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Génétique des Prédispositions aux Anévrismes de l'Aorte Thoracique et/ou Dissections Aortiques (AAT/DA)

Philippe KHAU VAN KIEN

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Université de Montpellier

Faculté de Médecine Montpellier - Nîmes

MEMOIRE

Pour obtenir l'Habilitation à Diriger des Recherches

Discipline: Génétique (4704)

Génétique des Prédispositions aux Anévrismes de l'Aorte Thoracique

et/ou Dissections Aortiques (AAT/DA)

Soutenu le 28 janvier 2022

Par Philippe KHAU VAN KIEN

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PRELIMINAIRE

Je déclare avoir respecté, dans la conception et la rédaction de ce mémoire d'HDR, les valeurs et principes d'intégrité scientifique destinés à garantir le caractère honnête et scientifiquement rigoureux de tout travail de recherche, visés à l'article L.211-2 du Code de la recherche et énoncés par la Charte nationale de déontologie des métiers de la recherche et la Charte d'intégrité scientifique de l'Université de Montpellier. Je m'engage à les promouvoir dans le cadre de mes activités futures d'encadrement de recherche.

RESUME

Génétique des Prédispositions aux Anévrismes de l’Aorte Thoracique et/ou Dissections Aortiques (AAT/DA)

Les AAT/DA sont des affections sévères associées à une mortalité élevée. En dehors de syndromes reconnaissables (Marfan, Loeys-Dietz, Ehlers-Danlos...), une composante héréditaire mendélienne, le plus souvent dominante est identifiable dans au moins 20% des AAT/DA. Environ 40 gènes, supports d’une partie de cette hérédité ont été identifiés (la majorité récemment) et peuvent être classés en 5 catégories selon leur physiopathologie associée et/ou supposée : 1-composants de la Matrice Extra-Cellulaire, 2-composants de la voie de signalisation du TGF-béta, 3-appareil contractile des Cellules Musculaires Lisses et Mécanotransduction du signal, 4- Bicuspidie de la Valve Aortique et hypoplasie de la voie gauche et 5- Autres. Identifier un défaut moléculaire dans une famille, permet une démarche de dépistage, de prévention du risque aortique efficace par une médecine et une chirurgie de précision (imagerie aortique régulière, éviction des facteurs/circonstances à risque, chirurgie programmée à un diamètre fonction du défaut génétique et, en fonction de celui-ci : traitement préventif par bêtabloquants et/ou sartans, voire autre). Cependant, ces découvertes soulèvent de multiples questionnements et controverses, concernant le rôle réel de ces gènes et de leurs défauts moléculaires, nécessitant la Recherche. Encadrant depuis 2005 (Montpellier puis Nîmes), une activité diagnostique à recrutement National, avec à ce jour plus de 680 familles analysées pour la plupart de ces gènes, mon projet consiste en : 1- exploiter les données cliniques et moléculaires, bien caractérisées en apportant de nouvelles informations ; 2- transférer en recherche les données justifiant d’études fonctionnelles (transcription, protéomique, modèles cellulaires, voire animaux) pour caractériser : des variant de signification incertaine (mais d’intérêt) ou l’effet réel de variant (probablement) délétères. Pour ce faire, le souhait d’encadrer des étudiants en Master 2 et en Thèse, de transmettre savoir et compétences, justifie mon engagement pour l’HDR.

Mots clefs : Aorte, Anévrismes, Dissections, Syndrome de Marfan, Gènes, Matrice Extra-Cellulaire, Voie de signalisation du TGF-béta, Cellules Musculaires Lisses, Mécanotransduction du Signal.

