital intestinal pseudo-obstruction) and intestinal polyposis, either by clinical study or by sequencing of candidate genes. Furthermore, my effort was addressed to develop a new strategy to stable trasduce intestine for future application such as gene therapy of gastrointestinal and no gastrointestional disorders.

CHAPTER 3.
GENETICS OF DISMOTILITY DISORDERS.

The enteric nervous system (ENS) is a collection of neurons in the wall of the entire gastrointestinal tract. It modulates and integrates the motility (1), microcirculation (2), secretion (3), and immune/inflammatory response (4). It consists in two major plexuses, the myenteric (Auerbach) and the submucosal (Meissner), otherwise associated neural elements connecting the ganglia and projecting to effector systems (muscles, interstitial cell of Cajal, glands, blood vessels and immune cells). The human ENS contains approximately as many neurons as the spinal cord (about 100 million) and is a specialized branch of the peripheral nervous system that can function independently from the central nervous system. The ENS is derived from specific axial levels of the neural crest (NC). Vagal, truncal and sacral NC cells colonize the gut in a rostral-to caudal progression during embryonic development. Truncal NC gives rise to ganglia in the esophagus and the proximal stomach, whereas the vagal NC gives rise to ganglia throughout the length of the gut. The entire colonization process is complete by approximately 13 week of gestation in humans. By the 24 weeks' gestation, infants seem to have a full complement of the ENS neurotrasmitters, and at least partial ENS function is established. However, new enteric nerve cells continue to differentiate

at least throughout the first couple of years of life, suggesting that the young ENS continues to develop and change. Normal ENS development requires survival of NC derived cells and their coordinated proliferation, movement and differentiation into neurons and glias. All of these processes are influenced by the microenvironment of the developing gut. Defects in the NC derived cells themselves or alterations in the gut microenvironment may result in abnormal development of the ENS, such as Hirschsprung's disease (HSCR), chronic intestinal pseudo-obstruction (CIP) and esophageal achalasia (EA). Although not yet fully elucidated, mutations in genes involved in these processes may also explain phenotypic variants of different motor disorder of the intestine, as those mentioned before, objects of this thesis.

3.1 HIRSCHSPRUNG'S DISEASE.

3.1.1 Definition, epidemiology, pathophysiology and clinics.

Hirschsprung's disease (OMIM 142623) is a heterogeneous genetic disorder, resulting from an anomaly of the enteric nervous system of neural crest cells origin, characterised by the absence of parasympathetic intrinsic ganglion cells in the submucosal and myenteric plexuses. It is regarded as the consequence of the premature arrest of the craniocaudal migration of vagal neural crest cells in the hindgut, between the fifth and twelfth week of gestation, to form the enteric nervous system and is therefore regarded as a neurocristopathy (5).

Hirschsprung's disease occurs in approximately 1 of each 5000 live births and with a male predominance of 4:1. It is generally sporadic, although in 3-7% of cases a genetic trasmission has been reported (5). The risk for short segment disease is 5% in brothers and 1% in sisters of index cases; for long segment disease the risk is 10%, regardless of sex. (6)

The embryonic disorder is a lack of the craniocaudal migration, differentiation and maturation of neuroblasts from the neural crests, the earlier the migration ceases, the longer the aganglionic segment will

be. The aganglionic segment is permanently contracted, causing dilatation proximal to it (7).

Hirschsprung's disease may be classified according to the lenght of the aganglionic segment: the classic form (short segment 70-75% of cases) is limited to the rectum and sigmoid colon; the long segment, or subtotal colonic disease (10-15%), generally involves the bowel up to the splenic flexure; total colonic aganglionosis (TCA: 3-6%) may extend to involve a variable amount of the short bowel. Total intestinal aganglionosis sometimes is associated with intestinal malrotation or volvulus (8). Ultrashort segment aganglionosis is considered a functional alteration, without any detectable histological finding. Although longer aganglionic segments tend to produce more drammatic symptoms, some patients with even short segment disease deteriorate rapidly (9).

The hallmark of diagnosis is the absence of ganglion cells from the myenteric and submucosal plexuses, as seen on a full-tickness or suction (mucosal-submucosal) biopsy of the rectum. Proximal contents fail to enter the unrelaxed, aganglionic segment. The lack of non-adrenergic-non cholinergic inhibitory innervation is responsible for a tonic contraction of the affected segment, with absence of peristalsis and dilatation of the gut proximally. (10)

Morphologically, ganglion cells are absent from the narrowed segment and for some distance (1 to 5 cm usually) into the dilated segment. The pattern of nerve fibers is abnormal also; they are hypertrophic with abundant, tickned bundles. Specific stains for acetylcholinesterase are used to highlight the abnormal morphology (11, 12).

In the last years new insights in the pathophysiology of Hirschsprung's disease have been given. There is a growing and emerging body of evidence that suggests that the disease can be the expression of a genetic alteration, as reported later on.

In the newborn, symptoms may appear during the first hours of life with failure to pass meconium, or in the first week with a picture of functional intestinal obstruction. However, the delay in the passage of meconium does not always occur and a percentage of children still present late or with complications despite a history of complications since birth. Enterocolitis, the commonest complication, is always severe and is an important cause of mortality in these young patients.

In infants and children the presentation is often less drammatic and may not mimic acute intestinal obstruction. Severe constipation and recurrent fecal impaction are more common. Physical examination reveals a distended abdomen and a contracted anal sphincter and rectum in most children. The rectum is devoid of stool except in cases of short-segment aganglionosis. As the finger is withdrawn, there may

be an explosive discharge of foul-smelling liquid stools, with decompression of the proximal normal bowel.

Abdominal radiographs show intestinal "cut-off" sign in rectosigmoid region with absence of air distally. Other common findings are small bowel dilatation in 74% and multiple air-fluid levels. (13) . Because of the risk of perforation, contrast enema should not be performed in the presence of clinical enterocolitis.

Rectal washouts should be the initial approach in the care of a child, regardless of age, who presents with enterocolitis. Along with washouts, intravenous antibiotics or oral metronidazole (in mild cases) should be used. Should the disease process fail to improve or the infants's condition deteriorate, the performance of a leveling colostomy should be considered (14,15).

When the history (early onset of constipation, absence of faecal soiling) and/or the physical examination (empty rectal ampulla) suggest an organic cause, anorectal manometry should be performed.

Anorectal manometry evaluates the response of the internal anal sphincter to inflation of a ballon in rectal ampulla (16). When the rectal ballon is inflated, there is normally a reflex relaxation of the sphincter. The rectoanal inhibitory reflex is absent in patients with Hirschsprung's disease; there is no relaxation, or there are may even be

paradoxical contraction of the internal anal sphincter. The anorectal manometry is particulary useful when the aganglionic segment is short and results of radiologic or pathologic studies are equivocal.

Barium enema is helpful in the assessment of a transition zone between aganglionic and ganglionic bowel and in giving an estimation of the lenght of an aganglionic segment. Demonstration of the transition zone is easier if no effort is made to clearance the bowel. In the newborn, dilatation of the proximal ganglionic bowel may not have developed and radiological diagnosis may be more difficult. The sensivity and specifity for recognition of a transition zone have been reported to be 80% and 76%, respectively (17). The barium enema may not show a transition zone in cases of total colonic Hirschsprung's disease, or may be indistinguishable from cases of functional constipation when ultra-short-segment Hirschsprung's disease is present.

Nevertheless, the diagnosis is based on histological evidence. Since the mid 1970s, demonstration of acetylcholinestease activity in mucosal biopsies has allowed the non invasive suction rectal biopsy thecnique to become the most reliable diagnostic method for aganglionosis (11,12).

The treatment of Hirschsprung's disease is to resect the aganglionic segment of the rectum and colon, pull down normally

innervated bowel, and anastomose this bowel at the anorectal region, while preserving the sphincter muscle. Recently minimally invasive approaches to the 1-stage pull-through have became popular. The one-stage approach, even in the newborn period, either by laparotomy or by combined laparoscopy and transanal dissection, has been advocated.

Some patients with Hirschsprung's disease continue to have problems postoperatively, this may be because of residual disease or association with neuronal dysplasia. If the definitive operation fails because of an impassable stricture, disruption or residual disease, further secondary surgery may be necessary and a different operation may then lead to a reasonable result.

3.1.2 Genetics of Hirschsprung's disease.

HSCR occurs as an isolated trait in 70% of patients, is associated with chromosomal abnormality in 12% of cases, trisomy 21 being by far the most frequent (>90%). Additional congenital anomalies are found in 18% of cases, including gastrointestinal malformation, cleft palate, polydactyly, cardiac septal defects and craniofacial anomalies. The higher rate of associated anomalies in familial cases than in isolated cases (39% vs 21%) strongly suggests syndromes with Mendelian inheritance (18). Isolated HSCR appears to be a multifactorial malformation with low, sex dependent penetrance,

variable expression according to the length of the aganglionic segment, and suggesting the involvement of one or more gene(s) with low penetrance (19). These parameters must be taken into account for accurate evaluation of the recurrence risk in relatives. Segregation analyses suggested an oligogenic mode of inheritance in isolated HSCR. With a relative risk of recurrence as high as 200, HSCR is an excellent model for the approach to common multifactorial diseases.

A large number of chromosomal anomalies have been described in HSCR patients. Free trisomy 21 (Down syndrome) is by far the most frequent, involving 2-10% of ascertained HSCR cases. Syndromes associated with HSCR can be classified as: (1) pleiotropic neurocristopathies, (2) syndromes with HSCR as a mandatory feature, (3) occasional association with recognisable syndromes.

The neural crest is a transient and multipotent embryonic structure that gives rise to neuronal, endocrine and paraendocrine, craniofacial, conotruncal heart, and pigmentary tissues. Neurocristopathies encompass tumours, malformations, and single or multifocal abnormalities of tissues mentioned above in various combinations. Multiple endocrine neoplasia type 2 (MEN 2), and Waardenburg syndrome are the most frequent neurocristopathies associated with HSCR (20).

Waardenburg syndrome (WS), an autosomal recessive condition, is by far the most frequent condition combining pigmentary anomalies and sensorineural deafness, resulting from the absence of melanocytes of the skin and the stria vascularis of the cochlea. The combination of HSCR with WS defines the WS4 type (Shah-Waardenburg syndrome). Indeed, mutations of the endothelin pathway and *SOX10* mutations have been identified in WS4 patients with CNS involvement including seizures, ataxia, and demyelinating peripheral and central neuropathies (21).

Eight genes are known to be involved in HSCR in humans, those encoding for the proto-oncogene *RET* (*RET*), glial cell line derived neurotrophic factor (*GDNF*), neurturin (*NTN*), endothelin B receptor (*EDNRB)*, endothelin 3 (*EDN3)*, endothelin converting enzyme 1 (*ECE1*), *SOX10*, and *SIP1* genes. RET and EDNRB signalling pathways were considered biochemically independent. However, an HSCR patient heterozygous for weak hypomorphic mutations in both *RET* and *EDNRB* has recently been reported (22). Each mutation was inherited from a healthy parent. SOX 10, otherwise, is involved in cell lineage determination and could be responsible of the reduced expression of EDNRB in the *dom* mouse.

The last de novo mouse model for the human WS4 is *dominant megalon* (*Dom*). The *Dom* gene is *Sox10*, a member of the SRY (sex

determining factor)-like, high mobility group (HMG) DNA binding proteins. Subsequently, heterozygous *SOX10* mutations have been identified in familial and isolated patients with WS4 (including de novo mutation) with high penetrance (23).

3.1.3 Aim of the project.

Aim of our study was first of all to clinically characterize the Hirschsprung disease in children trying to identify new syndromic phenotype associated with HSCR; secondly to make a mutational analysis of some genes (SOX10 and ZFX1B) already involved in syndromic HSCR and to make a possible phenotype-genotype correlation. My personal contribution was on clinical examination of HSCR patients.

3.1.4 Paper in submission.

Clinical and genetic characterization of syndromic HSCR.

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Introduction.

Hirschsprung disease (HSCR) is a congenital malformation characterized by the absence of neurons in the distal colon with symptoms ranging from neonatal intestinal obstruction to chronic constipation[1]. HSCR is attributed to the failure of neural crest cells to completely colonize the intestinal tract during the 4th to 12th weeks of gestation, and hence is termed a neurocristopathy[2]. The absence of the intramural ganglion cells is at level of both the myenteric and submucosal plexuses . Patients can be classified as rectosigmoid HSCR (RS, 78% cases) when the aganglionic segment does not extend beyond the upper sigmoid, long segment HSCR (LS, 32% of cases), when aganglionosis extends proximal to the sigmoid and total colonic aganglionosis (TCA, 3-8% of cases), when aganglionosis extends within 30 cm proximal to the terminal ileum[3]. In most cases, the diagnosis of HSCR is made in the newborn period owing to intestinal obstruction, such as failure to pass meconium within the first 48 hr of life, abdominal distension, vomiting[4, 5]. Some patients are diagnosed later with severe constipation and/or diarrhea, chronic abdominal distension, vomiting, and failure to thrive[4]. The diagnosis of HSCR is based on the absence of relaxation of internal anal

sphincter, after the 12th day of life, revealed by the anorectal manometry[6]. Suction rectal biopsy confirms the diagnosis in most cases, but a full thickness rectal biopsy is needed for diagnosis of HSCR[7]. The following criteria, obtained on suction rectal biopsy specimens, represent the gold standard of the diagnosis of HSCR: the absence of ganglion cell, replaced by hypertrophic nerves with no network. These hypertrophic nerves are responsible for increased acelylcholinesterase in the aganglionic bowel[8, 9]. The incidence of HSCR is estimated at 1/5000 live births with a 4:1 male predominance[10]. HSCR occurs as an isolated trait in 70% of cases[11]. A chromosomal abnormality is associated with it in 12% of cases, trisomy 21 being far the most frequent (>90%)[11]. Associated congenital anomalies have been found in 18% of HSCR patients[11]. In many cases of HSCR associations, neurocristopathies and syndromes, the underlying genetic links are already known or suspected.

Because it is generally accepted that the pattern of associated lesions may throw some light on possible genetic associations of the disease[12, 13], aim of our study was to clinically characterize the Hirschsprung's disease in children, to make a mutational analysis of some genes (SOX10 and ZFX1B) already involved in syndromic HSCR and to correlate phenotype to genotype.

Patients and methods.

Patients and clinical evaluation.

From January 2004 and June 2005 we have enrolled 48 HSCR patients (mean age.....), diagnosed by the absence of the anorectal inhibitory reflex revealed by the manometry and by the presence of the two histological criteria, absence of ganglion cells and presence of acetilcholinesterase fibers, on a suction rectal biopsies. A complete clinical evaluation was performed by using a specific questionnaire for the anamnesis, collecting data on time of beginning of symptoms, type of symptomatology, length of the aganglionic segment, familial anamnesis, symptoms associated. To get a better clinical characterization all the patients underwent an oculistic, audiometric, orthopedic

and neurological examination and, after parental consens, a blood sample was obtained for karyotype and DNA extraction for genetic analysis.

Anorectal manometry.

This test was carried out without sedation and after complete clearance of the lower bowel. A Dent sleeve catheter with at least three silicon lumens with side holes (outside diameter, approximately 2.0 mm), perfused with a low compliance pneumohydraulic system at a rate of 0.5 ml/min, was used. A latex balloon (2.5 cm x 3.0 cm) was attached to the end of a thin silicon lumen and tied to the tip of the motility probe, 5 cm above the distal transducer. Pressures from the latex balloon and from the side holes were transmitted via the silicon tubes to pressure transducers. The outputs for all transducers were fed into a poligraph (MMS UPS-2020 Medical measurement system for investigation of gastrointestinal motility) and graphed on running paper. The anorectal pressure profile and the effect of rectal distention were evaluated. The response of the anal canal to rectal distention was evaluated with the base of the distending balloon 11 cm above the anal verge, one pressure transducer in the rectum 6 cm above the anal verge, and another in the anal canal 1 cm above the anal verge. With sudden rectal distention, in normal children, the external sphincter contracts and the internal anal sphincter relaxes. This relaxation of the internal sphincter results in reduced anal pressure. This phenomenon is referred to as anorectal inhibitory reflex, and it is absent in HSCR.

Suction rectal biopsy.

In all patients, diagnosis of HSCR was based on both hematoxylin-eosin (H&E) and histochemical acetylcolinesterase (AchE) staining on two suction rectal biopsies performed by Noblett capsule. Suction rectal biopsies were taken at least 2 cm above the pectinate line and were kept moist on saline-soaked filter paper in a small Petri dish. One biopsy was embedded in O.C.T. (Bio-Optica), snap frozen for cryostat sectioning in a plane perpendicular to the mucosal surface, and stored at -

80°C. Ten micrometer-thick sections, oriented to obtain longitudinal sections, were cut at -23°C with a cryostat microtome (Cryostat HM560MU), until submucosa was visible, then mounted on slides and stained with H&E and AchE stainings. In the AChE staining iso-OMPA was used instead of OMPA. The other biopsy, kept in formalin, was wax-embedded, cut and stained with H&E. Histochemical criteria to establish HSCR diagnosis include: absence of ganglion cells in sub-mucosa, presence of nerve trunks in sub-mucosa, increased AChE-positive fibers in muscularis mucosae and sub-mucosa.

Analysis of sequences of the genes SOX10, ZFX1B (fare correggere a Sebastio)

Specific primers for SOX10 not available from the literature, were designed in the intronic regions in order to amplify, by PCR on genomic DNA, both the coding regions and the intron-exon junctions. These primers were designed by the analysis of the genomic sequences of the two genes deposited in Genbank and optimized for a single condition of amplification. Briefly: denaturation for 1 min at 94°C, hybridization for 1 min. at 52°C, elongation for 1 min. at 72°C. The primers for the amplification of the exonic regions and of the intron-exon junctions of ZFX1B were already reported[11, 14]. The PCR products were purified and direct sequenced, on both directions, using an ABI PRISM Dye Terminator Cycle DNA sequencing Kit and an automatic sequencer (ABI PRISM 377, Applied Biosystems). Primers used for the amplification are available on request.

Results.

