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## **An update on the Marfan syndrome and its pathological aspects at the protein and molecular level**

**Gwenaëlle Collod-Bérout<sup>1</sup> & Catherine Boileau<sup>2</sup>**

<sup>1</sup>INSERM U383, Université Paris V, Hôpital Necker-Enfants Malades, 149-161 rue de sèvres, 75743 Paris Cedex 15, France; <sup>2</sup>Laboratoire de Biochimie, d'Hormonologie et de Génétique Moléculaire Hôpital Ambroise Paré, 9 av Charles de Gaulle, 92104 Boulogne Cedex, France

### **Abstract**

*Marfan Syndrome (MFS) was the founding member of the "heritable disorders of connective tissue". The cardinal features of this autosomal dominant syndrome are prominently observed in three systems: skeletal, ocular and cardiovascular. It is now well established that defects in the fibrillin gene, the major component of extracellular microfibrils, located on chromosome 15 (FBN1) cause not only classic MFS, but also a large range of milder, overlapping phenotypes.*

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Correspondence/Reprint request: Dr. Catherine Boileau, Laboratoire de Biochimie d'Hormonologie et de Génétique Moléculaire, Hôpital Ambroise Paré, 9 av Charles de Gaulle, 92104 Boulogne Cedex, France. Fax: (33 1) 47 83 32 06  
E-mail: boileau@necker.fr

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## Introduction

A hundred years have now elapsed since *Dr. Antonin Marfan* [1] reported on the case of Gabrielle P. thus describing some of the skeletal features that today define the syndrome that carries his name. Since then, substantial progress has been made with respect to the description of the pleiotropic manifestations of this disease, the understanding of underlying pathophysiological mechanisms and the availability of prevention and treatment of major complications.

### 1. Nosology: what is Marfan syndrome today?

Marfan syndrome (MFS, OMIM #154700) is an autosomal dominant connective tissue disorder that has an estimated incidence of 1/5 000 with probably over 25 % of sporadic cases. The syndrome involves many systems (skeletal, ocular, cardiovascular, pulmonary, skin and integument, and dura) but its more prominent manifestations are skeletal, ocular and cardiovascular. In 1986, an international group of experts agreed upon diagnostic criteria to distinguish classic Marfan syndrome from many related disorders. These criteria constitute what is currently referred to as the "Berlin nosology" [2]. Patients are diagnosed based on involvement of the skeletal system and two other systems with at least one major manifestation (ectopia lentis, aortic dilation/dissection, or dural ectasia). Patients with an affected first degree relative are required to have involvement of at least two other systems with one major manifestation preferred but not required.

This nosology has been found wanting in many individual cases and revised criteria have been recently proposed that constitute the new "Ghent nosology" [3]. This new formulation requires involvement of three systems with two major diagnostic manifestations. It provides for major skeletal manifestations and considers affected first-degree relatives or molecular data as major diagnostic criteria.

Finally, development of surgical prevention and therapy for aortic aneurysms and dissection have lead to treatment of life-threatening cardiovascular complications associated with the Marfan syndrome and have considerably altered life expectancy for patients. Interestingly, the review of the medical problems of surviving patients has revealed possible unidentified pleiotropic manifestations of the Marfan syndrome or manifestations that could be related to aging of this population. These medical problems include the onset of arthritis at an early age, varicose veins, ruptured or herniated discs, and prolapse of the uterus or bladder in women. These medical problems now need to be properly investigated and monitored.

The continued efforts to redefine diagnostic criteria emphasize persistent shortcomings. The phenotype of the Marfan syndrome remains incompletely defined. Most manifestations are age-dependant and are difficult to quantify. The Ghent nosology has been field-tested in The National Institutes of Health [4]. Their study shows that 19% of patients diagnosed under Berlin criteria failed to meet the Ghent standard. In the absence of molecular data, it is difficult to determine the genetic risk and natural history of patients who meet the Berlin but not the Ghent diagnostic criteria.