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Mon cursus professionnel, mes travaux et recherches, n'auraient pu être présentés et résumés dans ce mémoire sans le soutien, l'aide et la formation apportés par de nombreuses personnes ou institutions. S'il est impossible de citer l'ensemble des composantes, contribuant à cette synergie et à ce développement personnel et collectif, je tiens particulièrement à remercier ici:

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- le Dr Annie Nivelon-Chevallier, à l'origine de mon orientation en Génétique Médicale et de mon investissement pour la Génétique des AAT/DA,
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- A mes Amis, à ma famille, à mes fils, Virgil et Victor,
- A Brigitte,

Liste des principales abréviations

- **AAT** : Anévrisme(s) de l’Aorte Thoracique
- **BVA** : Bicuspidie de la valve Aortique
- **CML** : Cellule(s) Musculaire(s) Lisse(s)
- **CRISPR/Cas9**: Clustered Regularly Interspaced Short Palindromic Repeats/clustered regularly interspaced short palindromic repeats-associated protein 9 nuclease
- **DA** : Dissection(s) aortique
- **ETT** : Echocardiographie Trans-Thoracique
- **MEC** : Matrice Extra-Cellulaire
- **NGS** : Next-Generation Sequencing
- **NMD** : Non-sens Mediated Decay
- **OI** : ostéogénèses Imparfaites
- **PCA** : Persistance du Canal Artériel
- **SED** : Syndrome(s) d’Ehlers-Danlos
- **SLD** : Syndrome(s) de Loeys-Dietz
- **SNP** : Single Nucléotide Polymorphism
- **SSG** : Syndrome de Shprintzen-Goldberg
- **TGF- β** : Transforming Growth Factor- β
- **WB**: Western-blot

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1- Qualification

Diplômes :

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- **2005 : Doctorat d'Université en Génétique Humaine**, Université de Bourgogne. Co-direction : Pr Wolf (Dijon), Pr Jeunemaitre (Paris). Sujet : anévrisme/dissection de l'aorte thoracique avec persistance du canal artériel : une nouvelle entité.
- **2001 : Doctorat en Médecine et spécialisation en Génétique Médicale**. Université de Bourgogne :

- **Thèse d'exercice** : « Une nouvelle forme d'anévrisme /dissection aortique familial avec persistance du canal artériel et anomalies ultrastructurales cutanées, non liée à *COL3A1* ni à *FBN1*. Revue de la littérature, à propos d'une famille ».

- Président : Pr Wolf (Dijon).

- Mention très honorable avec félicitations du Jury.

- **Diplôme d'études spécialisées** : Génétique Clinique, Chromosomique et Moléculaire (DES 48) : inter-région Nord-Est, Coordonateur régional, Pr Mandel (Strasbourg).

- Mémoire : « Paragangliomes familiaux, à propos de deux familles ».

- **2000 : DEA de Génétique Humaine, Paris VI (Pr Brice, Pr Fellous, Pr Hors-Cayla).**

- Responsable, Pr X Jeunemaitre.

- Laboratoire : INSERM U36, Collège de France (Paris V)

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1999 : Diplôme des cours de Génétique moléculaire et cellulaire de l'Institut Pasteur.

1998 : Maîtrise des sciences biologiques et médicales (MSBM) :

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1987 : Baccalauréat série D, Mention Bien (biologie).

Titres : Docteur en médecine, spécialiste en Génétique Médicale (Clinique, Chromosomique et Moléculaire), Docteur d'Université en Génétique Humaine. Ancien Assistant Hospitalo-Universitaire des Hôpitaux de Besançon. Praticien Hospitalier.

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TRAVAUX

I- BREVET

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IV- CHAPITRES DE LIVRES – OUVRAGES DIDACTIQUES

1. Rousseau H, Verhoye JP, Heautot JF, Editors. *Les Syndromes Aortiques Aigus*. 1st ed. Paris (France): Springer-Verlag France; c2009. Chapter 1, Khau Van Kien P, Demaria R. Aspect Génétiques; p.3-16.
ISBN P: 978-2-287-79928-0, ISBN O : 978-2-287-79928-0.
2. Claustres M, Editor. *Molecular Genetic Analysis of Rare Diseases in 2007: Selected Examples*. 1st ed. Kerala (India): Research Signpost; c.2007. Khau Van Kien P, Pallares-Ruiz N, Plancke A, Claustres M. Genetics of Aortic Dissection: Hope and Promises; p.79-102.
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V- PRINCIPALES COMMUNICATIONS ORALES (Congrès internationaux)