We included in our study patients, who received the diagnosis of HSCR based on absence of the anorectal inhibitory reflex revealed by the manometry and on the presence of the two histological criteria, absence of ganglion cells and presence of acetilcholinesterase fibers, on a suction rectal biopsies. From January 2004 to June 2005 we enrolled 48 HSCR patients. The oculistic examination was performed in 43/48 patients, the audiometric exam in 37/48 patients. The orthopaedic evaluation was done in twelve patients and the neurologic visit in only patient. The

karyotype analysis was performed in 39/48 patients, the DNA was extracted by 46/48 patients and from 6 pairs of parents in the familiar and syndromic cases. We found in our population 10 cases of familial HSCR (20.8%) in 5 different families. One of these families have 3 subjects affected by Shah-Waandenburg's syndrome. In the remaining patients, 21 were sporadic HSCR (43.75%), 3 (6.25%) are Down's patients and 15 (31.25%) had one or more anomalies associated with HSCR. In particular, skeletal-muscle-limb anomalies were found in 20.3%, dysmorphysms in 25%, skin and integumentary and urogenital anomalies in 6.25%, RPSM, cardiac and gastrointestinal anomalies in 4.1% and ophthalmic and hearing problems in 2.08%, (Fig.1). We have also found that the longest traits of aganglionic colon have been found in patients with HSCR associated with other anomalies more frequently than in simple sporadic HSCR (39% vs 6%) (Fig.2, panel A and panel B). Five patients on 15 with HSCR with associated anomalies have an affected familiar (33%), as already reported in the literature. This means that the mendelian transmission is confirmed. Sequencing of the coding regions and the exon-intron boundaries of ZFX1B did not show nucleotidic changes, whereas analysis of the SOX10 gene revealed 2 polymorphisms, C566A in exon 3 (Gly188GLy), T1205C in exon 5 (His401His), respectively, and a nucleotide substitution G2154A in the 3' untranslated region.

Discussion.

Associated congenital anomalies occur in at least 5-32% of HSCR patients. These associations are of significance due to at least two reasons: firstly, the majority may be attributed to abnormal genetic development signalling, yielding clues as to the genetic background of HSCR and its pathogenesis and, secondly, the influence of associated anomalies on the long-term prognosis. Essentially, HSCR is caused by defects at a molecular level of normal signalling during enteric nervous system's development. As a consequence, the cues controlling the migration of the neural crest cells go awry resulting in aganglionosis of the distal bowel. The disorder is complex, as is shown by the number of genes implicated in its pathogenesis (at least 11). The pattern of conditions

associated with HSCR have already been of great value in revealing many of the genetic associations of the disease (Spounge, 1985) with known genetic variations being identified in at least 12% of HSCR cases (Bolk S 2000, Carrasquillo MM 2002) which is higher than what is expected in the normal population. In addition, these genetic variations are associated with more than 50% of the observed abnormalities associated with HSCR. On the other hand, it must be borne in mind that certain observed associations may not be higher in HSCR than in general population and may have little to do with HSCR per se. By looking at associated birth defects of a genetic condition, information can be gleaned not only on how the genes control development work but also on how they interact and crosstalk through gene-gene interaction whilst remaining genetically distinct. The incidence of associated congenital anomalies has been noted to be relatively low in a number of series and are interestingly enough, not frequently associated with familial transmission (Moore SW 1991, Brown RA 2000).

In our study, the associated physical anomalies were less frequent in the familial than in the sporadic HSCR (33% vs 67%). Individual anomalies vary in incidence from 2.08% to 25% (Fig.1), the most frequent being dysmorphysms and skeletal-muscle-limb anomalies (25 and 20.3%, respectively). Less frequent are genitourinary and skin anomalies (6.25%) closely followed by those related to heart, central nervous system and bowel (4.1%). Although ocular and hearing abnormalities are important, they represent only the 2.08%.

Possible genetic associations for HSCR disease could be hypothesized considering that major RET mutations may give rise to HSCR by haploinsufficiency (20-25% of cases), the majority of HSCR probably arise from the multiplicative combined effects of other susceptibility genes and mutations (11) and the final phenotypic expression seems to depend on the combined cumulative effects of the susceptibility loci at critical genes that control the mechanisms of cell proliferation, differentiation and maturation (Mc Callion AM, 2003). Study of known critical sites should include the potential modifying role of significant genes flanking the critical areas which may affect the final phenotypic expression of HSCR. Our study on the anomalies associated with HSCR can contribute much

towards the identification of candidate genes and the underlying of complex mechanisms of cell proliferation, differentiation and maturation of enteric neuroblasts and of the pathogenesis of HSCR.

As an important consequence, assessment of all HSCR patients should include a careful clinical and genetic evaluation.

Bibliography

1. Puri P, Ohshiro K, Wester T. Hirschsprung's disease: a search for etiology. *Semin Pediatr Surg* 1998;7:140-7.
2. Taraviras S, Pachnis V. Development of the mammalian enteric nervous system. *Curr Opin Genet Dev* 1999;9:321-7.
3. Burns AJ, Douarin N.M. *Development,* 1998.
4. Klein MD, Coran AG, Wesley JR, Drongowski RA. Hirschsprung's disease in the newborn. *J Pediatr Surg* 1984;19:370-4.
5. Jung PM. Hirschsprung's disease: one surgeon's experience in one institution. *J Pediatr Surg* 1995;30:646-51.
6. Emir H, Akman M, Sarimurat N, Kilic N, Erdogan E, Soylet Y. Anorectal manometry during the neonatal period: its specificity in the diagnosis of Hirschsprung's disease. *Eur J Pediatr Surg* 1999;9:101-3.
7. Kurer MH, Lawson JO, Pambakian H. Suction biopsy in Hirschsprung's disease. *Arch Dis Child* 1986;61:83-4.
8. Meier-Ruge W, Lutterbeck PM, Herzog B, Morger R, Moser R, Scharli A. Acetylcholinesterase activity in suction biopsies of the rectum in the diagnosis of Hirschsprung's disease. *J Pediatr Surg* 1972;7:11-7.
9. Tam PK, Boyd GP. Origin, course, and endings of abnormal enteric nerve fibres in Hirschsprung's disease defined by whole-mount immunohistochemistry. *J Pediatr Surg* 1990;25:457-61.
10. Badner JA, Sieber WK, Garver KL, Chakravarti A. A genetic study of Hirschsprung disease. *Am J Hum Genet* 1990;46:568-80.
11. Amiel J, Lyonnet S. Hirschsprung disease, associated syndromes, and genetics: a review. *J Med Genet* 2001;38:729-39.

12. Spouge D, Baird PA. Hirschsprung disease in a large birth cohort. *Teratology* 1985;32:171-7.

13. Cohen IT, Gadd MA. Hirschsprung's disease in a kindred: a possible clue to the genetics of the disease. *J Pediatr Surg* 1982;17:632-4.

14. Goldberg RB, Shprintzen RJ. Hirschsprung megacolon and cleft palate in two sibs. *J Craniofac Genet Dev Biol* 1981;1:185-9.

15. AS Brooks MB, C Meijers. Spectrum of phenotypes associated with Hirscsprung disease: an evaluation of 239 patients from a single institution., In The Third International Meeting: Hirschsprung disease and related neurocristopathies., Evian, France, 1998.

16. Bodian M CC. A family study of Hirschsprung disease. *Ann Hum Genet* 1963;26:261.

17. Hofstra RM, Osinga J, Buys CH. Mutations in Hirschsprung disease: when does a mutation contribute to the phenotype. *Eur J Hum Genet* 1997;5:180-5.

18. Shah KN, Dalal SJ, Desai MP, Sheth PN, Joshi NC, Ambani LM. White forelock, pigmentary disorder of irides, and long segment Hirschsprung disease: possible variant of Waardenburg syndrome. *J Pediatr* 1981;99:432-5.

19. Sham MH, Lui VC, Chen BL, Fu M, Tam PK. Novel mutations of SOX10 suggest a dominant negative role in Waardenburg-Shah syndrome. J Med Genet 2001;38:E30.

20. Shim WK, Derieg M, Powell BR, Hsia YE. Near-total intestinal aganglionosis in the Waardenburg-Shah syndrome. *J Pediatr Surg* 1999;34:1853-5.

21. Southard-Smith EM, Angrist M, Ellison JS, Agarwala R, Baxevanis AD, Chakravarti A, Pavan WJ. The Sox10(Dom) mouse: modeling the genetic variation of Waardenburg-Shah (WS4) syndrome. *Genome Res* 1999;9:215-25.

22. Pingault V, Bondurand N, Kuhlbrodt K, Goerich DE, Prehu MO, Puliti A, Herbarth B, Hermans-Borgmeyer I, Legius E, Matthijs G, Amiel J, Lyonnet S, Ceccherini I, Romeo G, Smith JC, Read AP, Wegner M, Goossens M. SOX10 mutations in patients with Waardenburg-Hirschsprung disease. *Nat Genet* 1998;18:171-3.

23. Fryer AE. Goldberg-Shprintzen syndrome: report of a new family and review of the literature. *Clin Dysmorphol* 1998;7:97-101.

24. Mowat DR, Croaker GD, Cass DT, Kerr BA, Chaitow J, Ades LC, Chia NL, Wilson MJ. Hirschsprung disease, microcephaly, mental retardation, and characteristic facial features: delineation of a new syndrome and identification of a locus at chromosome 2q22-q23. *J Med Gene*t 1998;35:617-23.

25. Zweier C, Albrecht B, Mitulla B, Behrens R, Beese M, Gillessen-Kaesbach G, Rott HD, Rauch A. "Mowat-Wilson" syndrome with and without Hirschsprung disease is a distinct, recognizable multiple congenital anomalies-mental retardation syndrome caused by mutations in the zinc finger homeo box 1B gene. *Am J Med Genet* 2002;108:177-81.

26. Garavelli L, Donadio A, Zanacca C, Banchini G, Della Giustina E, Bertani G, Albertini G, Del Rossi C, Zweier C, Rauch A, Zollino M, Neri G. Hirschsprung disease, mental retardation, characteristic facial features, and mutation in the gene ZFHX1B (SIP1): confirmation of the Mowat-Wilson syndrome. *Am J Med Genet* A 2003;116:385-8.

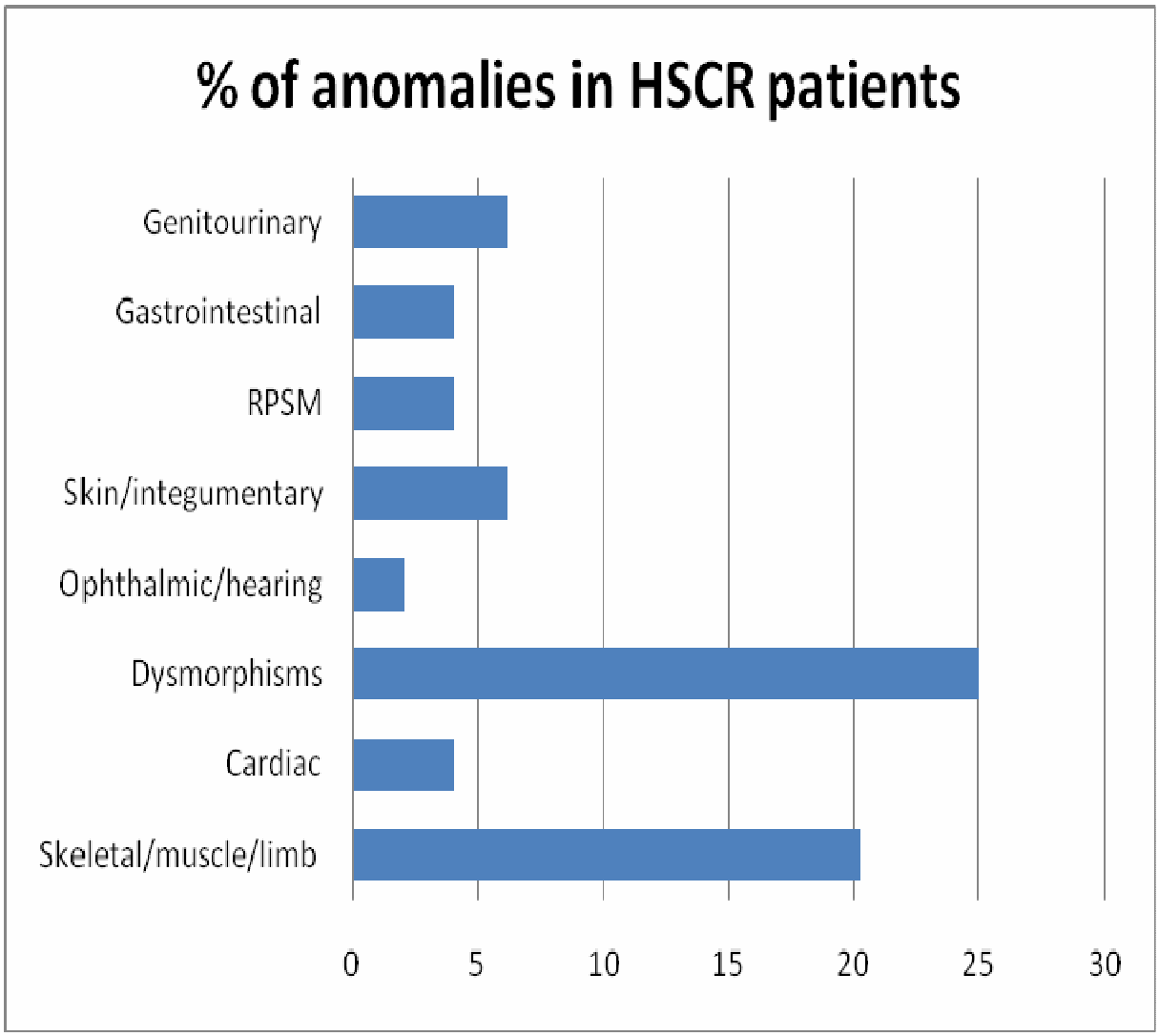
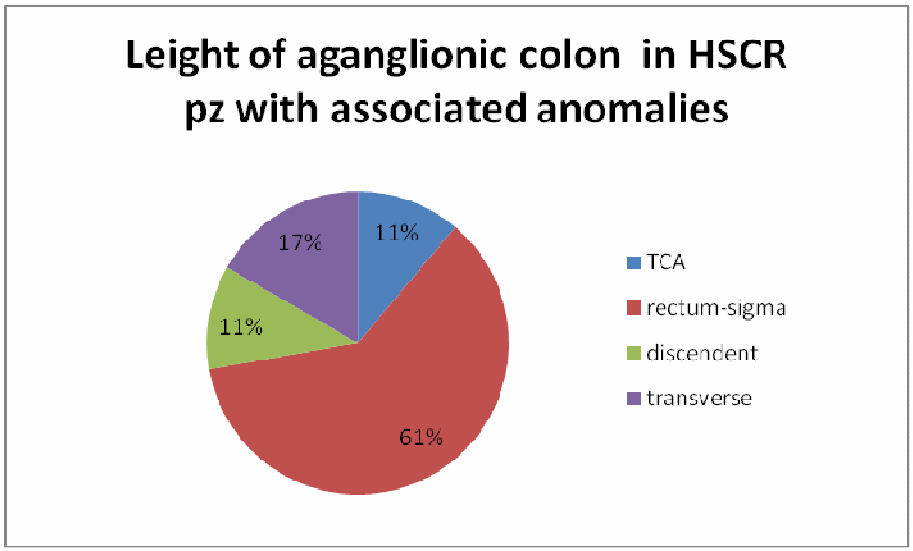
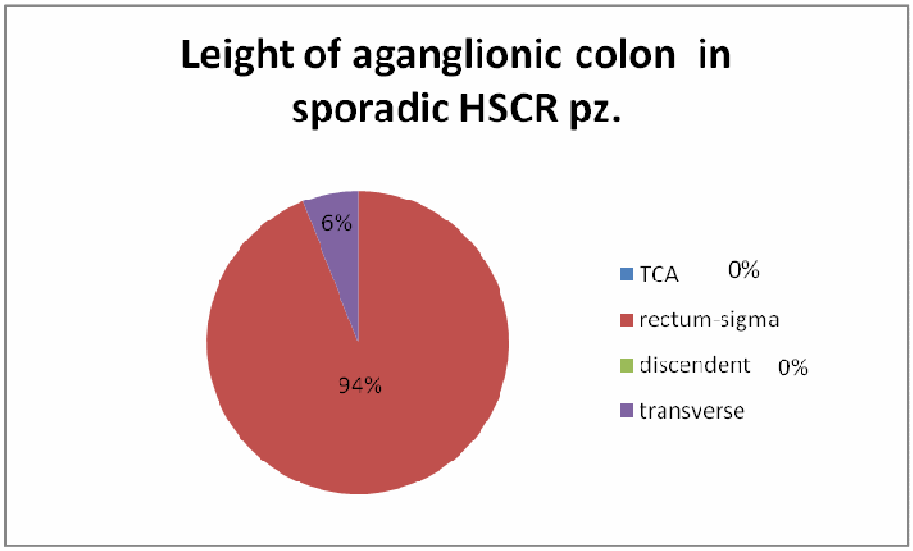

% of anomalies in HSCR patients
Genitourinary
Gastrointestinal
RPSM
Skin/integumentary
Ophthalmic/hearing
Dysmorphisms
Cardiac
Skeletal/muscle/limb
0
5
10
15
20
25
30

FIG.1



Panel A



Panel B

FIG. 2

3.2 CONGENITAL INTESTINAL PSEUDO-OBSTRUCTION.

3.2.1 Definition, epidemiology and clinics.

The term chronic intestinal pseudo-obstruction (CIP) describes a disorder of gastrointestinal motility characterized by failure of the intestine to propel its contents through an unobstructed lumen (24). It has been estimated that approximately 100 infants with congenital CIP are born each year (24). Symptoms include persistent nausea, vomiting, abdominal distention, weight loss and constipation (25,26). Diarrhea caused by either bacterial overgrowth or a hypersecretory state may also occur (27). Symptoms may be intermittent or continuous and in the most severe forms often there are signs of an intestinal obstruction (such as dilated bowel loops or air-fluid levels) in the absence of a fixed lumen-occluding obstruction (28). In most cases, CIP results in a irreversible disorder of intestinal motor function, with the management consisting of nutritional support, use of prokinetics agents and prevention and treatment of bacterial overgrowth. Gastrostomies and jejunostomies may also be used for slow administration of enteral formulas. Referral for intestinal transplantation should be considered in the presence of frequent septic episodes, limited intravenous access for nutritional support and impending liver failure (29).