### 2. The Marfan syndrome and *FBNI*

Scientists, as early as 1931, suggested that the basic defect in Marfan syndrome lay in a defect in the mesoderm [5]. In 1955, *Victor McKusick* considered the syndrome as a



prominent member of the new nosologic group he named "the heritable disorders of connective tissue" [6]. The Marfan syndrome was long considered to be due to a defect either in one of the collagens or elastin since their abnormalities are a prominent feature of the disease. However, protein and gene studies conclusively demonstrated that neither was involved. In 1986, *Sakai* and co-workers identified a new extracellular matrix protein that they named "fibrillin" [7] (OMIM#134797). This protein is the major component of microfibrils that are structures found in the extracellular matrix either as isolated aggregates or closely associated with elastin fibers. Ultrastructurally, microfibrils display a typical "beads-on-a-string" appearance consisting of a long series of globules connected by multiple filaments. In 1990, *Hollister* et al. using a monoclonal antibody against fibrillin, reported abnormalities of the microfibrillar system in the Marfan syndrome [8]. The following year, the gene encoding fibrillin-1 (*FBNI*) was cloned and the first mutations in the gene were identified in Marfan syndrome patients [9-11]. Interestingly, the year before the *FBNI* gene was cloned, *Kainulainen* et al. [12] demonstrated through linkage analysis that the gene involved in classic complete forms of the Marfan syndrome was located on human chromosome 15 precisely where the *FBNI* gene was later located. Therefore the identification of the gene defect in Marfan syndrome is a rare example in which both positional and functional cloning strategies converged rapidly to identify a disease gene.

### 3. The *FBNI* gene and other members of the fibrillin family

The gene encoding type 1 fibrillin (*FBNI*) lies on the long arm of chromosome 15 at 15q15-q21.1. This very large gene [first estimated at 110 kb, now at over 230 kb (Human Genome Sequencing Project, NT\_034890 sequence)] is highly fragmented into 65 exons, transcribed in a 10 kb mRNA that encodes a 2871 amino acid protein [10, 11, 13, 14]. Three additional alternatively-spliced exons, likely untranslated, were found upstream of exon 1 [15]. Conservation of nucleotide sequences within this region between human, mouse and porcine suggests that this region of the gene may harbor important regulatory elements. This region is GC-rich, contains a CpG island, and lacks conventional TATA or CCAAT boxes.

The deduced primary structure reveals a highly repetitive protein that contains essentially three repeated modules (figure 1):

- The first repeated module is the **EGF-like module** that is homologous to one found in the

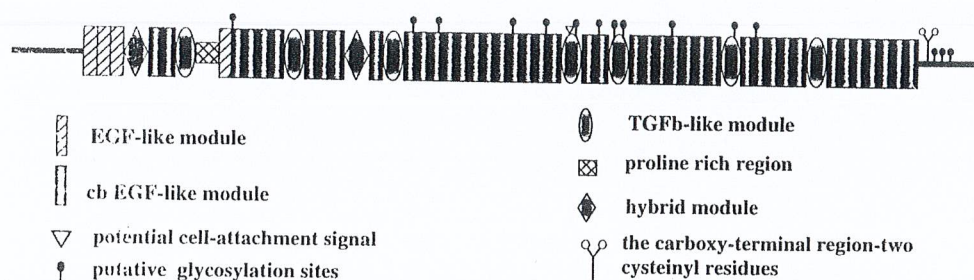
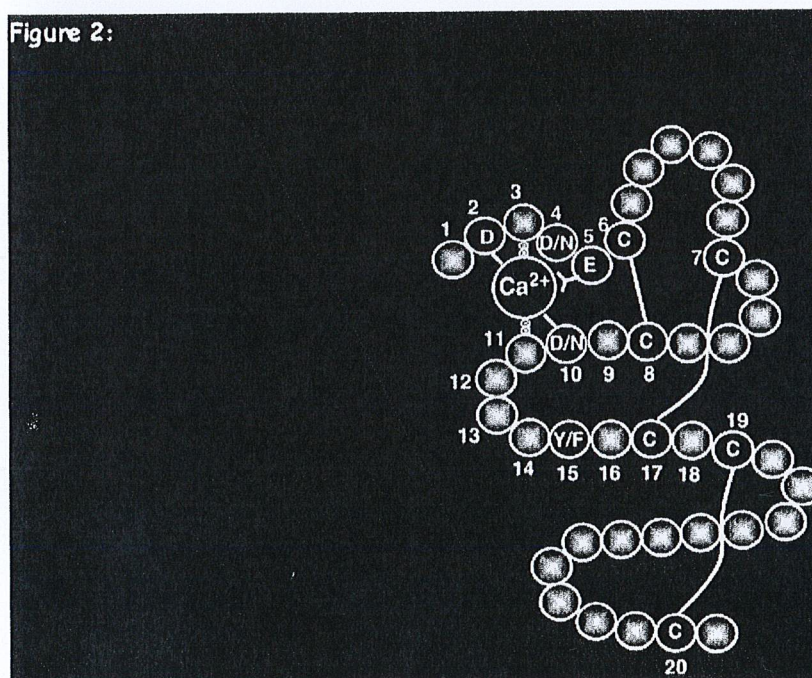