1. Les tests diagnostiques dans les maladies rares : évolutions technologiques nécessaires. **Khau Van Kien P** (La Presse Médicale, 2010 39(5):69-70, doi :10.1016/j.lpm.2010.02.022.).1^{er} Colloque « Maladies Rares et Orphelines, Entreprises et Laboratoires au cœur de l'innovation » Pôle Eurobiomed. Montpellier, 29 et 30/10/09.
2. L'annonce d'une Maladies Vasculaire rares d'origine Génétique : gestion psychologique, sociale, conseil génétique. **Khau Van Kien P**, Khau Van Kien A, Alric P, Demaria R, Sarda P, Quéré I. 43^{ème} Congrès du Collège Français de Pathologie Vasculaire. Paris, 18-20/03/2009 *Journal des Maladies Vasculaires*,2009,34(2):105.
3. Des mutations de la chaîne lourde de myosine 11 responsables d'un syndrome associant anévrisme de l'aorte thoracique et/ou dissection aortique (AAT/DA), persistance du canal artériel et rigidité aortique. **Khau Van Kien P**, Zhu L, Vranckx R, Lalande A, Boisset N, Mathieu F, Wegman M, Glancy L, Brunotte F, Gasc JM, Bruneval

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4. A unique locus on chromosome 16p12.2-16p13.13 responsible for thoracic aortic aneurysm/dissection, ductus arteriosus and aortic stiffness. **Khau Van Kien P**, Mathieu F, Zhu L, Lalande A, Brunotte F, Wolf JE, Jeunemaitre X.
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 7. Aorte du syndrome de Marfan et des autres dystrophies du tissu conjonctif. **Khau Van Kien P**
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 9. Familial Aortic Dissection/Aneurysm Associated With Patent Ductus Arteriosus: A New Entity? **Khau Van Kien P**, Lalande A, Bonnet C, Dellinger A, Petit A, Nivelon-Chevallier A, Brunotte A, Jeunemaitre X, Wolf JE. Journal of the American College of Cardiology, 2002; 39(5):264. 51th annual session of American College of Cardiology. Atlanta, Georgie, USA 17-20/03/02
 10. Difficultés du Conseil Génétique dans une famille de Dissections Aortiques. **Khau Van Kien P**, Lesca G, Wolf JE, Zabot MT, Peyrol S, Jeunemaitre X, De Paepe A, Plauchu H et Nivelon-Chevallier A (Prix de la meilleure Communication, avec G. Lesca – publié dans les annales de génétique 1998). XXIII^{èmes} Journées du Club de Conseil Génétique de Langue Française. Lyon 23-26/09/ 1998

11. Le rhumatisme articulaire aigu (R.A.A) dans le Service de Pédiatrie générale de l'Hôpital Principal de Dakar. -Etude épidémiologique, clinique, biologique, échographique et évolutive. **Khau Van Kien P**, Candito D, Imbert P, Bertocchi C, Favre E, Ka AS et Cloatre G. Xèmes rencontres franco-africaines de Pédiatrie. Journées parisiennes de Pédiatrie. Paris 11/10/1997

VI- PRINCIPALES COMMUNICATIONS SUR TABLEAU

- European Human Genetics Virtual Conference 2020.

Chesneau B, Plancke A, Rolland G, Marcheix B, Dulac Y, Edouard T, Aubert-Mucca M, Julia S, Langeois M, Gaston V, **Khau Van Kien P**. Familial aortic aneurysm syndrome caused by a +3 variant at a donor splice site of *MYH11*.