3.2.2 Pathogenesis and genetics of CIP

There is no single pathogenic cause for CIP in children. It is classified in primary and secondary CIP. Based on histopathology or manometric findings, patients with primary form have traditionally been subdivided into neuropathic (affecting primarily the nerves in the intestinal tract or demonstrating a disorganized pattern of intestinal motility) and myopathic form (affecting primarily the muscle of the intestinal tract or demonstrating diminished amplitude of coordinated contractions) (30). But the list of conditions and possible causes that have been associated with symptoms of CIP continues to expand (Tab.1)

Primary forms • Visceral neuropathy: sporadic or familial • Visceral myopathy: sporadic or familial Secondary forms • Muscular dystrophy • Scleroderma and other connective tissue disease • Postischemic neuropathy • Postviral neuropathy • Hypothyroidism • Diabetic neuropathy • Severe IBD • Organ Transplantation • Amyloidosis • Chagas' disease • Prenatal alcohol exposure • Chromosomal abnormalities • Multiple endocrine neoplasia IIB • Radiation enteritis

Tab 1. Classification of CIP

A genetic basis is suggested by some family clustering of CIP. Tanner reported one genetic form of CIP resulting from qualitative abnormalities of the enteric ganglia and nerve fibers. This histological phenotype suggests the presence of a differentiation defect. An autosomal recessive inheritance was supposed for this type of CIP occurring as a syndrome presenting with short small bowel, malrotation and pyloric hypertrophy associated with morphologic defects of argyrophil neurons in the myenteric plexus (31).

In 1996, it was described a family in which the chronic idiophatic intestinal pseudo-obstruction appeared to be segregating as an X linked recessive trait (CIIPX- OMIM 300048) and in which the disease locus was mapped to Xq28. The microsatellite markers, located in the distal part of Xq28, showed no recombination with a maximum lod score of 2.32. On the analysis of recombinants, the authors concluded that the critical region for the disease gene was limited by DXS15 toward the centromere and by DXYS154, the pseudoautosomal boundary toward the telomere (32). One year later, it was described another family with two brothers and a maternal uncle affected with CIP. All three had a patent duct arteriuos and chronic thrombocytopenia (33). One of the brothers and maternal uncle had gut malrotation, already described in the Italian family by Auricchio, suggested that gut malrotation may be a useful phenotypic marker for

an X-linked form of CIP. DNA analysis of this pedigree demonstrated cosegregation of the maternal grandamother Xq28 haplotype with the disease, confirming Auricchio's results.

3.2.3 Aim of the project

Aim of this part of the project has been the identification of the gene mutated in CIIPX and located in the CIIPX critical region in Xq28.

4.2.4. Paper published.

ARTICLE

Filamin A Is Mutated in X-Linked Chronic Idiopathic Intestinal Pseudo-Obstruction with Central Nervous System Involvement

Annagiusi Gargiulo,* Renata Auricchio,* Maria Vittoria Barone, Gabriella Cotugno, William Reardon, Peter J. Milla, Andrea Ballabio, Alfredo Ciccodicola, and Alberto Auricchio

We have previously reported that an X-linked recessive form of chronic idiopathic intestinal pseudo-obstruction (CIIPX) maps to Xq28. To select candidate genes for the disease, we analyzed the expression in murine fetal brain and intestine of 56 genes from the critical region. We selected and sequenced seven genes and found that one affected male from a large CIIPX-affected kindred bears a 2-bp deletion in exon 2 of the *FLNA* gene that is present at the heterozygous state in the carrier females of the family. The frameshift mutation is located between two close methionines at the filamin N terminus and is predicted to produce a protein truncated shortly after the first predicted methionine. Loss-of-function *FLNA* mutations have been associated with X-linked dominant nodular ventricular heterotopia (PVNH), a central nervous system (CNS) migration defect that presents with seizures in females and lethality in males. Notably, the affected male bearing the *FLNA* deletion had signs of CNS involvement and potentially has PVNH. To understand how the severe frameshift mutation we found can explain the CIIPX phenotype and its X-linked recessive inheritance, we transiently expressed both the wild- type and mutant filamin in cell culture and found that filamin translation can start from either of the two initial methionines in these conditions. Therefore, translation of a normal shorter filamin can occur in vitro from the second methionine downstream of the 2-bp insertion we found. We confirmed this, demonstrating that the filamin protein is present in the patient's lymphoblastoid cell line that shows abnormal cytoskeletal actin organization compared with normal lymphoblasts. We conclude that the filamin N terminal region between the initial two methionines is crucial for proper enteric neuron development.

Chronic idiopathic intestinal pseudo-obstruction (CIIP [MIM %300048]) is a clinical syndrome caused by a heterogeneous group of enteric neuromuscular diseases that result in a severe abnormality of gastrointestinal motility.[1] CIIP may present at any age and is diagnosed by radiological, surgical, or manometric evidence of abnormal bowel motility causing intestinal obstruction in the absence of any mechanical occlusion.[1] CIIP is an uncommon, often fatal, syndrome in infancy, which may occur because of primary intrinsic visceral neuromuscular disorders or secondary to a variety of conditions, such as drug toxicity, ischemia, inflammatory or autoimmune diseases, myopathies, or viral infections (e.g., Epstein-Barr or cytomegalovirus [CMV]).[1] Primary forms of CIIP are neurogenic (28%), myopathic (36%), or unclassifiable (36%).[2] Neuropathic abnormalities of enteric innervation cause neurogenic CIIP and may be quantitative (hypo-, hyper-, and aganglionosis) or qualitative in nature.[2] In Hirschsprung disease (HSCR [MIM #142623]), the most common neuronal CIIP, lack of migration of enteric ganglion cells during development results in aganglionosis along gastrointestinal segments of variable length.[2] In other cases, migration of enteric neurons is not affected, but enteric ganglia and nerve fibers show qualitative abnormalities, suggesting the presence of a differentiation defect. This may be the case for the patients described by Tanner[3]—affected by a short intestine, malrotation, and hypertrophic pyloric stenosis—who were reported to have absence of argyrophilic neurons in the myenteric plexus.[3] The condition was thought to be inherited as an autosomal recessive trait.[3] More recently, we reported an Italian family with 10 affected males in 4 generations, all related through healthy females; of the 10, 9 died in the first months of life. Two affected subjects (IV-1 and IV-5) in the last generation (fig. 1*A*) had severe CIIP associated with a short small intestine, malrotation, and hypertrophic pyloric stenosis.[4] Subject IV-1 survived repeated surgery and is still alive. Histological analysis of full-thickness ileal and colonic biopsies of samples from the two subjects evidenced abnormal neurons in the myenteric and submucosal plexuses.[4] Like the patients described by Tanner et al.,[3] there were abnormalities of argyrophilic neurons. In our study family, the condition appeared to be inherited with a clear X-linked recessive pattern—that is, X-linked chronic idiopathic intestinal pseudo-obstruction (CIIPX)—and we mapped the disease locus to Xq28 between *DXS15* and *DXYS154*.[4] There was an additional report by FitzPatrick et al.[5] about neurogenic CIIP with patent ductus arteriosus

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* These two authors contributed equally to this work.

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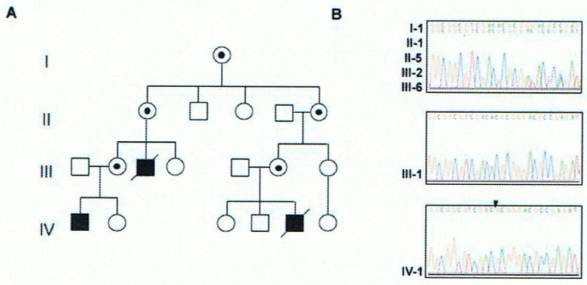

Figure 1. *A,* Pedigree of the CIIPX-affected family. *B,* Chromatogram showing *FLNA* exon 2 sequence around c.65-66delAC. *Top,* Sequence of the obligate carriers (I-1, II-1, II-5, III-2, and III-6,). *Middle,* Sequence of healthy male (III-1). *Bottom,* Sequence of the affected male (IV-1). The arrow indicates c.65-66delAC.

and large platelet thrombocytopenia, apparently inherited in an X-linked recessive manner. By April 2003, 63 genes were annotated in the CIIPX critical region (Human Genome Browser Gateway). We analyzed their expression in fetal murine gut and brain, to select candidate CIIPX genes, and we found a frameshift mutation in the filamin A gene (*FLNA* [MIM +300017]) that was associated with CIIPX in the Italian kindred.

Material and Methods

Clinical History

The index patient (IV-1), second cousin to subject IV-5, is a male and the first offspring of healthy, nonconsanguineous parents (fig. 1*A*). He was born at term after an uneventful pregnancy. On the 3rd d of life, he presented with bilious vomiting, and laparatomy showed a short small bowel with intestinal malrotation, pyloric hypertrophy, and an ileal volvulus. Fifteen days later, he required additional surgery for intestinal obstruction, and an ileostomy was raised. Histological examination of full-thickness ileal and colonic biopsies showed abnormal argyrophilic neurons in the myenteric plexus, as well as nerve fibers in the lamina propria in the colon.[4] Findings similar to the myenteric plexus were present in the submucosal plexus. At age 3 mo, continuity of his bowel was restored with an ileoileal anastomosis, but the bowel once again became obstructed. Manometric studies suggested a neuropathic disorder of his gut. A further ileostomy was raised, and full-thickness material was taken for further histology. At age 3 years, a Lester-type ileorectal anastomosis was performed to restore bowel continuity. In addition to the severe CIIP, by age 7 years, an asymmetrical spastic diplegia with impairment of fine finger movements also became apparent. Magnetic resonance imaging (MRI) of his brain showed an abnormal intermediate signal in the peritrigonal white matter. The patient and two carrier females in the family declined to undergo additional brain MRI after the identification of the *FLNA* mutation. The patient required lengthening operations for both Achilles tendons. He also had seizures: the first time after surgery during his 1st mo of life, then again at ages 8 and 18 years. Since the last episode, he has received carbamazepine treatment. He continues to require supplemental parenteral nutrition to maintain good health.

The second patient (IV-5) was a male, the third offspring of healthy, unrelated parents (fig. 1*A*). His older brother and sister have no gastrointestinal disorders and are completely well. He was born at term, weighed 4.2 kg, and measured 52 cm in length. On his 1st d of life, he had abdominal distension and bilious vomiting. Laparotomy demonstrated a small-intestinal malrotation with CIIP. Because of ongoing subocclusive symptoms, two intestinal resections were performed during the following weeks. He required total parenteral nutrition and an additional operation with ileoileal anastomosis. Intestinal histology of the ileum showed abnormalities of the myenteric and submucosal neurons similar to those of subject IV-1. Subject IV-5 died shortly after surgery, at age 8 mo.

Genomic DNA Extraction and PCR Amplification and Sequencing

Genomic DNA was isolated from peripheral–whole-blood lymphocytes and from paraffin-embedded sections by use of standard protocols (Qiagen Italy) or as described elsewhere.[4] The complete coding sequence of seven genes selected for direct sequencing, including the exon-intron boundaries, was amplified by PCR with Taq Gold DNA Polymerase (Roche). These genes are (from X chromosome centromere to telomere): *ZNF275*, *ATP2B3* (MIM *300014), *DUSP9* (MIM *300134), *SLC6A8* (MIM *300036), *ABCD1* (MIM *300371), *L1CAM* (MIM *308840), and *FLNA*. PCR was performed as follows: 35 cycles with 50 ng of genomic DNA at 94°C for 1 min, at the appropriate primer annealing temperature for 1 min, and at 72°C for 1 min. Primers and reaction conditions for PCR amplification are available on request.

Amplicons were screened for mutations by direct sequencing with an ABI PRISM Big Dye terminator cycle sequencing kit, and the reactions were analyzed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The sequenced exon and intron-exon boundaries were compared with consensus sequences obtained from the National Center for Biotechnology Information Database with use of standard software for DNA sequencing analysis (Autoassembler v. 2.1 [Applied Biosystems]). Sixty-six females and 32 males from the general Italian population were used as controls, for a total of 164 X chromosomes.

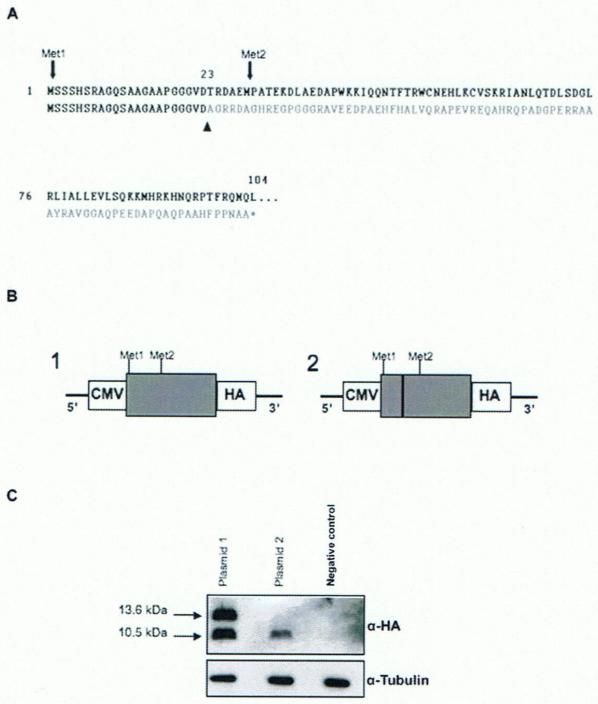

Figure 2. Effect of c.65-66delAC on filamin A translation. *A,* Sequence of the filamin A NH_2 terminus. Black letters indicate wild-type protein sequence; gray letters indicate predicted amino acid changes caused by c.65-66delAC; the asterisk (*) indicates a stop codon; "Met1" and "Met2" indicate the first and second filamin A methionines, respectively. *B,* Schematic representation of plasmids 1 and 2 expressing filamin A NH_2 terminus (amino acid residues 1–124) fused to the HA under the control of the CMV promoter. The line between Met1 and Met2 in plasmid 2 indicates the position of c.65-66delAC. *C,* Western blot analysis of FLNA expression in HEK 293 cells transfected with plasmids 1 and 2 and in an untransfected control. Protein molecular weight is shown on the left. The primary antibodies used are shown on the right.

RT-PCR Analysis of Gene Expression from the CIIPX Critical Region

Total RNA from C57BL/6 mice (Harlan Italy), from embryonic day–18 (E18) mouse brain or gut, from 5-wk-old mouse gut, and from V-1 individual lymphoblastoid cells was extracted using the Trizol reagent (Invitrogen Italy) according to the manufacturer's instructions. After DNAse treatment, 5 μg of total RNA was used as a template for cDNA synthesis, with use of random primers and SuperScript III (Invitrogen Italy). Subsequently, PCR amplification was performed using oligonucleotides specific for each of the 63 genes included in the CIIPX critical region and was designed in the region of homology between human and murine RNAs with use of the Oligo 4.0 software (National Biosciences). PCR was performed using Taq Gold DNA polymerase (Roche), with 35 cycles at 94°C for 1 min, at the appropriate primer annealing temperature for 1 min, and at 72°C for 1 min. Primer sequences and reaction conditions for each gene are available on request.

Generation of the Plasmid Constructs Expressing Filamin A NH_2 Terminus

We generated two plasmids that express the first N-terminal 124 aa of FLNA by fusing to a hemoagglutinin tag (HA) under the transcriptional control of the ubiquitous CMV promoter. In plasmid 1, the wild-type *FLNA* sequence was included, whereas, in plasmid 2, the *FLNA* sequence corresponding to c.65-66delAC was introduced. To do so, *FLNA* exon 2 sequence from the initial ATG codon to genome position 3688 was amplified from control and the patient's genomic DNA, with use of primers *Not*I, 5′-ATG AGT AGC TCC CAC TCT CGG GCG GGC CAG-3′, and *Bam*HI-TTA-HA epitope tag, 5′-GAT GGA CAC CAG TTT GAT GCT CTC GCG GT-3′. The amplification was performed with Pfu DNA Polymerase (Promega Italy). The PCR product was inserted into PCR 2.1-TOPO plasmid (Invitrogen) and then was digested with *Not*I and *Bam*HI restriction enzymes. The corresponding fragments from control and patient were then cloned into pAAV2.1-CMV-EGFP[6]

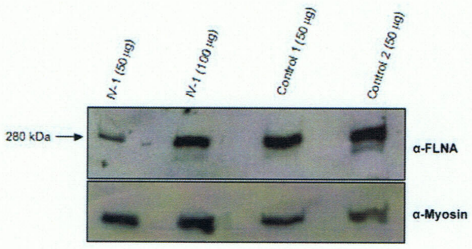

Figure 3. Western blot analysis of filamin A expression in patient IV-1 lymphoblasts. The amount of total protein loaded for each individual is shown above the lanes. Protein molecular weight is shown on the left. The primary antibodies used are shown on the right.

by removing the EGFP-coding sequence, to obtain plasmid 1 and plasmid 2, respectively.

Transfection of HEK 293 Cells with Constructs Expressing Filamin A and Western Blot Analysis

Human embryonic kidney (HEK) 293 cells were plated in 6-well plates containing 3×10^5 cells/well. After 24 h, the cells were separately transfected with 2 μg/well of plasmid 1 and plasmid 2 with use of FUGENE 6 (Roche), according to the manufacturer's instruction. Transfection with pAAV2.1-CMV-EGFP was used as a control. After 48 h, proteins from transfected cells were extracted with Lysis buffer (50 mM NaCl, 25 mM Tris [pH 8.0], 0.5% NP40, 0.1% SDS, 1 mM protease inhibitors [Roche], and 1 mM phenylmethylsulphonyl fluoride) and were kept on ice for 20 min. Samples were spun at 13,000 rpm for 20 min, and supernatants were collected. Protein concentrations were determined with the Bio-Rad dye protein assay. The proteins were denatured by heating at 98°C for 3 min and were separated on a 16% SDS-polyacrylamide gel electrophoresis with 4% stacking gel in 1 × Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS [pH 8.3]) in a miniprotean cell (Bio-Rad) at 130 volts for 2 h. The separated proteins were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane with transfer buffer (25 mM Tris base, 0.2 M glycine, and 20% methanol [pH 8.5]) in a minitransfer cell (Bio-Rad) at 100 volts at 4°C for 1 h. Membranes were incubated at room temperature for 1.5 h in blocking buffer containing 1 × TBS and 0.05% Tween 20 with 5% dried nonfat milk and then were probed with an anti-HA antibody (Sigma-Aldrich) for 2 h at room temperature. This was followed by incubation with a peroxidase-conjugated secondary anti-rabbit IgG for 1 h at room temperature. Signals were detected by chemoluminescence with the Pico Enhanced Chemiluminescence Kit (Pierce Chemical). A prestained molecular-weight ladder (Fermentas) was used to determine protein size.