Figure 1. Schematic representation of the deduced primary structure of fibrillin-1.

epidermal growth factor. A large number of extracellular proteins with diverse biological functions possess EGF-like domains. They include molecules such as thrombomodulin and fibulin, and the vitamin K-dependent blood coagulation factors VII, IX and X, protein S and protein C [16]. These modules contain six cysteine residues that form three intra-domain disulfide bonds. There are 47 of these throughout the fibrillin-1 protein. Among these, 43 contain a conserved consensus sequence for calcium binding and are called cb EGF-like modules. In these domains, the residues putatively involved in calcium binding are numbered sequentially in figure 2 as in Dietz and Pyritz [16]. They include the aspartic acid at position 2, glutamic acid at position 5, asparagine at position 10 and tyrosine or phenylalanine at position 15.

- The second repeated module, found 7 times interspaced with cb EGF-like in the protein, is called **TGF- $\beta$ 1-binding protein-like module** (TGF  $\beta$ 1-BP-like module) since it is homologous to modules found in the Transforming Growth Factor  $\beta$ 1 binding protein. This domain appears to be limited to proteins that localize to matrix fibrils [fibrillins and latent transforming growth factor  $\beta$ -binding proteins (LTBPs)]. These modules contain eight cysteine residues. The fourth TGF- $\beta$ 1-BP like module contains the RGD sequence which can interact with cell receptors [17]. No specific function has yet been ascribed to these modules. However, some evidence suggests that these domains mediate specific protein-protein interactions [18].

**Figure 2:**



**Figure 2. Schematic diagram of a normal cb EGF-like module.**

In white are represented the cysteine residues which are disulfide-bonded and stabilize the native fold of the domain, in grey without a black border, the residues implicated in calcium binding.



• Finally, the protein contains a third module consisting of approximately 65 amino acids, and found twice in the protein. These are called “**hybrid modules**” since they combine features of the EGF-like and the TGF- $\beta$ 1-BP-like modules. This module is also found in LTBP<sub>s</sub>, which have a single hybrid domain.

Finally the protein contains three unique regions: a **proline-rich region** that may act as a “hinge-like” region [13] and **the amino and carboxy terminal domains**. The N- and C-terminal domains of the fibrillins display two prominent features : the presence of an even number of cysteine residues, four in the N-terminal and two in the C-terminal domains and the presence of the basic consensus sequence for processing by furin-types enzymes BXBB (B=basic amino acid residue, K or R) in each domain. The 4-cysteine domain in the N-terminus of fibrillins is homologous to similar 4-cysteine domains in the N-terminal extended forms of the LTBP<sub>s</sub>. The C-terminal domains of the fibrillins are homologous to the C-terminal domain of all four members of the fibulin family, and thus a new type of extracellular module of approximately 120 amino acid residues in length has been proposed [19]. This type of homology is not shared by the LTBP<sub>s</sub>. The cysteine residues in the N- and C-terminal domains are candidate residues for intermolecular disulfide bonding.