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Plancke A, Chesneau B, Rolland G, Arnould C, Dautheville Guibal C, Audema C, Lavabre-Bertrand T, Chiesa J, **Khau Van Kien P**. Apport de la Génétique Moléculaire pour le diagnostic différentiel de sévices à enfants, de syndrome d'Ehlers-Danlos de type classique et de syndrome de l'X fragile.

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- Assises de Génétique Humaine et Médicale 2014. Bordeaux 29-31/01/2014 :

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- European Human Genetics Conference 2007. Nice, France 16-19/06/07:

Khau van Kien P, Baux D, Pallares-Ruiz N, Claustres M. Analysis of three Glycine substitutions in loop-regions of calcium-binding Epidermal Growth Factor-like domains of fibrillin-1: possible key positions for domain folding.

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Anévrismes de l'Aorte Thoracique et/ou Dissections Aortiques (AAT/DA) Familiaux : n'oublions pas le syndrome d'Ehlers-Danlos vasculaire ! **Khau Van Kien P**, Pallares-Ruiz N, Khau Van Kien A, Quéré I, Claustres M (résumés publiés dans Med Sciences HS n°2, vol 22 2006, p:90 et 136)

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- American Heart Association (AHA) - Scientific sessions 2005. Dallas, Texas, USA, 13-16/11/05:

Identification of a chromosome 16p12.2-13.13 locus for Familial Aortic Dissection with Ductus Arteriosus. **Khau Van Kien P**, Mathieu F, Zhu L, Lalande A, Brunotte F, Wolf JE, Jeunemaitre X.

Should Aortic Stiffness be Evaluated in Thoracic Aortic Aneurysm/dissection Relatives to Prevent Risks? Lalande A, **Khau Van Kien P**, Zhu L, Steinmetz E, Vandroux D, Bouchot O, Walker PM, Legrand L, Jeunemaitre X, Brunotte F, Wolf JE.

- European Human Genetics Conference 2005. Prague, République Tchèque, 7-10/05/05

Solving genetic counselling dead-end in two Duchenne muscular dystrophy families with the help of three novel microsatellite markers. Khau Van Kien P, Thorel D, Saquet C, Claustres M, Tuffery-Giraud S (publié dans *Eur J Hum Genet* 2005;13(supp1):376)

- 7th annual Society for Cardiovascular Magnetic Resonance Scientific session/ Euro Cardiovascular Magnetic Resonance 2004 Meeting. Barcelona, Spain, 13-15/02/04:

Screening of asymptomatic relatives in aortic inherited diseases. A comparative study between aortic MRI and transthoracic echocardiography. Khau Van Kien P, Lalande A, Salvé N, Brunotte F, Wolf JE (published in *J Cardiovasc Magn Reson* ;6(1):267-8

- 21ème congrès du Groupe de Réflexion sur la Recherche Cardiovasculaire (GRRC). La Baule, France, 22-23/04/04 :

Anévrisme/dissection de l'aorte thoracique familiale et persistance du canal artériel : une nouvelle entité physiopathologique. Khau Van Kien P, Zhu L, Mathieu F, Lalande A, Brunotte F, Lathrop M, Bruneval P, Wolf JE, Jeunemaitre X (publié dans *Arch Mal Coeur Vaiss* 2004 ;97(4) :450)

- 8th Annual Meeting of the European Council for Blood Pressure and Cardiovascular Research (ECCR). Seeheim, Germany, 10-12/10/03:

Familial aortic dissection/aneurysm with patent ductus arteriosus. Genetic arguments for a new pathophysiological entity. **Khau Van Kien P**, Mathieu F, Zhu L, Wolf JE, Nivelon-Chevallier A, Brunotte F, Jeunemaitre X. (published in *Hypertension* Volume 42, pages 627-648)

- 15th international meeting of echocardiography. Paris, France 11-13/06/03:

Screening and follow-up in familial thoracic aortic aneurysm/dissection. Should we screen for patent *ductus arteriosus*? **Khau Van Kien P**, Salve N, Lalande A, Brunotte F, Wolf JE