Western Blot Analysis of Filamin A Expression in Patient Lymphoblasts

Lymphoblasts from patient IV-1 and from two control individuals were grown in Roswell Park Memorial Institute PMI 1640 plus medium with 20% fetal bovine serum (FBS) in 5% CO_2 at 37°C, were harvested, precipitated, and lysed with Lysis buffer (50 mM NaCl, 25 mM Tris [pH 8.0], 0.5% NP40, 0.1% SDS, 1 mM protease inhibitors [Roche], and 1 mM PMSF) on ice for 20 min. Samples were spun at 13,000 rpm for 20 min, and supernatants were collected. Protein concentrations were determined with the Bio-Rad dye protein assay. The proteins were denatured by heating to 98°C for 3 min and then were separated on a 7% SDS-polyacrylamide gel electrophoresis with 4% stacking gel in 1 × Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS [pH 8.3]) in a miniprotean cell (Bio-Rad). The separated proteins were electrotransferred onto PVDF membrane with a transfer buffer (25 mM Tris base, 0.2 M glycine, and 20% methanol [pH 8.5]) in a minitransfer cell (Bio-Rad) at 4°C for 1 h. Membranes were incubated overnight in blocking buffer containing 1 × TBS and 0.1% Tween 20 with 5% dried nonfat milk and then were probed with anti–filamin A antibody (Cell Signaling) for 3 h at room temperature. This was followed by incubation with the peroxidase-conjugated secondary anti-rabbit IgG for 1 h at room temperature. Signals were detected by chemoluminescence through use of the Pico Enhanced Chemiluminescence Kit (Pierce Chemical). A prestained molecular-weight ladder (Fermentas) was used to determine protein size.

Immunofluorescence Analyses

Lymphoblasts were obtained from two unaffected subjects and from patient IV-1. Cells were cultured in serum-free RPMI 1640 medium plus 20% FBS (Invitrogen). Cells (~3,000) were centrifuged with Cytospin Centrifuge for Cells Suspensions (Shandon Cytospin 3 Cytocentrifuge [Global Medical Instrumentation]) at 800 rpm for 3 min and were transferred to the corresponding glass slides. Cells were fixed with 3% paraformaldehyde (Sigma) for 10 min at room temperature. After one wash with 1 × PBS (Invitrogen), Triton 0.2% (Bio-Rad) was applied to the slides, which were then incubated at room temperature for 5 min. After another wash with 1 × PBS, slides were incubated for 45 min in the dark with phalloidin-Texred (Sigma) (diluted 1:500 in 1 × PBS), with mouse antibodies directed to γ-tubulin (Sigma) (diluted 1:50 in 1 × PBS), or with rabbit antibodies to filamin A (Cell Signalling) (diluted 1:250 in 1 × PBS). Fluorochromes can directly bind the phalloidin, which in turn links F-actin, revealing F-actin without antibodies. Secondary anti-mouse and anti-rabbit antibodies (Invitrogen) conjugated to rhodamine were used at concentrations of 1:1000 and 1:200 dilutions, respectively, in 1 × PBS. Slides were covered with Mowiol gel (Calbiochem) and then were analyzed by fluorescence microscopy (Laser Scanner Microscopy 510 Zeiss).

Results

Mutation of FLNA *in CIIPX*

To identify the gene mutated in CIIPX, we analyzed, by reverse transcriptase, the expression pattern of the genes from the CIIPX critical region on Xq28 in c57/BL6 fetal (E18) brain and gut. Of the 63 genes located in the CIIPX critical region, 56 are homologous in mouse and human and therefore were analyzed. We found that 7 of 56 genes are expressed only in intestine, 13 are expressed only in brain, and 28 are expressed in both intestine and brain. Eight genes are not expressed in either tissue but are ex-

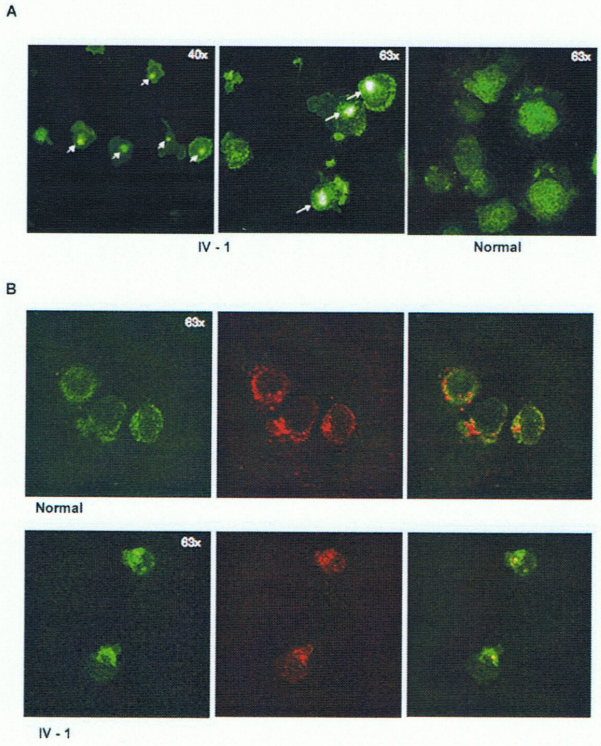

Figure 4. Actin organization and γ-tubulin distribution in patient IV-1 lymphoblasts. *A,* Actin immunofluorescence with phalloidin-Texred in patient IV-1 and control lymphoblasts. The white arrows indicate the phalloidin spots, which are more abundant in patient IV-1 than in control lymphoblasts. *B,* Staining for actin with phalloidin-Texred (*green staining*) and for γ-tubulin (*red staining*) in patient IV-1 and control lymphoblasts. The merged image on the right shows that the phalloidin spots are on the centrosomal side of patient IV-1 lymphoblasts.

pressed in lymphoblastoid cell lines (data not shown). We selected seven genes (*ZNF275, ATP2B3, DUSP9, SLC6A8, ABCD1, L1CAM,* and *FLNA*) from those expressed in both tissues for direct sequencing analysis of exon and intron-exon boundaries. *ABCD1, L1CAM, SLC6A8,* and *FLNA* were selected as candidate genes because of their involvement in already-known inherited disorders of the nervous system[7–10]; *ATP2B3* is a plasma-membrane protein possibly involved in the regulation of physiological ions homeostasis[11]; *DUSP9* was selected because of its protein's interactions with members of the extracellular signal-regulated kinase family of mitogen-activated protein kinases, its possible involvement in regulation of gene expression in neurons of neuroenteric system, and its contribution to pain caused by inflammation[12]; finally, *ZNF275,* because of its protein's high similarity to a Zinc-finger protein, was selected as a possible uncharacterized novel putative transcriptional activator.[13]

We found that the index patient (IV-1) bears a 2-bp deletion in exon 2 of *FLNA* (c.65-66delAC). Segregation analysis of the *FLNA* mutation confirms that all obligate carriers, by pedigree or established by linkage analysis,[4] are heterozygous for the 2-bp deletion (fig. 1*B*). The mutation is absent in 164 control X chromosomes.

Recently, a wide spectrum of developmental anomalies has been shown to be caused by mutations in *FLNA*.[14] Null mutations in the *FLNA* gene result in bilateral periventricular nodular heterotopia (PVNH [MIM #300049]), an X-linked dominant neuronal migration disorder clinically characterized by seizures.[15] The association between a potential severe loss-of-function *FLNA* mutation and an X-linked recessive disease involving enteric neuron develop-

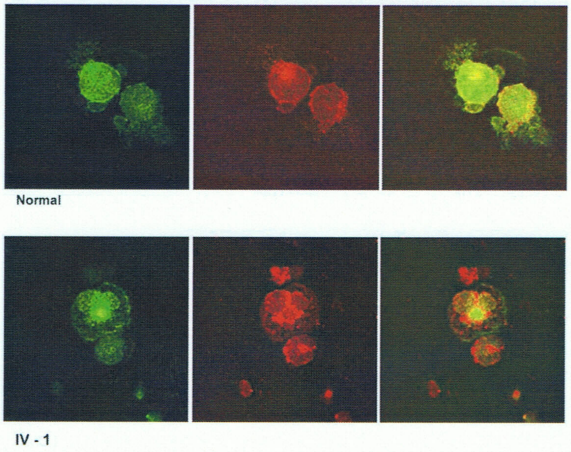

Figure 5. Actin organization and filamin A distribution in patient IV-1 lymphoblasts. Staining for actin with phalloidin-Texred (*green staining*) and for filamin A (*red staining*) in patient IV-1 and control lymphoblasts. The merged image on the right shows that the actin organization and filamin A distribution in the cells are similar.

ment prompted us to investigate the effect of *FLNA* c.65-66delAC at the protein level.

ATG Downstream of FLNA *c.65-66delAC Allows Production of a Shorter Filamin A Form*

The c.65-66delAC deletion found in patients with CIIPX is predicted to cause a frameshift and a premature stop codon at filamin A amino acid position 103 (fig. 2*A*). The predicted mutant protein retains the initial 22 aa identical to wild-type filamin A, followed by 81 different aa before a premature stop codon. This is the most severe *FLNA* loss-of-function mutation described to date,[16] predicted to cause lethality in males and PVNH phenotypes in females. Interestingly, the c.65-66delAC deletion is located 22 codons downstream of the initial *FLNA* ATG (Met1) (fig. 2*A*) and 5 codons upstream of a second ATG (Met2) (fig. 2*A*). If filamin A translation occurs from either methionine and results in the synthesis of two proteins differing in 27 aa at the NH_2 terminus, the c.65-66delAC deletion would affect only the translation of the longer form of filamin A. To determine whether this is the case, we transiently transfected HEK 293 cells with two different eukaryotic expression plasmids containing the CMV promoter, as shown in figure 2*B*. In both plasmids, the *FLNA*-coding sequence from the first methionine to amino acid residue 124 (corresponding to the end of exon 2) was fused to an HA peptide at its 3′ end, resulting in peptides of 124 aa of predicted length if translation occurs from Met1 or of 96 aa if it occurs from Met2. Whereas the wild-type *FLNA* sequence was included in plasmid 1, that bearing the c.65-66delAC deletion was present in plasmid 2. As shown in figure 2*C*, cells transfected with plasmid 1 express both filamin A forms, whereas those transfected with plasmid 2 express only the shorter form of the protein, albeit at lower levels, possibly because of nonsense-mediated decay. This suggests that (1) filamin A translation can start from either of its two initial methionines, (2) translation of the shorter filamin A form alone occurs because of the c.65-66delAC deletion. To confirm this, we analyzed endogenous filamin A expression in control and patient IV-1 lymphoblasts. Western blot with anti–filamin A antibodies shows that filamin A expression in the patient's lymphoblasts is similar to expression in control lymphoblasts (fig. 3*A*). Although, because of filamin A's high molecular weight (280 kDa), we cannot discriminate whether the protein present in the patient's lymphoblasts is of lower molecular weight than that in control lymphoblasts, these data confirm that c.65-66delAC results in filamin A translation despite the early frameshift. Finally, we investigated the impact of c.65-66delAC on cytoskeletal organization in the patient's lymphoblasts. Whereas actin immunofluorescence staining with phalloidin-Texred is predominantly diffused in the cytoplasm in lymphoblasts from healthy control individuals, in the cells derived from the patient, actin appears mainly concentrated in large dots localized on the centrosome side of the cells (fig. 4; the percentage of cells containing phalloidin dots in patient vs. control lymphoblasts was 78% vs. 9.4%). We con-

firmed, by immunofluorescence, the presence of filamin A in the patient's lymphoblasts and that it has a distribution similar to that of actin (fig. 5).

We additionally screened a series for but did not find any *FLNA* mutations in 12 individuals (8 unrelated sporadic and 4 familial cases, 8 males and 4 females, including 1 of the affected males described by FitzPatrick et al.[5]) affected with isolated non-HSCR CIIPX. In addition, the observed higher penetrance of HSCR in males than in females[17] prompted us to hypothesize that CIIPX might represent an additional susceptibility locus for HSCR. We therefore sequenced the *FLNA* region containing the two adjacent ATG codons in 37 unrelated patients affected with HSCR (27 males and 10 females), but we did not find any sequence variants.

Discussion

In this article, we show that CIIPX with CNS involvement is associated with a 2-bp deletion in the *FLNA* gene. This expands the wide spectrum of developmental anomalies caused by *FLNA* mutations. The *FLNA* loss-of-function mutations found in PVNH are embryonic lethal in males,[18] although there are reports of males with PVNH who harbor hypomorphic *FLNA* mutations.[16] *FLNA*-targeted disruption in mice causes male embryonic lethality, cardiac malformation, and midline skeletal defects.[19] Interestingly, Hehr et al.[20] described a male with PVNH without epilepsy but with severe constipation and facial dysmorphisms who bears an *FLNA* splice-site mutation resulting in the generation of both normal and aberrant *FLNA* mRNA. The male patient in our CIIPX-affected family had seizures and potentially has PVNH. Different from the male with PVNH and constipation described by Hehr and colleagues,[20] the CIIPX phenotype in our family is most distinguished by severe CIIP, present at birth, that is lethal unless promptly corrected by surgery.

FLNA encodes for a large cytoskeletal protein (2,639 aa) that cross-links the actin cytoskeleton into orthogonal networks and modulates the cellular response to chemical and mechanical environmental factors by regulating changes in their shape and motility.[21] In mammals, there are three highly homologous filamins: filamin A, B, and C. An actin-binding domain is located at the NH_2 terminus of these proteins and is composed of two calponin homology domains: CH1 and CH2. The remainder of the filamin molecule is composed of 24 filamin repeats that are predicted to adopt structurally homologous β-sheet configurations despite significant divergence at the protein level.[22] The chain of repeats is interrupted by two hinge regions that are proposed to confer flexibility to the Y-shaped filamin dimer. Homodimerization is mediated by the most C-terminal repeat 24 and possibly by other protein domains. We show that, rather than causing a early frameshift, the c.65-66delAC deletion found in the CIIPX-affected patient results in translation of a shorter filamin A from the second ATG. We initially observed this after western blot analysis of lysates from cells transfected with plasmids expressing either the wild-type or the c.65-66delAC cDNA, and we then confirmed the presence of detectable filamin A in the patient's lymphoblasts by both western blot and immunofluorescence analyses. Interestingly, the presence of the mutant filamin A results in peculiar cytoskeletal organization, reflected by a larger amount of phalloidin dots in the patient's than in the control lymphoblasts, which are located on the centrosomal side of the cells. Others have described this large actin dots as "constriction rings."[23] Since cell motility is based on the periodic oscillatory activity of the actin system in association with ring formation and movement across the cell,[23] this ring can contribute to an abnormally polarized cell-surface motility.

This partial *FLNA* loss of function may explain the recessive CIIPX inheritance in our family. What remains to be elucidated is why this is associated with severe CIIP rather than with a classic PVNH phenotype. In CIIPX, abnormal neurons are present in the myenteric and submucosal plexuses, whereas, in classic PVNH, normally differentiated cortical neurons fail to migrate properly. The association between frameshift and missense heterozygous *FLNA* mutations with PVNH but not with CIIP may suggest that filamin A dose impacts more severely on cortical neuron migration than on enteric-neuron development. Conversely, since we show that *FLNA* c.65-66delAC is associated with CIIPX, it is possible that the first 27 FLNA aa residues between Met1 and Met2, adjacent to filamin A actin-binding site[21] and absent in filamin A shorter form, are crucial for proper enteric neuron structure and function.

In conclusion, we show that *FLNA* c.65-66delAC causes CIIPX with CNS involvement in our family and results in the synthesis of a shorter filamin A form responsible for cytoskeletal abnormalities, which suggests a crucial role for FLNA in enteric-neuron structure and function.

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Web Resources

The URLs for data presented herein are as follows:

Human Genome Browser Gateway, http://genome.ucsc.edu/cgi-bin/hgGateway (for the CIIPX critical region)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CIIP, HSCR, *FLNA, ATP2B3, DUSP9, SLC6A8, ABCD1, L1CAM,* and PVNH)

References

1. Milla PJ (1994) Intestinal pseudo-obstruction in children. Wrightson Biomedical, Petersfield, United Kingdom, and Bristol, PA
2. Staiano A, Tozzi A, Auricchio A (1996) Aetiology and assessment of constipation in children. In: Barbara L, Corindolesi R, Gizzi G, Stanghellini V (eds) Chronic constipation. Saunders Company, Philadelphia, pp 153–168
3. Tanner MS, Smith B, Lloyd JK (1976) Functional intestinal obstruction due to deficiency of argyrophil neurones in the myenteric plexus: familial syndrome presenting with short small bowel, malrotation, and pyloric hypertrophy. Arch Dis Child 51:837–841
4. Auricchio A, Brancolini V, Casari G, Milla PJ, Smith VV, Devoto M, Ballabio A (1996) The locus for a novel syndromic form of neuronal intestinal pseudoobstruction maps to Xq28. Am J Hum Genet 58:743–748
5. FitzPatrick DR, Strain L, Thomas AE, Barr DG, Todd A, Smith NM, Scobie WG (1997) Neurogenic chronic idiopathic intestinal pseudo-obstruction, patent ductus arteriosus, and thrombocytopenia segregating as an X linked recessive disorder. J Med Genet 34:666–669
6. Auricchio A, Kobinger G, Anand V, Hildinger M, O'Connor E, Maguire AM, Wilson JM, Bennett J (2001) Exchange of surface proteins impacts on viral vector cellular specificity and transduction characteristics: the retina as a model. Hum Mol Genet 10:3075–3081
7. Sarde CO, Mosser J, Kioschis P, Kretz C, Vicaire S, Aubourg P, Poustka A, Mandel JL (1994) Genomic organization of the adrenoleukodystrophy gene. Genomics 22:13–20
8. Jouet M, Rosenthal A, Armstrong G, MacFarlane J, Stevenson R, Paterson J, Metzenberg A, Ionasescu V, Temple K, Kenwrick S (1994) X-linked spastic paraplegia (SPG1), MASA syndrome and X-linked hydrocephalus result from mutations in the *L1* gene. Nat Genet 7:402–407
9. Hahn KA, Salomons GS, Tackels-Horne D, Wood TC, Taylor HA, Schroer RJ, Lubs HA, Jakobs C, Olson RL, Holden KR, et al (2002) X-linked mental retardation with seizures and carrier manifestations is caused by a mutation in the creatine-transporter gene (*SLC6A8*) located in Xq28. Am J Hum Genet 70:1349–1356
10. Robertson SP, Twigg SR, Sutherland-Smith AJ, Biancalana V, Gorlin RJ, Horn D, Kenwrick SJ, Kim CA, Morava E, Newbury-Ecob R, et al (2003) Localized mutations in the gene encoding the cytoskeletal protein filamin A cause diverse malformations in humans. Nat Genet 33:487–491
11. Brown BJ, Hilfiker H, DeMarco SJ, Zacharias DA, Greenwood TM, Guerini D, Strehler EE (1996) Primary structure of human plasma membrane Ca^{2+}-ATPase isoform 3. Biochim Biophys Acta 1283:10–13
12. Ji RR, Befort K, Brenner GJ, Woolf CJ (2002) ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. J Neurosci 22:478–485
13. Mallon AM, Platzer M, Bate R, Gloeckner G, Botcherby MR, Nordsiek G, Strivens MA, Kioschis P, Dangel A, Cunningham D, et al (2000) Comparative genome sequence analysis of the *Bpa/Str* region in mouse and man. Genome Res 10:758–775
14. Robertson SP (2005) Filamin A: phenotypic diversity. Curr Opin Genet Dev 15:301–307
15. Fox JW, Lamperti ED, Eksioglu YZ, Hong SE, Feng Y, Graham DA, Scheffer IE, Dobyns WB, Hirsch BA, Radtke RA, et al (1998) Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. Neuron 21:1315–1325
16. Parrini E, Ramazzotti A, Dobyns WB, Mei D, Moro F, Veggiotti P, Marini C, Brilstra EH, Dalla Bernardina B, Goodwin L, et al (2006) Periventricular heterotopia: phenotypic heterogeneity and correlation with filamin A mutations. Brain 129: 1892–1906
17. Badner JA, Sieber WK, Garver KL, Chakravarti A (1990) A genetic study of Hirschsprung disease. Am J Hum Genet 46: 568–580
18. Eksioglu YZ, Scheffer IE, Cardenas P, Knoll J, DiMario F, Ramsby G, Berg M, Kamuro K, Berkovic SF, Duyk GM, et al (1996) Periventricular heterotopia: an X-linked dominant epilepsy locus causing aberrant cerebral cortical development. Neuron 16:77–87
19. Hart AW, Morgan JE, Schneider J, West K, McKie L, Bhattacharya S, Jackson IJ, Cross SH (2006) Cardiac malformations and midline skeletal defects in mice lacking filamin A. Hum Mol Genet 15:2457–2467
20. Hehr U, Hehr A, Uyanik G, Phelan E, Winkler J, Reardon W (2006) A filamin A splice mutation resulting in a syndrome of facial dysmorphism, periventricular nodular heterotopia, and severe constipation reminiscent of cerebro-fronto-facial syndrome. J Med Genet 43:541–544
21. Popowicz GM, Schleicher M, Noegel AA, Holak TA (2006) Filamins: promiscuous organizers of the cytoskeleton. Trends Biochem Sci 31:411–419
22. Gorlin JB, Yamin R, Egan S, Stewart M, Stossel TP, Kwiatkowski DJ, Hartwig JH (1990) Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. J Cell Biol 111:1089–1105
23. Bornens M, Paintrand M, Celati C (1989) The cortical microfilament system of lymphoblasts displays a periodic oscillatory activity in the absence of microtubules: implications for cell polarity. J Cell Biol 109:1071–1083