When the *FBN1* gene was cloned, a second gene sharing a high degree of homology was identified and located on chromosome 5. This gene was named *FBN2* and the protein it encodes **fibrillin-2** [10]. *FBN2* has been genetically linked to a rare disorder that shares features of Marfan syndrome: congenital contractural arachnodactyly (CCA) (OMIM#120150). The clinical manifestations of CCA are essentially found in the skeleton and associated with distinctive manifestations including crumpled ears and campodactyly. Several mutations were identified in this gene in CCA patients [20].

Ikegawa et al. described the structure and chromosomal assignment to 2p16 of a “**fibrillin-like**” gene (*FBNL*), that is highly homologous to fibrillin [21]. The *FBNL* gene is expressed in many tissues but it is not expressed in brain and lymphocytes. The amino acid sequence of the *FBNL* gene is 36.3% identical to *FBN1* (OMIM#134797) and 35.4% identical to *FBN2*. *FBNL* contains 1 EGF-like module and 5 repeated cb EGF-like module. The gene spans approximately 18 kb of genomic DNA and contains 12 exons. The *FBNL* gene was thought to possibly be involved in Marfan-like conditions such as hypermobility syndrome or mitral valve prolapse. In 1999, Stone *et al.* identified a single nonconservative mutation in the *FBNL* gene, also named *EFEMPI* (EGF-containing fibulin-like extracellular matrix protein 1) in 5 families with Doyme honeycomb retinal dystrophy (DHRD; OMIM#126600), or malattia Leventinese (MLVT) [22]. This autosomal dominant disease is characterized by yellow-white deposits known as drusen that accumulate beneath the retinal pigment epithelium.

#### 4. The fibrillin proteins

The fibrillins are a new family of extracellular matrix glycoproteins containing a large number of cysteine residues. They show a wide distribution in both elastic and non-elastic tissues and are integral components of 10 nm diameter microfibrils [7, 23]. Fibrillin-1 is synthesized as profibrillin and proteolytically processed to fibrillin. The cleavage site has been mapped to the carboxy-terminal domain of profibrillin-1. The propeptide started at position S<sup>2732</sup> directly C-terminal to the R<sup>2728</sup>KRR sequence. Wild type profibrillin is not incorporated into extracellular matrix until it is converted to fibrillin [24]. The N-terminal region of each protein directs the formation of homodimers within a few hours after



secretion and disulphide bonds stabilize the interaction [25]. Dimer formation occurs intracellularly, suggesting that the process of fibrillin aggregation is initiated early after biosynthesis of the molecules. Fibrillin is post-translationally modified by  $\beta$ -hydroxylation and N- and O-linked carbohydrate formation [26].

The solution structure of the TGF- $\beta$ -like module from human fibrillin-1 identified a novel fold which was globular in nature [27] and appears to break up linear regions within fibrillin-1 molecules after rotary shadowing electron microscopy. If these linker regions are effectively flexible, the kinks and bends observed in fibrillin-1 molecules would be required for proper alignment of molecules within the assembled microfibril [18].

Baldock et al. have derived a model of fibrillin alignment in microfibrils based on automated electron tomography, immunolocalization in directionally orientated untensioned microfibrils, mass changes on microfibril extension, immunofluorescence studies and published observations [28]. Their model predicts maturation from a parallel head-to-tail alignment to an approximately one-third stagger that is stable as a 56-nm folded form, but not as an  $\sim$ 100-nm form. This model accounts for all microfibril structural features, suggests that inter- and intramolecular interactions drive conformation changes to form extensible microfibrils, and defines the number of molecules in cross section.

Fibrillin-1 and -2 co-distribute in elastic and non-elastic connective tissues of the developing embryo, with a preferential accumulation of the *FBN2* gene product in elastic fiber-rich matrices [23]. Mouse study of the developmental expression of the fibrillin genes has revealed different patterns. Except for the cardiovascular system, in which *fib-1* gene activity is early and always higher than *fib2*, fibrillin-2 transcripts appear earlier than fibrillin-1 transcripts and accumulate for a short period of time just before overt tissue differentiation i.e. a window of time immediately preceding elastogenesis. In contrast, the amount of fibrillin-1 transcripts increases at an apparently gradual rate throughout morphogenesis and is mainly expressed during late morphogenesis and well-defined organ structures. Furthermore, *fib-1* is predominantly represented in stress- and load-bearing structures like aortic adventitia, suspensory ligament of the lens, and skin. Spatio-temporal patterns of gene expression thus suggest distinct but related roles in microfibril physiology. Fibrillin-1 would provide mostly force-bearing structural support whereas fibrillin-2 would predominantly regulate the early process of elastic fiber assembly [29]. Fibrillins would contribute to the structural and functional heterogeneity of microfibrils.