Screening and follow-up in familial thoracic aortic aneurysm/dissection with patent ductus arteriosus, a new disease. An echocardiography and MRI study of a large family. Salve N, **Khau Van Kien P**, Lalande A, Brunotte F, Wolf JE

- European Human Genetic Conference 2002. Birmingham, Royaume-Uni. 03-06/05/03:

Familial aortic dissection/aneurysm with patent ductus arteriosus. A new entity. **Khau Van Kien P**, Mathieu F, Wolf JE, Nivelon-Chevallier A, Brunotte F, Jeunemaitre X- **XV colloque de l'association des cytogénéticiens de langue française 2002. Toulouse 09/2002 :**

Chromosome "15po" sans marquage centromérique. Anomalie chromosomique ou polymorphisme? **Khau Van Kien P**, Collonge-Rame MA, Fellmann F, Bresson JL

- **European Society of Cardiology Congress 2002. Berlin (Allemagne) 31/08-04/09/2002:**

A third family with aortic A third family with aortic dissection/aneurysm and patent *ductus arteriosus* association. Is there a new syndrome? **Khau Van Kien P**, Lalande A, Brunotte F, Jeunemaitre X, Wolf JE

- **X^{ième} Colloque de l'Association des Cytogénéticiens de Langue Française. Tours 21 et 22 Septembre 1998 :**

Réarrangement familial de la région 14q31 avec expression phénotypique variable : région soumise à empreinte génomique ? **Khau Van Kien P**, Nadal N, Luquet I, Favre B, Geneste B, Nivelon-Chevallier A et Mugneret F.

Enseignement – Encadrement – Vie scientifique

I- ACTIVITE D'ENSEIGNEMENT

- Co-responsable d'une Unité d'Enseignement Master 1- UMBS203 : « Génétique Médicale et Conseil Génétique » Université Montpellier 1 et Montpellier 2 (2005-2010). 10h/an.
- Enseignement formation initiale médecine : UE de Génétique Médicale – DFGSM3 depuis 2010. 4h/an.
- Enseignement post-universitaire : DIU « formation complémentaire en Gynécologie-Obstétrique pour le Médecin Généraliste » et DU « Prévention et suivi en Gynécologie pour les Sages-Femmes ». Depuis 2011, 3h/an.
- Enseignement formation initiale Maïeutique. Depuis 2011, 2h/an.
- Participation à l'enseignement DES de Génétique Médicale, DES de Médecine Interne, Capacité d'angiologie...
- Formation Médicale Continue : participation à différentes réunions.
- Lors de mon Assistanat au CHU de Besançon (Pr JL BRESSON), enseignement en PCEM1 :4h, DCEM3 :4h et Unité de Master 1 : 18h.
- Enseignement à l'Université Montpellier 2 (2007-2010) : ULBIO54-Génétique et Evolution de l'Homme (3h)

II- CO-ENCADREMENT :

Co-encadrement du travail de post-doctorant du Dr Aliya ISHMUKAMETOVA, financement bourse post-doctorat AFM (CHU de Montpellier) :

1- Ishmukhametova A, Chen JM, Bernard R, de Massy B, Baudat F, Boyer A, Méchin D, Thorel D, Chabrol B, Vincent MC, Khau Van Kien P, Claustres M, Tuffery-Giraud S.

Dissecting the structure and mechanism of a complex duplication-triplication rearrangement in the *DMD* gene. *Hum Mutat.* 2013;34(8):1080-4.

2- Ishmukhametova A, Khau Van Kien P, Méchin D, Thorel D, Vincent MC, Rivier F, Coubes C, Humbertclaude V, Claustres M, Tuffery-Giraud S. Comprehensive oligonucleotide array-comparative genomic hybridization analysis: new insights into the molecular pathology of the *DMD* gene. *Eur J Hum Genet.* 2012;20(10):1096-1100.