3.3 ESOPHAGEAL ACHALASIA

3.3.1 Definition, epidemiology and clinics.

The esophageal achalasia is the most recognized motor disorder of the esophagus . The disorder is characterized by the partial or complete loss of lower esophageal sphincter (LES) relaxation after physiological stimulus and loss of esophageal peristalsis. An increase in LES resting pressure may also be present (34). This disease produces characteristic manometric and radiographic features (35,36). Achalasia remains an uncommon but worldwide disorder. The incidence of the disease is about 1/200000 per year and it has an equal frequency in men and women in all age groups; however, fewer than 5% of all patients with achalasia manifest symptoms, such as disphagia, before the age of 15 years (0.11 case per 100000 children)(37).

3.3.2 Pathogenesis and genetics of esophageal achalasia.

Numerous theories exist regarding pathogenesis, but a degenerative lesion involving intramural nitric oxide-containing inhibitory neurons in the distal esophagus and LES appears to be central to the disease. Lack of the inhibitory influence prevents the

normal sequencing of the distal contractions (resulting in aperistalsis) and normal relaxation of the LES with swallowing (38).

Pharmacological and histological studies have shown that neuron degeneration is due to inflammatory and fibrotic process (39). The etiology of these phenomena remains unknown, but both genetic and immunologic factors presumably are relevant. Some data support an immunological basis for achalasia. Infact, early achalasia patients and end-stage achalasia patients, who underwent esophagomyotomy and esophageal resection respectively, showed a myenteric inflammation resulting from an immunologic process as the major cause of the myenteric neuronal degeneration (40). The majority of myenteric inflammatory cells in patients with achalasia are CD3-positive T cells, most of which are also CD8-positive, although the relative percentage of such cells appears to decrease with disease progression. Furthermore, many of the CD3-positive/CD8-positive myenteric lymphocytes also express activation markers, suggesting they are resting or activated cytotoxic T cells. The immunohistochemical demonstration of the granzyme B in a subpopulation of these cells supports the contention that achalasia is an immune-mediated disease, although the inciting antigen remains an enigma (40). Also, in animal models with megaesophagus it has been shown that the muscolar layer of the dilated portions shows a focal inflammation and single fiber

necrosis (41). The stimulus for this inflammatory reaction is still not completely understood but may be driven by autoimmune factors. Presumably participating in the process, autoantibodies against M2 muscarinic acetylcholine receptors have been found in patients with achalasia. These antibodies increase the contractile activity of the LES through specific activation of the receptors (42).

In many cases, the achalasia have a clear genetic component, for example in early diagnosis case (before 6 months of age) and in familial cases, in which autosomal recessive inheritance is suggested (OMIM 200400). In literature, it has been described about 8 families with 21 affected members, in the majority they were pediatric patients with achalasia diagnosis before the age of 10. Four of these patients were also syndromic (43-49). In the same families, there were also some relatives with esophageal symptoms without a clear achalasia diagnosis (50).

An achalasia-like picture also accompanies a variety of genetically based syndromes, where in the pathophysiologic mechanisms responsible for the esophageal manifestations may vary but reveal a genetic basis. For example, achalasia is associated with the autoimmune poliendocrine syndrome (APS1) (51), the megaduodenum (52,53), the multiple endocrine neoplasia (MEN2B) (54), the peripheric motor neuropathy with disautomomy (55), the

vulvar leiomiosis with or without nefropathy(56), the Down's syndrome, the Sjogren's syndrome, the Allgrove's syndrome (achalasia, alacrima, adrenal insufficiency). Maybe, these last two syndrome might be caused by the same genetic mutation in the aladin gene, located on chromosome 12q13 (57-59).

One important genetic factor for the pathogenis of achalsia is the HLA-DQ, in particular alleles DQA1-0103 and DQB1-0603 (60-62). Furthermore, in those patients with this HLA alleles it has been found a greater prevalence of the myentric anti-plexus antibodies (42).

Recently, it has been shown that collagenous networks play a critical role in the morphogenesis of the embryo and in the maintenance of the tissue architecture of adult tissues. The 19 genetically distinct types of collagen molecules are expressed in developmental and tissue-specific manners (63-66). In particular, type XIX collagen is a poorly characterised extracellular matrix component thought to be involved in the formation of specialized basement membrane zones (vessels, nerves and mesenchima). XIX collagen is important for the cell-to-cell interaction, the communication between cells and the local concentration of signalling molecules. This gene expression was for the first time studied in the mouse (Col19a1) and it was demonstrated the transient expression quite only in the embryonic muscle (67). Like the myogenic regulatory factor myf-5, Col19a1 transcription gradually

decrease in differentiating skeletal muscle progenitors and concomitantly to increased myogenin gene expression (68). This results show that Col19a1 could be involved in the muscle differentiation and function. The transient expression of Col19a1 in muscular tissue is confined to a few sites of the developing embryo, such limbs, tongue and smooth muscle layer of the stomach and esophagus, but also in skin and cerebral cortex and hippocampus of the new born brain. Unlike all other tissues, expression of Col19a1 in the central nervous system gradually increases after birth (68).

3.3.3 Animal models for esophageal achalasia.

More recently, two animal models have been created, one harbouring null and the second structural mutations of Col19a1, to reveal the critical contribution of this matrix protein to muscle physiology and differentiation(69). The null mice died in the majority after 3 week of postnatal life, showing signs of malnourishment. The few mice that survived past weaning stage revealed a dilated esophagus (megaesophagus) with retention of ingesta, immediately above the diaphragm level. The embryonic Col19a1 expression coincided with the formation and growth of the gastroesophageal junction. Specifically, in situ hybridizations revealed high Col19a1 expression in the lower-third portion of the embryonic esophagus destined to

become the abdominal segment; thereafter Col19a1 activity becomes gradually restricted to the mature LES, while decreasing in the muscle layer of the proximal stomach. Furthermore, intraluminal pressure recorded at the LES level of adult Col19a1 null mice showed significantly higher basal tone (three to eightfold) than wild-type mice. It also documented severely impaired or absent relaxation upon swallowing; even when present, relaxation was abnormally brief. The results of the manometric tests were remarkably similar to those reported for achalasic patients. The phenotype includes also an impaired nitric oxide-dependent relaxation of the sphinteric muscle, correlated with a disorganizied matrix and a normal complement of the enteric neurons and interstitial cells of Cajal. Null mice showed also an impaired smooth-to-skeletal muscle cell conversion in the abdominal segment of the esophagus, accounted for by failed activation of myogenic regulatory factors that normally drive esophageal muscle transdifferentation. In conclusion, all the data demonstrate that COL19A1 is an important gene for the LES development and function, and for this reason it could be involved in the pathogenesis of esophageal achalasia in men.

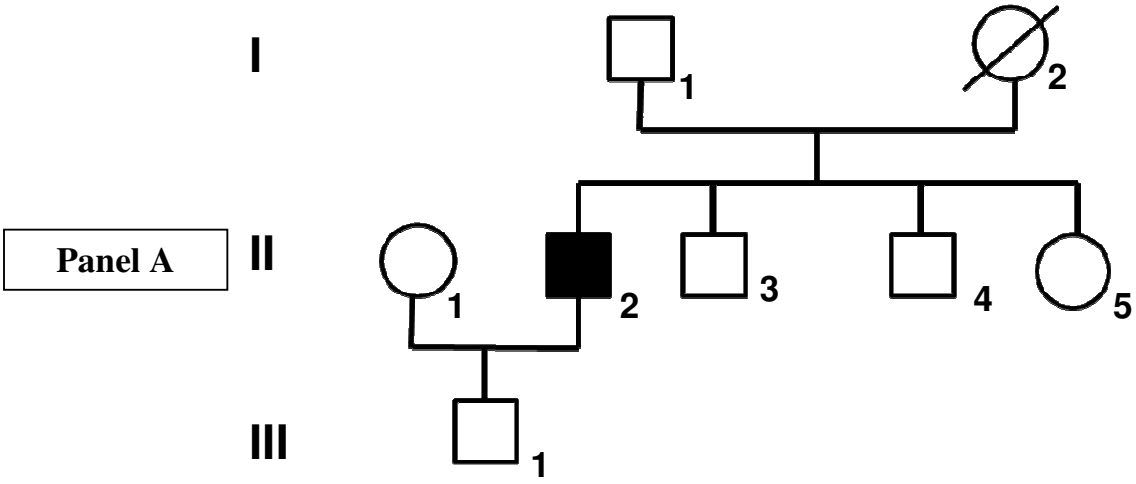
3.3.4 Aim of the project.

Aim of this part of the project was to analyze the potential involvement of mutation at the COL19A1 locus in the pathogenesis of esophageal achalasia.

Patients and Methods.

Patients.

44 adult patients (mean age: 27 years) and one pediatric patient (9 years old) affected by sporadic esophageal achalasia and one adult patient with a familial form of EA (also the father was affected by EA) have been enrolled in this study. Diagnosis have been made by clinical and manometric criteria. A blood sample was collected from each patient for genetic analysis. In two cases, in which mutation analysis have been successufully concluded, a blood sample was obtained from relatives (Fig. 3, Panel A and Panel B).



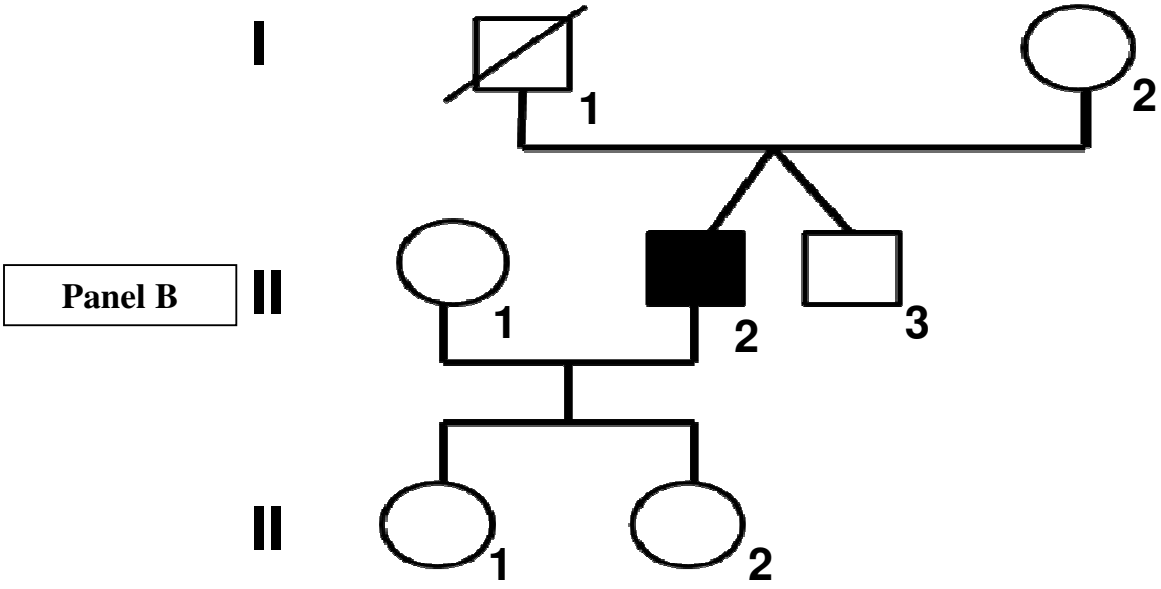


Fig.3 Pedigrees of the two patients carriers of COL19A1 mutations

Sequencing of COL19A1 gene.

First, we have performed genomic DNA extraction from peripheral blood collected from all patients affected by EA using standard protocol. Specific primers for COL19A1, not available from the literature, have been designed in the intronic regions in order to amplify, by the PCR, both the coding regions and of the intron-exon junctions (Tab.2). These primers have been designed by the analysis of the genomic sequences of the gene deposited in the GenBank and optimized for a single condition of amplification and to produce fragments of about 500 bp. Briefly, the PCR protocol will be: denaturation for 1 min at 94°C, hybridation for 1 min at 54°C, elongation for 1 min at 72°C. The PCR products have been direct sequenced using an automatic sequencer (ABI PRISM 377, Applied Biosystem). The sequences have been analyzed by Autoassembler 4.1 or SeqMan programs.

Fragment	Exon	Primers	Lung. Fram.	T_m calc.	T_m eff.
COLg1	1	5' TGCATCCAACCGTCATTTTATC 3' GAACACGAGCAGATGACATAG	350	62/62	58
COLg2	2	5' CGTTGATTGGTTTTGCTTTGGT 3' CTATGGCCTTTTCAATCTTAAC	287	62/62	58
COLg3	3	5' AAGTAACCACATGTAACCCTCA 3'GCTATGATCTTAAATTGCAGTAT	342	62/60	58
COLg4	4	5' GTTTCTCAATAGCATCATCCTC 3' CACTGTAGAGCCAAGATTAGATT	309	64/62	62
COLg5	5	5' TTTCTTGCAATCTCCAGTTTCC 3' CCATCCTAACTACGAGAATACT	319	62/62	58
COLg6	6	5' TTTTGTAGTGGTGTTTGCTGTT 3' CTCTTTCACAACTAACATCACA	462	60/60	58
COLg7	7	5' TCCACAAAACAATGAATCCTAC 3' AATTACCCACCTGGCTTCAAC	348	60/62	54
COLg8	8	5' GGAAAGTGCAAAAGTGTGATAA 3' AAACACTGAATGGCAAGACACA	380	60/62	58
COLg9	9	5' CCAGCTTCACATTTATAAACCA 3' ACACCAGCCTACCAATATGAAT	325	60/62	58
COLg10	10	5' GAGAAATAAGGAACAGATGGAA 3' TGTGATAAAGCTTCCTCCTAGA	382	60/62	58
COLg11	11	5' GGTTTAGAGCTTACATGTTAGA 3' AGGGAATGCACAAAACAAGTTAA	252	60/62	58
COLg12	12	5' TTGGTTTTACGACTCAGGATTG 3' AAACTAAAAACACTGGGCCTCT	365	62/62	58
COLg13	13	5' TTATCATTTTAGTGCCAGTTGT 3' TTTAGAATGCTTTTAGGAGATA	293	58/56	58
COLg14	14	5' GAAAGCTAAAAGGGACATGATA 3' TGGATAATAAACAGAATAATGGA	248	60/58	58
COLg15	15	5' CAAAGAAGCCAGTCAAAGAAGG 3' ATATGCCCATCTGCTTGCTCTA	324	64/64	62
COLg16	16	5' TTTAAAAGTCATGTGGAATTGG 3' AAACCAGTCATTCTAAACAGTA	300	58/58	52
COLg17	17	5' ATATTTGTCTTTGATTTGTTTA 3' ACTGAACAGAATACTTTTTTTA	254	52/54	52
COLg18	18	5' ACTTGAGAATAGGGAGAGCAGAT 3' TTATAGTGAGCCAAGATTGTGCC	489	66/66	66
COLg19	19	5' AGTTAGCAGGAGAGCAAGCAAT 3' AACCTCTTTATGCCTTACTTTCA	397	64/62	58
COLg20	20	5' TGGGTATTTTTGGAGGTCTGTT 3' CAATGTATTAGAAAACTCAAACC	376	62/60	58
COLg21	21-22	5' ATGAACTCTCCTTGATTTTATTG 3' CCAAAAGACAGTTACTATGCGT	490	60/62	58
COLg22	23	5' GATAAAGTGGGAAAGTGGGATG 3' TTAGGTATGATCAGTTTTTGTGG	319	64/62	58
COLg23	24	5' AAACAACAAACCACGTAGAATCA 3' TCTCTTCCTCCTATCTACTTAAC	219	62/64	58
COLg24	25	5' AATGTAAATGGCACCCCCTAGA 3' GCAGACCTAAACTTATTCCCAT	463	64/62	62
COLg25	26	5' CTCCCCGTTCCTTATTCTTTTAT 3' CCTTTCTATTCTTTTCTCCTTGT	438	64/62	58

Tab. 2. COL19A1 PCR primers used.