## 5. Role of $\text{Ca}^{2+}$ in fibrillin

The implication of the variable calcium binding affinities observed in fibrillin fragments is biologically significant. A number of studies have shown that the presence of calcium ions significantly protects full-length or recombinant fragments of fibrillin-1 from proteolysis by trypsin, elastase, endoproteinase Glu-C, plasmin and matrix metalloproteinases [30-33]. Moderate to high affinities for calcium suggest that fibrillin cb EGF modules would be close to fully saturated in vivo. Particular regions of fibrillin may need to be rigid for appropriate function. For example, cb EGF-like 12-13, located in the neonatal Marfan syndrome region (see paragraph 7) where mutations leading to severe phenotypes cluster, may be part of a region where rigidity is required for function. Fully saturated calcium binding sites may be required for stabilization of the microfibril against proteolytic degradation, when low-affinity sites not fully saturated in vivo may contribute to flexibility of the polypeptide chain or to biomechanical function. It may be advantageous to



allow some degree of extensibility of assembled microfibrils in tissues subjected to mechanical forces. The importance of domain context for modulating the structural effects of calcium binding mutations suggests an explanation why MFS phenotypes associated with apparently similar mutations may be diverse [33].

## **6. *FBNI* gene mutations in Marfan syndrome and related disorders**

To date over 500 mutations have been identified in the *FBNI* gene in Marfan syndrome patients and related diseases (figure 3) [34] (*Collod-Bérout* et al., In preparation). No major rearrangements have been identified except for three cases of multi-exon deletions [35, 36]. Three categories of mutations have been described: 1) missense mutations, 2) small in-frame insertions or deletions, mutations causing premature termination of translation and 3) exon-skipping mutations.

*FBNI* gene mutations have been identified in complete and incomplete forms of Marfan syndrome but also in various disorders: severe neonatal Marfan syndrome, dominantly inherited ectopia lentis [37], isolated skeletal features of MFS [38], the Shprintzen-Goldberg syndrome [39] and, more recently, familial or isolated forms of aortic aneurysms [40]. These results define the new molecular group of "type 1 fibrillinopathies" that comprises a spectrum of overlapping diseases. Presently no genotype/phenotype correlations have been identified except for neonatal mutation (see paragraph 7). To facilitate their identification, a "Marfan database" has been developed that includes not only molecular but also clinical data. The database is attached to a software that provides various tools for its analysis and allows optimized multicriteria research [34, 41-43]. It is only through a large collaborative international effort that genotype/phenotype correlations will be eventually identified.

No case of incomplete penetrance has ever been demonstrated for patients carrying fibrillin-1 mutations associated with Marfan syndrome. However, patients with the same mutation can show a wide degree of phenotypic variability. This has been exemplified in large pedigrees with sharp differences in clinical severity of musculoskeletal and cardiovascular features of the syndrome [44].

## **7. Neonatal Marfan syndrome and *FBNI* gene mutations**

Neonatal Marfan syndrome is the most severe form of the disorder. Affected newborns display severe cardiac valve regurgitation and dilatation of the proximal aorta which usually lead to heart failure and death in the first year of life. Skeletal manifestations such as arachnodactyly, dolichostenomelia, and pectus deformities are typically present. Such infants may also display congenital flexion contractures, crumpled ears, loose redundant skin, and a characteristic "senile" facial appearance [45]. The mean life span is usually low (approximately 1 year [46]). The primary cause of death is congestive heart failure associated with mitral and tricuspid regurgitation. Family investigation usually reveals that the Marfan patients with the severe neonatal phenotype are sporadic cases: *Buntinx* et al. reported that 37 of 44 cases with neonatal manifestations were sporadic [45]. For a longtime it was generally thought that the neonatal phenotype could be explained by mutations in a distinct gene than that involved in the classic "adolescent-adult" form of the syndrome as the observed symptoms were extremely severe and overlapped with congenital contractural