3- Khelifi MM, Ishmukhametova A, Khau Van Kien P, Thorel D, Méchin D, Perelman S, Pouget J, Claustres M, Tuffery-Giraud S. Pure intronic rearrangements leading to aberrant pseudoexon inclusion in dystrophinopathy: a new class of mutations? *Hum Mutat.* 2011;32(4):467-475.

Encadrement de stagiaire MASTER 1 :

- Loriane GUTERMANN (2011) - Université Montpellier 1.
- Naoual Boulisfane (2006) - Université Montpellier 1.
- Audrey BADOUIN (2008) - Université Montpellier 1
- Lise VOGEELEER (2008) - Université Montpellier 1
- Gérard CHAPELLE (2007) - Haute Ecole Charleroi Europe- Fleurus- Belgique.

Rapporteur Thèse d'exercice Médecine:

- Dr Lisa MEISER- Université Montpellier 1-2007
- Dr Nicolas SALVE- Université de Bourgogne-2003

III- EXPERTISE/REVIEWING DANS DES REVUES A COMITE DE LECTURE.
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- Activité régulière de reviewer pour l'European Journal Of Human Genetics (<https://orcid.org/0000-0001-7754-969X>).

- Expertise pour évaluation des bourses de Thèse de la Société Française d'Hypertension Artérielle (Génétique).

IV- SOCIETES SAVANTES- ASSOCIATIONS.

- Membre de l'Association Nationale des Praticiens de Génétique Moléculaire (depuis 2004).
- Membre de l'Association Francophone de Génétique Clinique (depuis 2001).
- Membre du Conseil Scientifique de l'Association Française des Syndromes d'Ehlers-Danlos (depuis 2001).
- Membre du Conseil Scientifique de l'Union Nationale des Syndromes d'Ehlers-Danlos (depuis 2010).

III- COORDINATIONS - COLLABORATIONS

- Coordinateur du Centre de Compétences Anomalies du Développement et Syndromes Malformatifs - Sud-Ouest – Occitanie - Réunion du CHU de Nîmes- Filière de Santé Maladies rares AnDDI.
- Coordinateur de la Fédération Inter Etablissement Montpellier-Nîmes Hospitalo-Universitaire de Génétique Médicale, Médecine Moléculaire et Génomique.
- Coordinateur de la Fédération Médicale Hospitalo-Universitaire Montpellier-Nîmes « Maladies Rares ».
- Centres de Référence « Marfan et Apparentés », CHU de Toulouse et « Maladies Vasculaires Rares », APHP- Filière de Santé Maladies rares FAVAMulti.

IV- INVESTIGATEUR PROJETS DE RECHERCHE / CONTRATS DE RECHERCHE :

- DiagnosticAnté-natal sur Cellules fœtales trophoblastiques Circulantes (CFTC) de Maladies à EXpansions de triplets à partir de sang maternel (DIACCIMEX)-Promoteur CHU de Montpellier- Investigateur Principal pour le CHU de Nîmes- n°AC : 2017-A00232-51.

- Diagnostic Prénatal Non Invasif (DPNI) de la Mucoviscidose par MEMO-PCR en temps réel- Promoteur CHU de Montpellier- Investigateur Principal pour le CHU de Nîmes- n°AC : 2012-A01183-40.
- Post doc fellow ship (#14178) - AFM 2010-2012 « Large rearrangements of the dystrophin gene : CGH-array study and analysis of intronic breakpoints". Porteur de la demande pour le Dr Ishmukhametova.
- Dépistage Génétique des Anévrismes de l'Aorte Thoracique et/ou Dissections Aortiques (AAT/DA) : séquençage haut débit de 6 gènes de prédisposition (étude principale) et étude cytogénétique (étude ancillaire) -Programme Hospitalier Recherche Clinique Régional 2007- Promoteur CHU de Montpellier- Investigateur coordonateur- N°ID RCB : 2007-A01026-47.
- Mise en place et évaluation médico-économique d'une stratégie innovante de détection de mutations dans le gène *COL3A1* dans le cadre du diagnostic du syndrome d'Ehlers-Danlos vasculaire. Programme de soutien aux innovations diagnostiques et thérapeutiques 2006- Promoteur APHP- Investigateur Principal (CHU de Montpellier).
- Bourse d'Etude, Fondation pour la Recherche Médicale- Diplôme d'Etudes Approfondies (DEA), 1999-2000.