4.3.4 Results.

We have found two mutations (changing in DNA sequence, absent in control chromosomes) and five polymorphisms (changing in DNA sequence that doesn't modify the aminoacidic sequence or is present also in control chromosome), as shown in tab. 3. The first mutation, found in patient II-2 of the first pedigree (Fig.3, panel A) is located in exon 10 and is a substitution of a glicine with a proline (A320P) in a repeated domain of the protein (Fig.4).

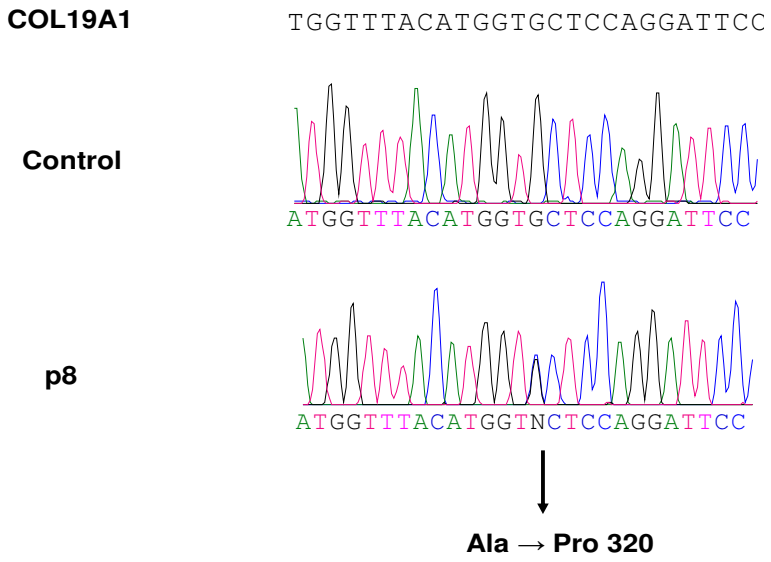


Fig. 4. Mutation in exon 10 found in one patient affected by esophageal achalasia

The sequencing of COL19A1 in the pedigree didn't show any mutation in the relatives (Fig. 3, panel A).

The second mutation, found in patient II-2 (Fig.3, panel B) is a deletion of three nucleotides, that cause the deletion of a glutamine at position 712 of the protein (Fig.5).

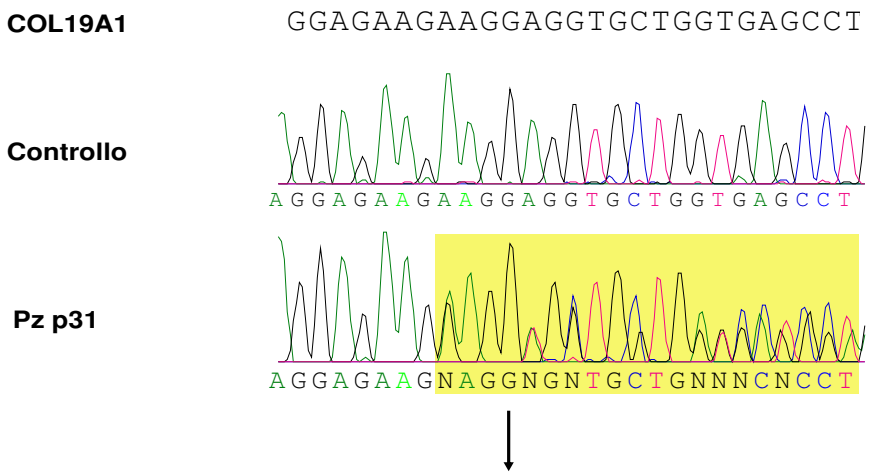


Fig. 5. Mutation in exon 32 found in one patient affected by esophageal achalasia and his homozygous twin.

In this second pedigree, nobody carried the mutation found except for subject (II-2), monozygous twin of our patient, but he wasn't apparently affected by EA (Fig.3, panel B). He will soon undergo an esophageal manometry to exclude any functional anomaly.

Both the mutations found have been tested in 400 control chromosome and no one was revealed.

Fragment	*Exon*	*Results*
10	10	One patient (II-2, Fig.3, panel A): with an heterozygous nucleotide substitution G>C (G958C) p.A320P *(1° mutation)*
12	12	Seven patients with an heterozygous nucleotide substitution C>G (C1055G) also present in the control chromosome p.G355A (polymorphism)
15	15	One patient with a double substitution in heterozygous GC>CT in intron 15 (ISV15+10-11GC>CT)
24	25	Five patients with an heterozygous nucleotide substitution G>A (G1689A) p.P563P (polymorphism) Eleven patients with an homozygous thymine insertion in position 279482 in intron 25 (ISV25+29insT)
28	32-33-34	One patient (II-2, Fig.3, panel B) with a three base deletion (AGA) in exon 32 (2136-2138delAGA) p.E712del *(2^a mutation)* The same patient carries an heterozygous nucleotide substitution in exon 34 C>T (C2289T) present also in control chromosome. p.G762G, (polymorphism)
39	49	Three patients with a thymine deletion in intron 48 (ISV48-50delT).
40	50-51	One patient with a thymine deletion in intron 50 (ISV50-12delT)

Tab.3. Polymorphisms and mutations found in COL19A1 gene in patients affected by EA.

Although, two aminoacidic changes found in the patients are absent in 400 normal chromosome, the involvement of COL19A1 in the pathogenesis of EA in humans still remains to be demonstrated. To this end, it will be necessary to increase number of patient screened and to analize the in vitro effects of our mutations maybe by using a knock-in animal model.

5. INHERITED POLYPOSIS SYNDROMES.

5.1 Clinical and genetics of polyposis in children.

The inherited polyposis syndromes are a group of conditions in which multiple gastrointestinal polyps occur in the lumen of the gastrointestinal tract. (Tab.4)

Familial adenomatous polyposis • Gardner syndrome • Turcot syndrome • FAP Hamartomatous syndrome • Familial juvenile polyposis • Peutz-Jeghers syndrome • Codwen syndrome • Others (MEN2B, neurofibromatosis, basal cell nevus)

Tab. 4 Classification of inherited polyposis syndromes

These conditions have an autosomal pattern of inheritance and exhibit an increased risk of colon cancer , otherwise benign and malignant extraintestinal tumors might be observed. In childhood, gastrointestinal polyps are common and are almost always isolated benign lesion, but it is very important to make an accurate diffential diagnosis between these form and the inherited ones.

A fundamental property of normal cellular growth regulation is the delicate counterbalance of growth promotion and growth inhibition. In the broadest sense, a gastrointestinal polyp results from a defect in this highly regulated process. This may occur by either aberrant “gain-of-function” in a growth-promoting protein (oncogene) or “loss-of-function” in a growth-inhibitory protein (tumor suppressor gene). Oncogene are most often activated and tumor suppressor gene are most often inactivated, although additional molecular and cellular mechanisms favoring polyp formation may occur. The genetic gastrointestinal polyposis syndromes most often result from a germline (inherited) inactivating mutation of a tumor suppressor gene. Eventual somatic mutation of the normal remaining allele results in loss of normal growth constraint and creates a selective growth advantage, unchecked growth and polyp formation. Progression of a polyp to a gastrointestinal cancer requires the sequential acquisition of additional genetic defects (fig.6). Recognition of this “adenoma-to-carcinoma” sequences has been a major advance in the biology of gastrointestinal neoplasia (70).

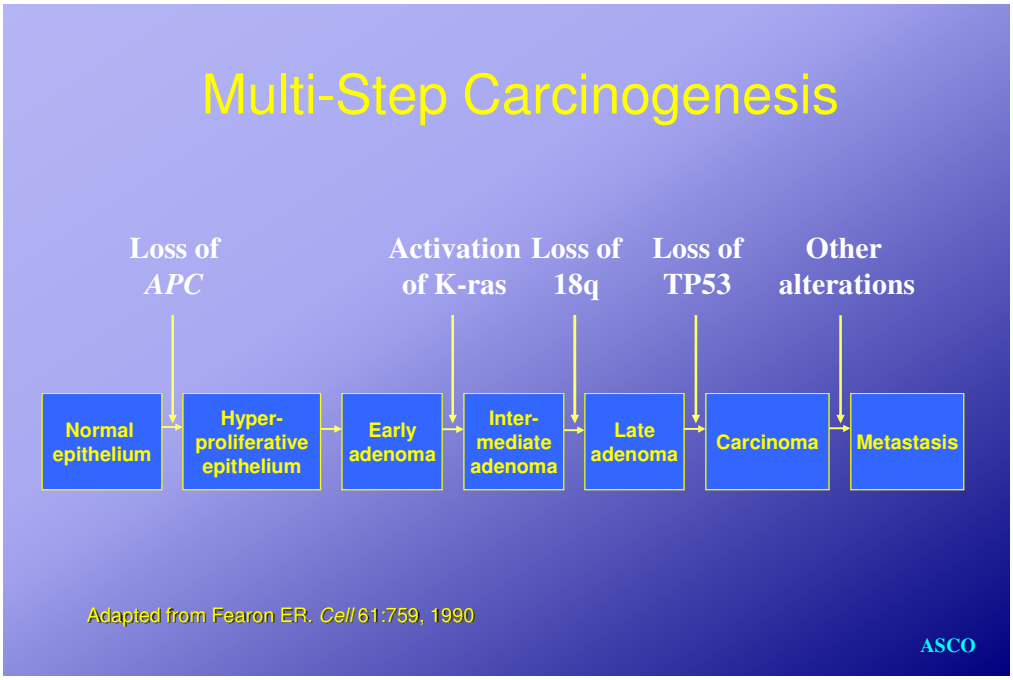


Fig. 6. Multi-step carcinogenesis hypothesis

Familial adenomatous polyposis (FAP, OMIM 175100) is the most common genetic polyposis syndrome. Inheritance is autosomal dominant, although spontaneous mutations account for approximately 30% of cases. The average age at the onset is 16 years. The occurrence of upper gastrointestinal adenomas confers a statically increased risk of duodenal and periampullary carcinomas. Progression to neoplasia is considered inevitable by the fifth decde in patients with FAP.

Extra-intestinal manifestations of FAP include desmoids tumors, epidermoid cysts, osteomas, congenital hypertrophy of

retinal pigment epithelium, fibromas and lipomas, all of which are benign, but may signal the presence of FAP in at-risk patients.

The genetic abnormality in FAP is germline defect in APC, a tumor suppressor gene that maps to chromosome 5q21 (71). APC is a cytoplasmic protein that binds and regulates the degradation of β-catenin, a protein with multiple functions, including regulation of cytoskeletal organization, organization of tissue architecture, cell migration and adhesion, intracellular signaling and gene transcription (72) (Fig. 7).

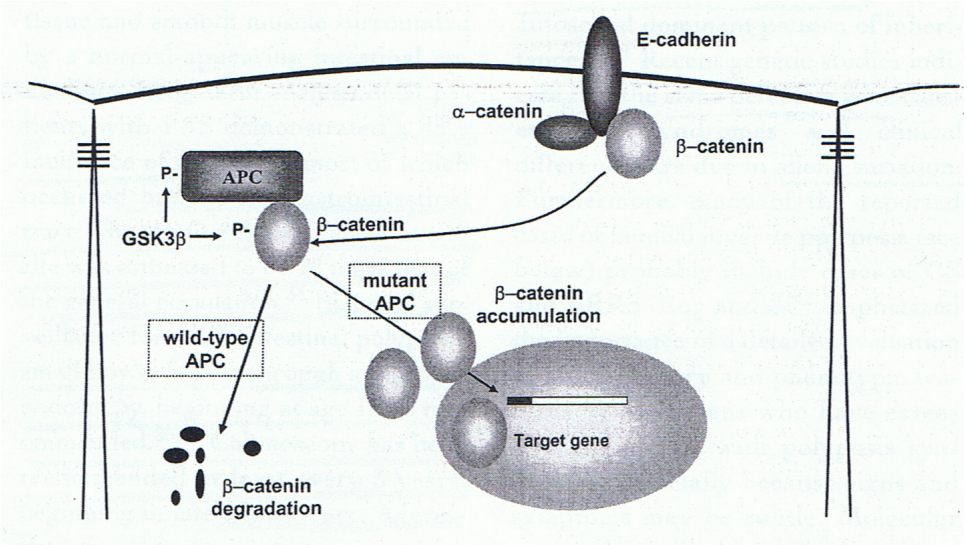


Fig. 7. Regolazione delle β-catenine da parte di APC normale e mutato

Although more than 400 APC mutations have been found, most occur in a mutation cluster region located upstream of domains responsible for β-catenin degradation. Thus the accumulation of β-catenin is the likely cellular event leading to tumor development.

Correlation exist between the location of mutation in the APC gene and the clinical presentation of FAP, as shown in the picture (Fig.8).

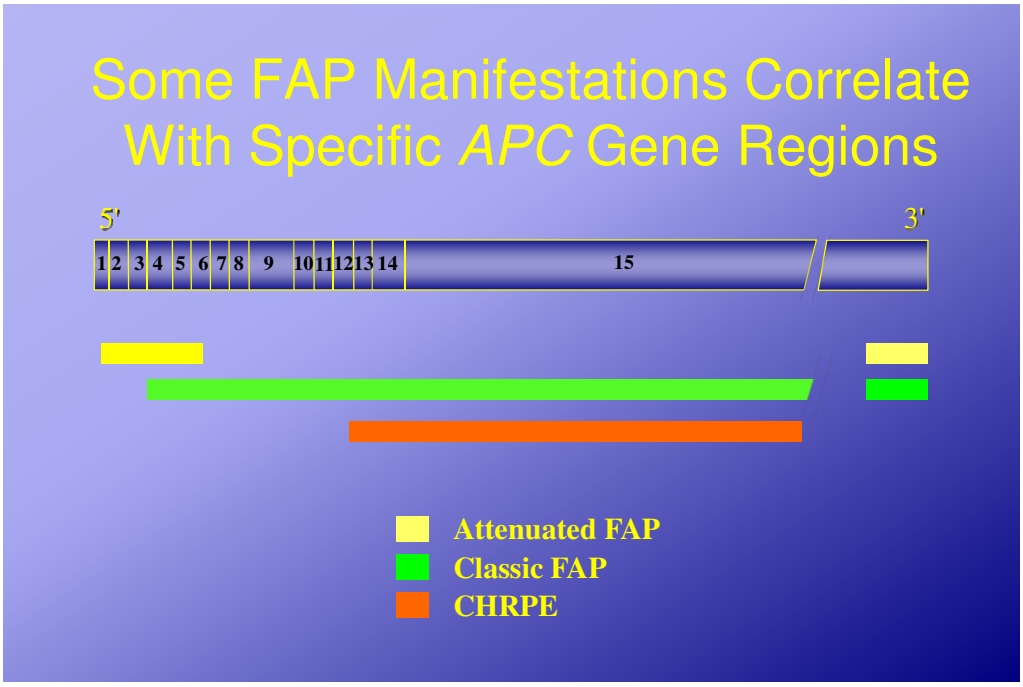


Fig. 8. Genotype-Phenotype correlation in FAP.

APC mutations can be directly detected in peripheral blood lymphocytes by the commercially available in vitro protein truncation assays (sensitivity and specificity 80-90%). Identification of an APC mutation merits genetic counseling and testing of first-degree relatives. Annual flexible sigmoidoscopy should be performed in person with a mutant allele. Individuals with negative test results but a family member with identified APC mutation can be relieved of the burden of annual examinations,

because of a cancer risk equivalent to that of the general population. Screening of at-risk children should occur at approximately age 8 to 10 years.

5.2 Case report published.

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Letter to the Editor

A dramatic case of early-onset familial adenomatous polyposis

To the Editor:

Familial adenomatous polyposis (FAP) is an inherited predisposition to the development of hundreds to thousands of adenomatous polyps in the colon. Symptoms usually arise in the third decade of life, and the median age for the diagnosis of colon cancer is 35–40 years (1).

FAP is caused by an autosomal dominant mutation in the adenomatosis polyposis coli (*APC*) gene, located on the long arm of chromosome 5 (5q21). More than 730 germline mutations have been identified; most of these are nonsense or frameshift mutations that result in a premature stop codon, thus producing a truncated, inactive APC protein (2). The preferred genetic test for FAP is the protein truncation test (PTT), a test that can detect more than 80% of all germline mutations.

Symptomatic young patients have been described previously, but always in the context of a positive family history (3, 4). We report a severe case of FAP with onset in the toddler years, which represents a *de novo* mutation.

A 10-year-old female was evaluated for anemia and an 8-year-long history of recurrent hematochezia. Family history was negative for FAP, other polyposis syndromes, or colon cancer. The result of physical examination was normal. Laboratory evaluation revealed a hemoglobin level of 10.9 g/l, MCV of 68, and ferritin of 8 mg/dl.

Total colonoscopy revealed hundreds of sessile polyps measuring up to 1.0 cm, with pancolonic involvement. Histology confirmed classic adenomatous polyps, with an evidence of moderate dysplasia in some biopsies. Esophagogastroduodenoscopy (EGD) revealed polyps in the gastric fundus and duodenum, and histological examination showed adenomatous polyps without extension beyond the muscularis mucosae. Because of dysplasia in some of the colonic biopsies, the patient was referred for total proctocolectomy and ileoanal anastomosis with mucosectomy. She has remained well since then, with the ongoing EGD surveillance of the upper gastrointestinal mucosa.

Mutation analysis was performed in the proband and both parents, with the APC PTT, single-strand conformational polymorphism (SSCP) and direct sequencing of amplified fragments of the gene, according to previously reported methods (4–6) (Fig. 1). With this method, we identified the causative mutation in the proband, i.e. a 5-bp deletion at nucleotides 3927–3931 (AAAGA) called '3927–3931delAAAGA' and previously described by Miyoshi et al. (7), which resulted in a premature stop after codon 1309. As shown in the Fig. 2, PTT of the *APC* gene (exon 15, fragment 15ET7-15J, including nucleotides from 3085 to 5103) showed an expected normal polypeptide of about 74 kDa, as well as a truncated polypeptide of about 31 kDa. SSCP analysis showed a variant conformer of exon 15 (fragment G), and direct sequencing of this fragment confirmed the AAAGA deletion at nucleotides 3927–3931 (Fig. 3). Molecular diagnosis of both parents showed that they were not carriers of the mutation. Therefore, the APC mutation in the proband is a *de novo* mutation. In fact, microsatellite analysis of 15 markers (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D1BS51, D5S818, and FGA) and the amelogenina locus, performed on DNA from the parents and proband, excludes the possibility of a non-paternity case.