arachnodactyly. Godfrey et al. showed an abnormal morphology of fibrillin microfibrils in fibroblast cultures from patients with the neonatal phenotype [46]. As in the classic "adolescent-adult" form, there was an apparent decrease in accumulation of immunostainable fibrillin, but they appeared shorter, fragmented and frayed. Molecular analyses revealed that the neonatal Marfan syndrome was also due to mutations within the *FBNI* gene. Furthermore a clustering of mutations in the protein region encoded by exons 24 to 32 was observed (figure 4), suggesting an unknown but critical function of these domains [47]. The severe phenotype associated with these specific mutations in this region of the gene represents, to date, the only genotype/phenotype relationship established. The observed clustering of mutations enables direct screening of this region of the *FBNI* gene to confirm diagnosis of neonatal Marfan syndrome in patients. Finally, confirmation of the sporadic nature of the mutation is important for genetic counseling since perinatal lethal Marfan syndrome can also result from compound heterozygosity [48] or potential homozygosity.

## 8. Pathogenic mechanisms

Fibrillin-1 is an important component of the microfibrillar system that acts as a scaffold for elastogenesis. Elastic fibers first appear in fetal development as aggregates of microfibrils. These microfibrils are arranged in parallel arrays on which elastin is deposited and appears as an amorphous material. Elastin-containing microfibrillar bundles aggregate to form true elastic fibers. These observations suggest that microfibrils determine the form and the orientation of elastic fibers, therefore directing fiber assembly as a scaffold on which elastin is deposited [29]. This model explains the typical fragmentation and disarray of elastic fibers observed in the media of Marfan patients. However, unlike elastin, fibrillin-1 is also highly expressed in the vascular adventitia. Therefore reduction of this protein in the adventitia is very likely also involved in the mechanism for dilatation and for increased risk of aneurysm since the role of the adventitia is to maintain the vascular diameter. The pleiotropic manifestations of the disease can be explained by the observation that numerous microfibrillar aggregates devoid of elastin are found in the zonule, as well as cartilage and the extracellular matrix of many organs. However, the actual pathogenic mechanisms in these tissues still remain speculative.

At the molecular level, two different mechanisms explain part of the variability observed in the Marfan syndrome. These two models explain the difference between the mild (first model) and severe (second model) forms of the disease. In the first case, the mutation is responsible for the appearance of a premature STOP codon that reduces the stability of the mutant transcript and consequently greatly reduces protein production from the mutated copy of the gene. In the affected subjects, the amount of fibrillin-1 protein produced is 50 % that of normal and is produced only from the normal gene copy. The second mechanism is the dominant-negative mechanism that is associated with the majority of the mutations identified in the *FBNI* gene (i.e. missense mutations). In this case, an abnormal protein is produced from the mutated copy that considerably interferes with the assembly (polymerization) of fibrillin molecules. In this model, the amount of fibrillin is greatly reduced (< 35 %). Among the numerous missense mutations identified, most are located in one of the cb EGF-like modules and either affect a highly conserved cysteine residue (therefore disrupting the intradomain structure through the disappearance of a disulfide bond) (figure 2) or affect one of the amino acids involved in calcium binding (that plays a major role in defining interdomain linkage) [49].



What is still unknown are the multiple consequences triggered by the various mutations and the effect of unknown modifier (enhancing or protecting) genes on the clinical expression. These mechanisms and the great number of mutations identified in the *FBNI* gene explain the great variability of the disease observed not only between families but also among affected individuals in a single family.