**Génétique des Prédispositions
aux Anévrismes de l'Aorte
Thoracique et/ou Dissections
Aortiques (AAT/DA)**

Introduction

Parmi les maladies cardiovasculaires, les Anévrismes de l’Aorte Thoracique et/ou les Dissections aortiques (AAT/DA) sont des évènements cardiovasculaires sévères, responsables de 1-2% des décès dans les sociétés occidentales (Verstraeten et al., 2017). Le plus souvent silencieux, les AAT évoluent à bas bruit et sont asymptomatiques dans 95% des cas avant la survenue de DA ou d’une rupture qui, lorsqu’elles surviennent ne sont diagnostiquées dans un contexte d’extrême urgence hospitalière que dans moins de la moitié des cas (Elefteriades et al., 2015). La mortalité est élevée dans ce contexte (>50% des cas de DA de type A, selon la classification de Stanford). Le dépistage des AAT et plus encore de la maladie sous-jacente amenant à leur survenue (que l’on regroupera sous le terme générique « d’aortopathies ») permet une prise en charge médico-chirurgicale efficiente en termes de survie mais aussi de qualité de vie, avec un risque relativement faible de complications (JCS Joint Working Group, 2013). Les progrès importants de cette prise en charge, reposent sur : la surveillance régulière des différents diamètres de l’aorte par imagerie (échocardiographie le plus souvent), le traitement préventif par bêtabloquants et la lutte contre l’hypertension artérielle, la chirurgie prophylactique de remplacement et l’éviction des circonstances à risque (efforts violents glotte fermée, activités sportives à risque...). Ces progrès ont été réalisés en grande partie grâce aux études menées à partir du syndrome de Marfan, véritable maladie héréditaire modèle pour les AAT/DA (Erbel et al., 2001; Shores et al., 1994). Par conséquent, dépister le plus précocement possible une aortopathie constitue un véritable challenge pour la prévention des AAT/DA.

Sur le plan histologique, l’analyse des pièces opératoires d’AAT/DA (hors aortites inflammatoires) était réputée d’une « grande monotonie phénotypique » ne permettant que très peu d’orienter vers la cause de l’aortopathie sous-jacente, acquise ou constitutionnelle. L’aspect constamment retrouvé est celui de la nécrose (ou dégénérescence) kystique de la média, caractérisée par : une élastolyse avec fragmentation des fibres et des lames élastiques, une accumulation de substance

mucoïde dans la Matrice Extra-Cellulaire (MEC) et une raréfaction des Cellules Musculaires Lisses (CML) et de leurs noyaux. Il existe toutefois de subtiles différences dans les proportions respectives de ces 3 caractéristiques histo-pathologiques permettant une classification et une gradation des lésions (Halushka et al., 2016; Seike et al., 2021; Waters et al., 2017). L'identification des gènes impliqués dans différentes entités où l'histologie et l'immunohistochimie de pièce-opératoires de chirurgie aortique fait ressortir des distinctions mais la recherche de celles-ci ne sont pas encore transférées en pratique clinique.