We have described a patient with severe FAP, caused by a deletion of bases AAAGA from sites 3927 to 3931 on the *APC* gene. Twenty percent of all FAP pedigrees are associated with this mutation, which typically results in severe and early-onset disease, often associated with congenital hypertrophy of the retinal pigment

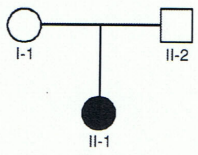

Fig. 1. Molecular diagnosis of familial adenomatous polyposis: Pedigree of the family.

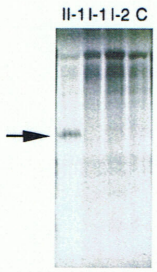

Fig. 2. Protein truncation test analysis of the adenomatosis polyposis coli gene (exon 15). The arrow indicates the truncated polypeptide present in the proband (II-1) and absent in her parents (I-1 and I-2) and normal control (C).

epithelium (CHRPE) (8). The development of polyps with dysplasia is rare in the first decade of life, but when it occurs, it is usually associated with the 3927–3931delAAAGA mutation (Fig. 4).

Distante et al. (3) reported an affected family in which a 5-year-old girl presented with hematochezia due to extensive colonic polyposis; her father had required colectomy for FAP at the young age of 23 years.

Phenotype is clearly dependent on genotype in FAP, in regard to both severity and extracolonic manifestations of the disease, commonly referred to as Gardner syndrome. However, the APC mutation site cannot explain observed differences in phenotype within families or between unrelated FAP patients with identical mutations. Although changes in environment and clinical practice probably account for some phenotypic variation, it is tempting to believe that the action of some modifier gene affects phenotype as well (9).

Genetic testing for FAP has become an important clinical tool in the past decade; indeed,

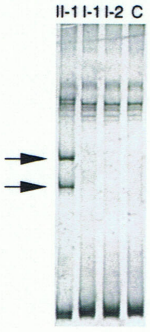

Fig. 3. Single-strand conformational polymorphism analysis of the adenomatosis polyposis coli gene (exon 15 fragment G). The arrows indicate the alterated conformers present in the proband (II-1) and absent in her parents (I-1 and I-2) and normal control (C).

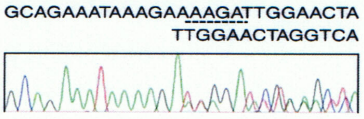

Fig. 4. Sequence analysis of the proband's amplified DNA (adenomatosis polyposis coli gene, exon 15 fragment G) reveals the presence of the 3927–3931delAAAGA mutation.

because of the risk of early-onset disease and precocious colorectal cancer, testing of family members is now the standard of care. When used appropriately, the APC-truncated protein product test can identify more than 80% of affected individuals; however, there are false-negative results, and most experts recommend genetic counseling to go along with the testing. Giardiello et al. (10) evaluated the appropriateness of *APC* gene testing, as well as the use of informed consent and genetic counseling prior to testing, and found that most testing was done for appropriate indications (clinical features of FAP or at risk of the disease due to affected first-degree relatives), but that informed consent or genetic counseling was rarely provided (<20% of patients). Even more concerning was the fact that more than 30% of the test results were incorrectly interpreted by the ordering physician.

The novelty of the present case report lies in the very young age at initial presentation (age of 2 years), as well as the fact that there was no family history of FAP, thus delaying the diagnosis for many years.

Therefore, it is important that a child with iron-deficiency anemia and a long history of rectal bleeding has an early and adequate endoscopic investigation in spite of a family history of FAP.

The severity of our patient's colonic disease, including the presence of dysplasia in the biopsy specimens, should prompt pediatricians to have a higher index of suspicion for FAP in children, even in the absence of family history.

All pediatric patients with FAP warrant upper gastrointestinal screening and surveillance endoscopy from the time of initial colonoscopy irrespective of referable symptoms. Patients with APC mutation between codons 1225 and 1694 may be more susceptible to aggressive gastroduodenal involvement in FAP (11).

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Letter to the Editor

References

1. Peterson G, Slack J, Nakamura Y. Screening guidelines and premorbid diagnosis of FAP using linkage. Gastroenterology 1991: 100: 1658.
2. Fearnhead NS, Britton MP, Bodmer WF. The ABC of APC. Hum Mol Genet 2001: 10: 721–733.
3. Distante S, Nasioulas S, Somers GR et al. Familial adenomatous polyposis in a 5 year old child: a clinical, pathological, and molecular genetic study. J Med Genet 1996: 33 (2): 157–160.
4. Eccles DM, Lunt PW, Wallis Y et al. An unusually severe phenotype for familial adenomatous polyposis. Arch Dis Child 1997: 77: 431–435.
5. Roest PA, Roberts RG, Sugino S et al. Protein truncation test (PTT) for rapid detection of translation-terminating mutations. Hum Mol Genet 1993: 2 (10): 1719–1721.
6. Groden J, Gelbert L, Thliveris A et al. Mutational analysis of patients with adenomatous polyposis: identical inactivating mutations in unrelated individuals. Am J Hum Genet 1993: 52 (2): 263–272.
7. Miyoshi Y, Ando H, Nagase H et al. Germ-line mutations of the APC gene in 53 familial adenomatous polyposis patients. Proc Natl Acad Sci USA 1992: 89 (10): 4452–4456.
8. Gayther SA, Wells D, SenGupta SB et al. Regionally clustered APC mutations are associated with a severe phenotype and occur at a high frequency in new mutation cases of adenomatous polyposis coli. Hum Mol Genet 1994: 3 (1): 53–56.
9. Houlston R, Crabtree M, Phillips R et al. Explaining differences in the severity of familial adenomatous polyposis and the search for modifier genes. Gut 2001: 48: 1–5.
10. Giardiello FM, Brensinger JD, Petersen GM. AGA technical review on hereditary colorectal cancer and genetic testing. Gastroenterology 2001: 121 (1): 198–213.
11. Attard TM, Cuffari C, Tajouri T, et al. Multicenter experience with upper gastrointestinal polyps in pediatric patients with familial adenomatous polyposis. Am J Gastroenterol 2004: 99 (4): 681–686.

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6. GENE THERAPY IN THE GUT

Gene therapy is an experimental approach to treat human diseases based on the transfer of genetic material to the cells. The transferred genetic material can be DNA or RNA. Most commonly it is a natural gene but it can also be a chimeric gene or a gene encoding for a regulatory RNA. A cell is said to be transduced when it has incorporated and expresses a foreign gene (73) To facilitate cell transduction, the genetic material is packaged into molecular constructs named vectors, which can be of viral (74) and non-viral nature(75).

For a gene to be expressed inside a cell its coding DNA sequence should be linked to appropriate regulatory DNA sequences. Gene promoters (and other regulatory elements) may allow transgene expression in every transduced cell (universal promoters) (76) or, alternatively, only in selected cells containing transcription factors able to interact specifically with the promoter (as in the case of tissue specific promoters)(77) On the other hand, promoters may determine a continuous and fixed expression of the transgene in the transduced cell or, alternatively, promoter activity can be sensitive to certain drugs that when given to the patient can modulate promoter function, permitting regulatable expression of the transgene (78) The most challenging issues for a successful

application of gene therapy to treat human diseases concern the choice of the relevant therapeutic gene, of appropriate promoter and regulatory sequences and of an effective vector for delivering the transgene or trangenes into target cells. Thus gene therapy is a matter of genes, vectors, promoters, and regulatory elements. Promoter and vector features determine transduction efficacy and specificity, duration of transgene expression, and eventually appearance of side effects.

Viral vectors are the most efficient vehicles for gene transfer. Different viruses have served to construct gene therapy vectors, including adenoviruses(79), retroviruses (80) (including lentivirus), AAV(81), herpesvirus (82), baculovirus(83), SV40 virus (84), vacciniavirus(85) and others. The list of viral vectors is still expanding and modifications of already existing systems will increase the number of potential applications of gene therapy.

Murine retroviruses are single stranded RNA viruses, which after interaction with a retrovirus receptor can integrate in the genome of a dividing cell. Cells that do not proliferate actively under physiological conditions, such as hepatocytes, are difficult to transduce with retroviral vectors. Furthermore, transduction efficacy is limited by the low titre of virus obtained with the production procedures currently used (80). The recent development

of human lentiviral (human immunodeficiency virus) based vectors offers promising perspectives for gene transfer into non-dividing cells

AAV are non-pathogenic human parvoviruses that, after deletion of all viral genes except ITR, have been used successfully as gene therapy vectors. AAV vectors do not induce significant immune response in animal models and are able to transduce dividing and non-dividing cells. The AAV genome persists mainly as episome in the target cell nucleus, although a minority of it can integrate in the host chromosome (86). After systemic injection AAV demonstrate significant liver tropism(87).

Why could be important to find a strategy to trasduce stably the gut? First of all there are a number of disorders of the intestinal epithelium that could be amenable to gene therapy. In addition, the intestine could be used as an alternative site for the production of proteins that need to be secreted to the blood for the correction of disorders such as haemophilia.

The intestinal tract has many features that make it an attractive target for therapeutic gene transfer: (a) easy accessibility via the intestinal lumen; (b) large surface area of the epithelium; (c) the possibility of in situ gene transfer by endoscopy; (d) known location

of stem cells within the intestinal crypt, (e) intestinal cells can secrete foreign protein into the circulation.

As the epithelium turns over rapidly (two to four days), the ideal targets for a stable gene transfer would be the intestinal stem cells. Permanent gene transfer is conceivable if the gene of interest can be integrated into intestinal stem cells. Accessibility of these cells to vectors delivered into the intestinal lumen is limited by their deep location and by the mucus that lines the epithelium. In vitro experiments have shown that intestinal mucus can be solubilised by a variety of agents including proteases, detergents, and sulfhydryl compounds (88).

Several methods of gene transfer to the GI tract have been used. Most of the techniques target the epithelium but a submucosal approach may transduce cells of the muscularis mucosae(89). It has been demonstrated in vivo gene transfer to various locations of the gastrointestinal tract such as oesophagus, stomach, and colon using cationic liposomes.(90,91). Gene transfer was achieved by luminal instillation using catheter infusion. A high efficiency of transfection was observed in colonic epithelium, with near 100% of epithelial cells expressing the transgene. Transgene expression was transient and did not persist beyond four days, a finding that is consistent with the normal turnover time of gut epithelium.

Genes can be transferred into the intestinal epithelium using retroviral vectors introduced intraluminally (92). Given the continual proliferation of this tissue, these vectors would be an appropriate choice because of their ability to transduce dividing cells. However, transduction efficiency is comparatively low in the intestine of rat and mice and thus retroviral vectors would not satisfy the requirements of gene therapy in humans.

Adenoviral vectors have been shown to transduce intestinal cells in vivo when administered through an oral-duodenal tube (93). The considerable transduction efficiency of adenoviruses most probably reflects the fact that the intestine is a normal site of infection for this virus. Transfer of genetic material with adenoviruses is more successful in the small intestine as compared with the colon. Interestingly, single or repeated challenge with adenoviral vector did not cause increased host immune responses to this virus, suggesting that this type of vectors could be a good candidate for gene therapy of intestinal diseases (93). Also it has been demonstrated that adenovirus mediated gene transfer to intestinal epithelial cells can effectively deliver proteins to the circulation indicating that intestinal cells have the potential to be used as heterotopic sites for production of peptidic hormones, cytokines, and proteins that should function in plasma (94).

Another promising vector for gene transfer to the gut is the one based on AAV. It has been demonstrated that an orally administered AAV vector leads to persistent expression of a β-galactosidase transgene in both gut epithelial and lamina propria cells, and that this approach results in long term phenotypic recovery in an animal model of lactose intolerance (95). These data were not confirmed by , but considering the hundred different AAV serotype today known, with different tropism, we can suppose that some of them could have an intestinal tropism and could be useful for intestinal gene therapy.

Another singularity that makes AAV especially suitable as an orally delivered vector is its hardiness being very resistant to changes in pH and temperature and to solvents. The persistent and stable gene expression for almost six months is also another interesting feature. As enterocyte turnover is three to five days, this prolonged expression indicates that progenitor cells lying within the crypts can be transduced by AAV vectors.

All these features make AAV an attractive vector for GI gene transfer with potential application for vaccination purposes and for maintained protein replacement, particularly when the release of the protein into the portal circulation is a desired goal.

6.1 Our experience.

To trasduce in a stable way the intestinal ephitelium, we performed two different strategies: the first was to use serotype of AAV different from that already described in the literature (AAV 2/2), particularly we used AAV 2/1, 2/5, 2/7, 2/8; the second one consisted in the injection of a modified lentivirus, with a Spike envelope, that showed a high intestinal tropism when parenteral injected.

In the AAV experiments, we performed a intraduodenal injection of of 50-100 x 10^5 particelles of viral vectors, carring EGFP, in C57/BL6 mice at 4 week of life. We sacrificed the animals 4 week after the injection and then we analized the expression of EGFP in the intestine (duodenum and colon). No differences in the protein expression was observed between animals injected with viral vector and PBS.

In the second type of experiments, we used the modified lentivirus vector expressing the LAC-Z protein. We performed again a intraduodenal injection (50-100 x 10^5 particelles) in the same condition of the first experiments and then we sacrified the animals at 3^{rd} , 21^{st} and 28^{th} days from the injection. We collected duodenum, ascendant colon and rectum from each animal to X-Gal detection.

At 3rd day in the duodenum and at 21th day in the rectum we observed an increased activity of X-Gal , but we were not able to reproduce these results in a second series of experiments, using EGFP modified lentivirus vector.

The most relevant difficulties for the viral vector to trasduce the intestinal has probably been the location of the undifferentiated cell, located in the deepest part of the crypts, and maybe the low intestinal tropism of the CMV promoter. So, we are now hypnotizing to reduce intestinal obstacles using some molecules that can increase intestinal permeability (AT-100) or reduce the intestinal mucus. Another aim of our future work will be to create a new AAV expressing a different promoter with an higher intestinal tropism, such as intestinal fatty acid binding protein (IFABP), that is expressed in the intestinal crypts.

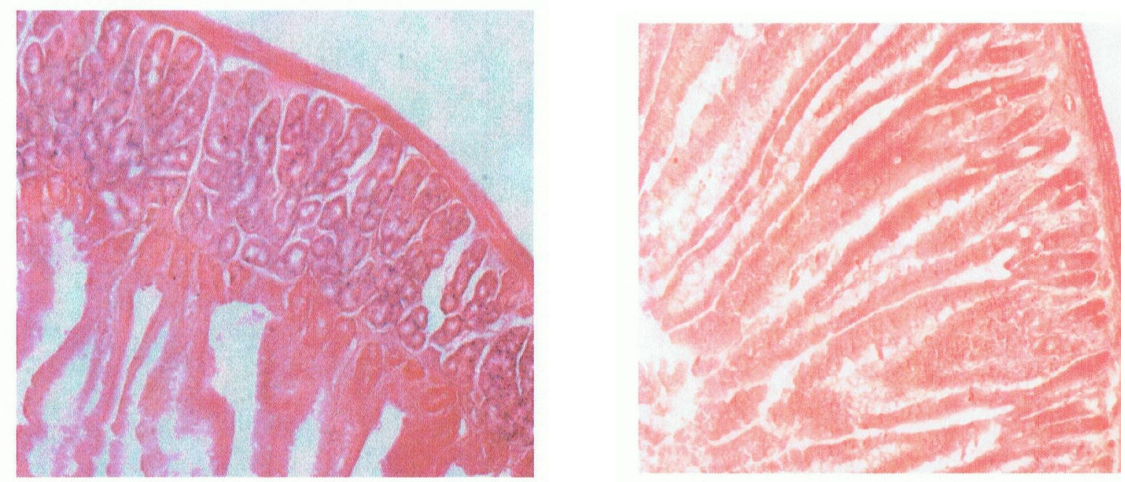

Fig. 9. Duodenum from C57/B6 mouse after 3 days of injection of modified lentivirus vector expressing LacZ (left) and injected of PBS (right).

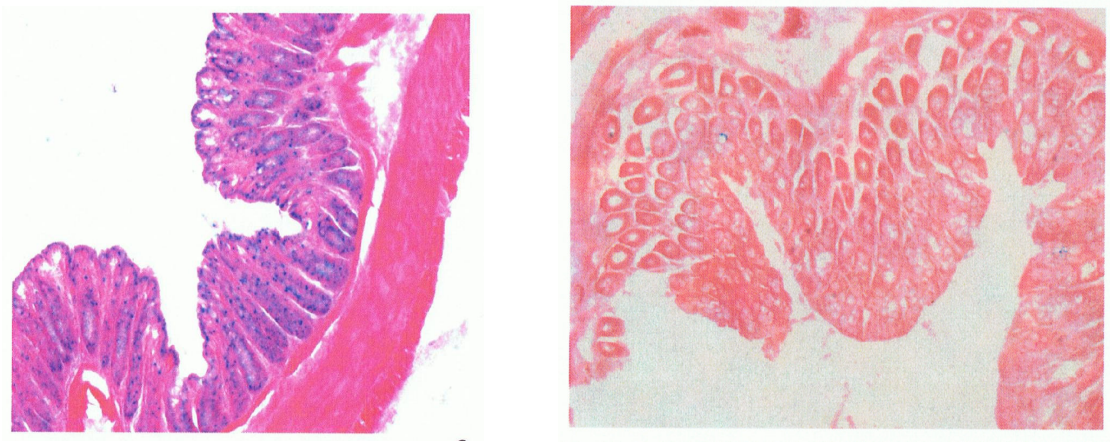

Fig. 10. Rectum from C57/B6 mice after 21 days of injection of modified lentivirus vector expressing LacZ (left) and injected of PBS (right).

7. CONCLUSION.

In this thesis we investigated about the clinical and genetic aspects of some intestinal dismotility disorders and of inherited poliposis syndromes. We have demonstrated the involvement of FLNA gene in the pathogenesis of an X-linked form of intestinal pseudo-obstruction. We have also hypnotized a possible rule of COL19A1 gene in the some form of esophageal achalasia. In the future, we would like to create animal models for the mutations found to elucidate more in details the involvement of these genes in the pathogenesis of the studied disorders.

Furthermore, we have described the clinical phenotype of HSCR pediatric patients and we performed the genetic screening of SOX10 and ZFX1B in these patients.

In the last part of the work, we tried to stably trasduce the intestinal epithelium using AAV and lentivirus vectors. We would like to perform future experiments using substances that are able to increase intestinal permeability and to reduce intestinal mucus, so that viral vector can easily arrive at the undifferentiated cell in the crypts. Otherwise, we will create new AAV vector with a promoter (IFABP) with an higher intestinal tropism.