## 9. Genetic heterogeneity in Marfan syndrome

The clinical variability of Marfan syndrome is only partly explained by the great number of mutations identified in the *FBNI* gene. In effect, we have demonstrated the existence of genetic heterogeneity, i.e. the involvement, in certain cases of Marfan syndrome of mutations located in another gene named *MFS2* (for Marfan syndrome type 2). Genetic heterogeneity was demonstrated through the study of a large French family in which affected individuals display an incomplete form of the syndrome: typical skeletal and cardiovascular features as well as involvement of the skin and integument. No ocular manifestations were observed until recently when one of the children developed ectopia lentis. We showed that fibrillin-1 was normal in several affected family members and excluded linkage between the *FBNI* gene and the disease in the family [50]. By exclusion mapping we located the *MFS2* gene on the short arm of chromosome 3 [51]. In this area is located the gene that encodes fibuline-2 (*FBLN2*), another microfibrillar component. Again through a double approach (genetic and protein) we showed that *MFS2* and *FBLN2* were not identical [52]. We are now identifying *MFS2* through positional cloning. Other teams have already identified families comparable to the French family in that they are not linked to or do not carry a mutation in the *FBNI* gene (*M. Boxer, L. Peltonen and B. Steinmann*, personal communications). Clinically these families are indistinguishable from other families linked to *FBNI*. Therefore, we are also trying to determine the percentage of Marfan syndrome cases that are associated with mutations in *MFS2* through genetic analyses as well as their clinical spectrum. Other teams, through protein studies have identified between 7 and 16 % of Marfan syndrome patients with normal fibrillin metabolism [53, 54]. The precise determination of this % is important for laboratories involved in diagnosis of Marfan syndrome since it will give the risk associated with investigation of only the *FBNI* gene.

## 10. Marfan syndrome is still an essentially clinical diagnosis

Although no specific therapy exists for Marfan syndrome, it is of great importance to confirm or firmly exclude the diagnosis in family members at risk as early as possible because of the potential fatal complications of the disease. At present, diagnosis is still based on thorough clinical examination, including measurements of body proportions, echocardiography of the aorta, slit-lamp ophthalmological evaluation and radio-graphs. A complete family history is also an essential part of the diagnosis. However in some cases the manifestations are not evident until adolescence and the clinical expression of the disease varies greatly between affected members of a single family. Therefore, there is an absolute need for an accurate diagnostic test.

The discovery of the involvement of fibrillin-1 has raised high hopes for a protein or DNA test applicable to Marfan syndrome patients. Immunofluorescence studies of cultured fibroblasts and skin sections of patients using monoclonal antibodies against fibrillin have



revealed that the amount of fibrillin deposition or of fibrillin microfibrils is greatly reduced [8]. Therefore, immunofluorescence analysis could be helpful in diagnosis. However the method has proven to be insufficiently sensitive and specific because of the existence of non-Marfan syndrome type 1 fibrillinopathies and of genetic heterogeneity. Therefore, an abnormal test result does not diagnose Marfan syndrome, and a normal test result does not exclude Marfan syndrome.

The identification of the *FBNI* gene has allowed the development of two types of diagnostic tests: either genetic family studies or mutation identification. Family studies can be performed with specific *FBNI* polymorphic markers to identify the mutation-bearing haplotype [55]. These studies are only reliable in families in which several affected individuals (at least 4) are available since the involvement of a *FBNI* mutation (and not that of another gene) must be clearly demonstrated. However, most family structures do not comply with this requirement. Furthermore, the method is inappropriate in sporadic cases. In practice, these instances represent over 40 % of the cases referred for biological diagnosis. The second molecular test is mutation identification. This test must only be performed after the protein analysis or the previously mentioned family study has conclusively shown the presence of a *FBNI* gene defect. Mutation identification is very costly and long. In effect, there is no quick and 100 % reliable method to investigate a large (~ 200 kb) and highly fragmented (10 kb of coding sequence fragmented in 65 exons) gene, knowing that almost each family has its own specific defect and that the mutations are essentially point mutations. Finally, this very costly analysis may fail to identify a mutation since only the coding sequence and closely surrounding regions are investigated. However, in the case of neonatal Marfan syndrome, where a clustering of mutations is found in a specific region, molecular diagnosis can be performed. In all other instances and until better molecular tools are available, mutation identification cannot be performed on a systematic basis. However, in a few cases where the family mutation had been identified, it was possible to perform prenatal diagnosis on chorionic villus samples or offer presymptomatic diagnosis in children at risk of affected subjects [56, 57].

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