8. ACKNOWLEDGEMENTS

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9. BIBLIOGRAPHY

1. Hansen MB. Neurohumoarl control of gastrointestinal motility. Physiol Res 2003;52:1-30.
2. Hansen MB, et al. Profile of neurohumoral agent on mesenteric and intestinal blood flow in health and disease. Physiol Res 1998;47:307-27.
3. Cooke A. Neurotransmitters in neuronal reflexes regulating intestinal secretion. N Y Acad Sci 2000;915:77-80.
4. Neurogastroenterology:From the basics to the clinics. Falk Symposium 112th. Freigburg, Germany:1999.
5. Taviras S, Pachinis V. Development of the mammalian enteric system. *Curr Opin Genet Dev* 1999; 9:321.
6. Lyonnet S, Bolino A, Pelet A, et al: A gene for Hirschsprung disease maps to the proximal long arm of chromosome 10. *Nat Genet*, 1993; 4: 346-350.

7. Gershon MD, Chalazonitis A, Rothman TP. From neural crest to bowel: development of the enteric nervous system *J Neurobiol* 1993; 24: 199-214.
8. Badner JA, Sieber Wk, Garver KL, et al: A genetic study Of Hirschsprung's Disease. *Am J Hum Genet* 1990; 46: 568-580.
9. Staiano A, Corazziari E, Andreotti MR, Clouse RE. Esophageal motility in choldren with Hirschsprung' s disease. *Am J Dis Child* , 1991; 145: 310-313.
10. Larsson LT, Shen Z, Ekbland E, Sundler F, Alm P, Andersson Ke. Lack of neuronal nitric oxide synthase in nerve fibers of aganglionic intestine: a clue to Hirschsprung's disease. *J Pediatr Gastroenterol Nutr* 1995; 20: 49-53.
11. Meier-Ruge W: Uber Ein Erkrankungsbild des colon mit Hirschsprung-Symptomatik.Vehr Dtsch Ges Pathol ,1971; 55:506-510.
12. Lake BD, Puri P. Nixon HH, Claireaux AE. Hirschsprung's disease. An appraisal of histochemically demonstrated acetylcholinesterase activity in suction rectal biopsy specimens as an aid

to diagnosis. *Arch Path Lab Med*, 1978; 26: 288-291.

13. Elhalaby EA, Coran AG, Blane CE, et al: Enterocolitis associated with Hirschsprung's disease: a clinical- radiological characterization based on 168 patient. *J Pediatr Surg*: 1995; 30: 1023-1027.
14. Swenson O, Fisher JH: Hirschsprung's disease during infancy. *Surg Clin North Am*, 1956; 36: 115-122.
15. Marty TL, Matlak ME, Hendrickson M et al: Unexpected death from enterocolitis after surgery for Hirschsprung's disease. *Pediatrics*, 1995; 96: 118-121.
16. Loening-Baucke V. Modulation of abnormal defecation dynamics by biofeedback treatment in chronically constipated children with encopresis. *J Pediatr* 1990; 116:214-222.
17. Taxman TI, Yulish BS, Rothstein FC. How useful is barium enema in diagnosis of infantile Hirschsprung's disease*? Am J Dis Child,* 1986; 140:881-884.

18. Brooks AS, Breuning MH, Meijers C. Spectrum of phenotypes associated with Hirschsprung disease: an evaluation of 239 patients from a single institution. *The Third International Meeting : Hirschsprung disease and the correlated neurocristopathies.* Evian, France.1998

19. Puffenberger EG, Kauffman ER, Bolk S, Matise TC, Washington SS, Angrist M, Weissenbach J, Garver KL, Mascari M, et al. Identity-by-descent and association mapping of a recessive gene for Hirschsprung disease on human chromosome 13q22. *Hum Mol Genet* 1994; 3: 1217-1225.

20. Decker RA, Peacock ML, Watson P. Hirschsprung disease in MEN 2A: increased spectrum of RET exon 10 genotypes and strong genotype-phenotype correlation. *Hum Mol Genet* 1998;7:129-134.

21. Edery P, Attie T, Amiel J, Pelet A, Eng C, Hofstra RM, Martelli H, Bidaud C, Munnich A, Lyonnet S. Mutation of the endothelin-3 gene in the Waardenburg-Hirschsprung disease (Shah-Waardenburg syndrome). *Nat Genet* 1996;12:442-444.

22. Auricchio A, Griseri P, Carpentieri ML, Betsos N, Staiano A, Tozzi A, Priolo M,Thompson H, Bocciardi R, Romeo G, Ballabio A, Ceccherini I. Double eterozygosity for a RET substitution interfering with splicing and an EDNRB missense mutation in Hirschsprung disease. Am J Hum Genet. 1999 Apr;64(4):1216-21.

23. Southard-Smith EM, Angrist M, Eleison JS,Et al. The Sox10 (Dom) mouse: modeling the genetic variation of Waardenburg-Shah (WS4) syndrome. *Genom Res* 1999; 9:215-225.

24. Di Lorenzo C. Pseudo-obstruction: current approaches. Gastroenterology 1999; 116:980-7.

25. Di Lorenzo prima, Mousa H, Hyman PE, Cocjin J, Flores AF, Di Lorenzo C. Long-term outcome of congenital intestinal pseudo-obstruction. Dig Dis Sci 2002;47: 2298-305.

26. Heneyke S, Smith VV, Spitz L, Milla PJ. Chronic intestinal pseudo-obstruction:treatment and long term follow-up of 44 patients. Arch Dis Child 1999;81-21-7.

27. Di Lorenzo C. Pseudo-obstruction: current approaches. Gastroenterology 1999; 116:980-7.

28. Rudolph CD, Hyman PE, Altschuled SM et al. Diagnosis and treatment of chronic intestinal pseudo-obstruction: report of consensus workshop. J Pediatr Gastroenterol Nutr 1997;24:102-12.

29. Sigurdsson L, Reyes J Kocoshis SA et al. Intestinal transplantation in children with chronic intestinal pseudo-obstruction. Gut 1999;45:570-4.

30. Stanghellini V, Camilleri M, Malagelada JR. Chronic idiopathic intestinal pseudo-obstruction:clinical and intestinal manometric findings. Gut 1987;28:5-12.

31. Tanner MS, Smith B, Llyod JK. Functional intestinal obstruction due to deficiency or argyrophil neurons in the myenteric plexus: familial syndrome presenting with short small bowel, malrotation and pyloric hypertrophy. Arch Dis Chil 1976;51:837-841.

32. Auricchio A, Brancolini V, Casari G, Milla PJ, Smith VV, Devoto M, Ballabio A. The locus for a novel syndromic form of neuronal intestinal

pseudo-obstruction maps to Xq28. Am J Hum Gnete 1996;58:743-748.

33. FitzPatrick DR, Strain L, Thomas AE, Barr DGD, Todd A, Smith NM, Scobie WG. Neurogenic chronic idiopathic intestinal pseudo-obstruction, patent duct arterious and thrombocytopenia segregation as an X linked recessive disorder. J Med Genet 1997;34:666-669.

34. Koshy SS, Nostrant TT. Pathophysiology and endoscopic/balloon treatment of esophageal motility disorders. 1997 *Surg Clin North Am* 77: 971-992.

35. Birgisson S, Richter JE. Achalasia: what's new in diagnosis and treatment? 1997 *Dig Dis Sci* 15:1-27.

36. Berquist WE, Byrne WJ, Ament ME, Fonkalsrud EW, Euler AR. Achalsia: diagnosis, management and clinical course in 16 children. 1983 *Pediatrics* 71:798-805.

37. Mayberry JB, Mayell Mj. Epidemiological study of achalsia in children. 1998 *Gut* 29:90-93.

38. Kahrilas Pj. Esophageal motilità disorders: current concepts of pathogenesis and treatment. 2000 *Can J Gastroenterol* 14:221-231.

39. Goldblum JR, Rice TW, Richter JE. Histopatologic features in esophagomyotomy specimens from patients with achalasia. 1996 *Gastroenterology* 111:648-654.

40. Clark SB, Rice TW, Tubbs RR, Richter JE, Goldblum JR. The nature of the myenteric infiltrate in achalsia: an immunohistochemical analysis. 2000 *Am J Surg Pathol*; 24 (8): 1153-8.

41. Baiocco A, Boujon CE, Hafeli W, Cherubini YD, Sestetti GE, Rossi GL. Megaoesophagus in rats: a clinical, pathological and morphometrical study. 1993 *J Comp Pathol* 108 (3): 269-281.

42. Ruiz-de-Leon A, Mendoza J, Sevilla-Mantilla C, Fernandez AM, Perez-de-la-Serna J, Gonzales VA, Rey E, Figueredo A, Diaz-Rubio m, De-la-Concha EG. Myenteric antiplexus antibodies and II HLA in achalasia. 2002 *Dig Dis Sci* 47 (1):15-19.

43. Bosher, L. P.; Shaw, A. Achalasia in siblings: clinical and genetic aspects. *Am. J. Dis. Child.* 135: 709-710, 1981.

44. Polonsky, L.; Guth, P. H. Familial achalasia. *Am. J. Dig. Dis.* 15: 291-295, 1970.

45. Thibert, F.; Chicoine, R.; Chartier-Ratelle, G. Forme familiale de l'achalasie de l'oesophage chez l'enfant. *Un. Med. Canada* 94: 1293-1300, 1965.

46. Vaughan, W. H.; Williams, J. L. Familial achalasia with pulmonary complications in children. *Radiology* 107: 407-409, 1973.

47. Westley, C. R.; Herbst, J. J.; Goldman, S.; Wiser, W. C. Infantile achalasia: inherited as an autosomal recessive disorder. *J. Pediat.* 87: 243-246, 1975.

48. Koivukangas, T.; Simila, S.; Heikkinen, E.; Rasanen, O.; Wasz-Hockert, O. Sjogren's syndrome and achalasia of the cardia in two siblings. *Pediatrics* 51: 943-945, 1973.

49. Dayalan, N.; Chettur, L.; Ramakrishnan, M. S. Achalasia of the cardia in sibs. *Arch. Dis. Child.* 47: 115-118, 1972.

50. O'Brien, C. J.; Smart, H. L. Familial coexistence of achalasia and non-achalasic oesophageal dysmotility: evidence for a common pathogenesis. *Gut* 33: 1421-1423, 1992.

51. McKusick, V. A. Personal Communication. Baltimore, Md., 1985.

52. Maldonado, J. E.; Gregg, J. A.; Green, P. A.; Brown, A. L., Jr. Chronic idiopathic intestinal pseudo-obstruction. *Am. J. Med.* 49: 203-212, 1970.

53. Schuffler, M. D.; Pope, C., Studies of idiopathic intestinal pseudoobstruction. II. Hereditary hollow visceral myopathy: family studies. *Gastroenterology* 73: 339-344, 1977.

54. Baylin, S. B. Personal Communication. Baltimore, Md., 4/22/1989.

55. Lisker, R.; Garcia-Ramos, G.; de la Rosa-Laris, C.; Diaz-Mitoma, F. Peripheral motor neuropathy associated with autonomic dysfunction in two sisters: new hereditary syndrome?. *Am. J. Med. Genet.* 9: 255-259, 1981.

56. Leichter, H. E.; Vargas, J.; Cohen, A. H.; Ament, M.; Salusky, I. B. Alport's syndrome and achalasia. *Pediat. Nephrol.* 2: 312-314, 1988.

57. Stratakis, C. A.; Lin, J.-P.; Pras, E.; Rennert, O. M.; Bourdony, C. J.; Chan, W.-Y. Segregation of Allgrove (triple-A) syndrome in Puerto Rican kindreds with chromosome 12 (12q13) polymorphic markers. *Proc. Assoc. Am. Phys.* 109: 478-482, 1997.

58. Tullio-Pelet, A.; Salomon, R.; Hadj-Rabia, S.; Mugnier, C.; de Laet, M.-H.; Chaouachi, B.; Bakiri, F.; Brottier, P.; Cattolico, L.; Penet, C.; Begeot, M.; Naville, D.; Nicolino, M.; Chaussain, J.-L.; Weissenbach, J.; Munnich, A.; Lyonnet, S. Mutant WD-repeat protein in triple-A syndrome. *Nature Genet.* 26: 332-335, 2000.

59. Weber, A.; Wienker, T. F.; Jung, M.; Easton, D.; Dean, H. J.; Heinrichs, C.; Reis, A.; Clark, A. J. L. Linkage of the gene for the triple A syndrome to chromosome 12q13 near the type II keratin gene cluster. *Hum. Molec. Genet.* 5: 2061-2066, 1996.

60. de la Concha EG, Fernandez-Arquero M, Coenejero L, Lazaro F, Mendoza JL, Sevilla MC, Diaz-Rubio M, Ruiz de Leon A. Presence of a protective allele for the achalsia on the central region of the major histocompatibility complex. 2000 *Tissue Antigens* 56 (2): 149-53.

61. Verne GN, Hahn AB, Pineau BC, Hoffman BJ, Wojciechowski BW, Wu WC. Association of HLA-DR and DQ alleles with idiophatic achalasia. 1999 *Gastroenterology* 117 (1): 26-31.

62. de la Concha EG, Fernandez-Arquero M, Mendoza JL, Coenejero L,Figueredo MA, Perez de la Serna J, Diaz-Rubio M, Ruiz de Leon A. Contribution of the HLA class II genes to susceptibility in achalasia. 1998 *Tissue Antigens* 52(4): 381-4.

63. van der Rest, M., and Garrone, R. (1991) *FASEB J.* 5, 2814-2823.

64. Kivirikko, K. I. (1993) *Ann. Med.* 25, 113-126.

65. Myers, J. C., Yang, H., D'Ippolito, J. A., Presente, A., Miller, M. K., and Dion, A. S. (1994) *J. Biol. Chem.* 269, 18549-18557.

66. Inoguchi, K., Yoshioka, H., Khaleduzzaman, M., and Ninomiya, Y. (1995) *J. Biochem. (Tokyo)* 117, 137-146.

67. Sumiyoshi H, Inoguchi K, Khaleduzzaman M, Ninomiya Y and Yoshioka H. Ubiquitous Expression of the α1(XIX) Collagen Gene (Col19a1) during Mouse Embryogenesis Becomes Restricted to a Few Tissues in the Adult Organism. 1997 *J Biol Chem* 272 (27): 17104-111.

68. Sumiyoshi H, Laub F, Yoshioka H, Ramirez F. Embryonic expression of type XIX collagen is transient and confined to muscle cells. *Dev Dyn.* 2001;220(2):155-62.

69. Sumiyoshi H, Mor N, Lee SY, Doty S, Henderson S, Tanaka S, Yoshioka H, Rattan S, Ramirez F. Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX-rich basement membrane zone. *J Cell Biol.* 2004 Aug 16;166(4):591-600.

70. Kinzler KW, Vogelstein B. Lesson from hereditary colorectal cancer. Cell 1996;87:159-70.

71. Groden J, Thliveris A, Samowitz W, Carlson M, gelbert L, Albertsen H et al. Identification and characterization of the familial adenomatous polyposis coli gene. Cell 1991;66:589-600.

72. O'Sullivan MJ, McCarthy T, Doyle C. Familial adenomatous polyposis: from bedside to benchside. Am J Clin Pathol 1998:109:521-6.

73. Blau HM, Springer ML. Gene therapy- a novel form of drug delivery. N Engl J Med 1995;333:1204-07.

74. Gunzburg WH, salmons B. Virus Vector design in gene therapy. Molecular Medicine Today 1995;1:410-17.

75. Ledley FD. Nonviral gene therapy:the promise of genes as pharmaceutical products. Hum Gnee Ther 1995;6:1129-44.

76. Wilkinson GW, Akrigg A. Constitutive and enhanced expression from CMV major IE promoter in a defective adenovirus vector. Nucleic Acids Res 1992;20:2233-9.

77. Saukkonen K, Hemminki A. Tissue-specific promoters for cancer gene therapy. Expert Opin Biol Ther. 2004 May;4(5):683-96.

78. Clackson T. Regulated gene expression systems. Gene Ther 2000;7:120-5.

79. Chengalvala MV, Lubeck MD, selling BJ et al. Adenovirus vectors for gene expression. Curr Opin Biotechnol 1991;2:_718-22.

80. Kalpana GV. Retroviral vectors for liver directed gene therapy. Semin Liver Dis 1999;19:27-37.

81. Bueler H. Adeno-associated viral vectors for gene transfer and gene therapy. Biol Chem 1999;380:613-22.

82. Kirn DH, McCormick F. Replicating viruses as selective cancer therapeutics. Molecular medicine Today 1996;2;519-27..

83. Boyce FM, Bucher NL. Baculovirus-mediated gene transfer into mammalian cells. PNAS 1996;93:23-48-52

84. Procapio A, Marinacci R, Martinelli MR et al. SV40 expression in human neoplastic and non-neoplastic tissues:perspective on diagnosis,

prognosis and therapy of human malignant mesotelioma. Dev Biol Stand 1998;94:361-7.

85. Grimm CF, Ortmann D, Mahr L, et al. Mouse alpha-fetoprotein specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. Gastroenterology 2000;119:1104-12.

86. Patjin GA, Kay MA. Hepatic gene therapy using adeno-associated virus vectors. Semin Liver Dis 1999;19:61-9.

87. Lau JY. Adenoassociated virus a gene delivery vector for liver cells. J Hepatol 1999;31:385.

88. Mantle M, Mantle D, allen A. Polymeric structure of pig small intestinal mucus glycoprotein. Dissociation by proteolysis or by reduction of disulphide bridges. Bichem J 1981;195:277-85.

89. Takehara T, Hayashi N, Yamamoto M et al. In vivo gene transfer and expression in rat stomach by submucosl injection of plasmid DNA. Hum Gene Ther 1996;7:589-93.

90. Schimd RM, Weicheinbach H, Draenert GF et al. Liposome mediated gene transfer into rat eosophageous. Gut 1997;41:549-56.

91. Westbrook CA, Chruma Sj, Arenas RB, et al. human APC gene expression in rodent colonic ephitelum in vivo using liposomal gene delivery. Hum Mol Genet 1994;3:2005-10.

92. Lau C, Soriano HE, Ledley FD et al. Retroviral gene transfer into the intestinal epithelium. Hum Gene Ther 1995;6:1145-51.

93. Cheng DY, Kolls JK, Lei D, et al. In vivo and in vitro gene transfer and expression in rat intestinal epithelial cells by E1-deleted adenoviral vector. Hum Gene Ther 1997;8:7555-64.

94. Foreman PK, Wainwright MJ, Alicke B et al. Adenovirus-mediated transduction of intestinal cells in vivo. Hum Gene Ther 1998;9:1313-21.

95. During MJ, Xu R, Young D et al. Peroral gene therapy of lactose intolerance using adenoassociated virus vector. Nat med 1998;4:1131-5.