L'imagerie médicale ne permet également que très peu d'orienter sur la cause précise de l'aortopathie sous-jacente avec toutefois quelques éléments d'orientation selon la localisation (sinus aortiques, aorte ascendante, horizontale ou descendante) et surtout la présence éventuelle d'une anomalie associée de la valve aortique (bicuspidie de la valve aortique, BAV)(Balistreri et al., 2013). L'imagerie fonctionnelle de l'aorte, notamment par ciné-IRM peut permettre d'évaluer des paramètres biomécaniques reflétant l'élasticité de l'aorte tel que la compliance aortique et la vitesse de l'onde de flux (Alain Lalande et al., 2002). Nous avons pu montrer avec d'autres équipes, pour certaines aortopathies génétiques (notamment en lien avec *MYH11*) que la détection d'une rigidité aortique anormalement précoce, précédant la dilatation et les AAT/DA chez des patients jeunes et asymptomatiques était corrélée à la présence d'une prédisposition génétique (Khou Van Kien et al., 2005; Lalande et al., 2008; Zhu et al., 2006). Ces techniques d'imagerie de pointe, ne sont cependant actuellement pas transférées en routine clinique pour le dépistage précoce des aortopathies génétiques.

L'analyse de l'ADN est indépendante de l'âge et du phénotype. Lorsque le défaut moléculaire responsable d'une prédisposition génétique est caractérisé à partir d'un patient, celui-ci peut permettre le dépistage familial préclinique de la prédisposition, la mise en place de la prévention du risque aortique ainsi que plus rarement des

possibilités de diagnostic prénatal, voire préimplantatoires dans des situations particulières.

Dans un modèle de Génétique formelle, les AAT/DA représentent un phénotype complexe et hétérogène où interviennent tout au long de la vie, un remodelage permanent et une multitude de facteurs, environnementaux (facteurs de risque), corporels (âge/vieillesse, sexe, grossesse...), biomécaniques (valve aortique, paroi et pression artérielle) et génétiques. Les différences dans la part respective de ces facteurs sont importantes selon la localisation de l'atteinte de l'aorte (segments) : sinus aortique, aorte ascendante, horizontale, descendante. En dehors de syndromes reconnaissables tels que le syndrome de Marfan (OMIM#154700) (ORPHA:558) et syndromes apparentés (syndromes de Loeys-Dietz), plus accessoirement le syndrome d'Ehlers-Danlos de type vasculaire (OMIM#130050) (ORPHA:286), les études d'agrégation familiale, reposant sur l'enquête généalogique complétée par échocardiographie ont depuis longtemps suggérées une composante héréditaire mendélienne forte avec un excès de plus de 20% d'apparentés AAT/DA au 1^{er} degré atteints d'AAT/DA ou avec mort subite inexplicée (Biddinger et al., 1997; Coady et al., 1999). Ces vingt dernières années, les découvertes issues de la caractérisation de variants touchant la fonction d'une trentaine de gènes dans les aortopathies héréditaires non syndromiques et de davantage dans des syndromes connus ou non, ont ouvert la voie au dépistage génétique, à une meilleure compréhension de leur physiopathologie moléculaire, à de nouvelles pistes de thérapies ciblées et plus globalement au développement d'une médecine moléculaire personnalisée, prédictive et préventive.

Première partie : Les 5 classes de gènes AAT/DA

Les formes syndromiques de prédispositions mendéliennes aux AATD/DA caractérisées à la fois sur le plan clinique et génétique ont été les premières décrites avec des gènes codant pour des composants de la trame des fibres élastiques (fibrilline de type 1 pour le syndrome de Marfan) ou collagénique (collagène de type III pour le syndrome d'Ehlers-Danlos de type vasculaire). Ces gènes impliqués dans des formes syndromiques, reconnaissables cliniquement ont également été les premiers à être impliqués dans des formes familiales non-syndromiques, souvent subcliniques d'AAT/DA. Ils constituent avec d'autres gènes identifiés plus récemment, un premier groupe de gènes de prédisposition codant pour des composants de la MEC. Par la suite, l'identification de gènes codant pour les sous-unités des récepteurs du TGF-béata a permis de décrire les syndromes de Loeys-Dietz dont les gènes sont également tous impliqués dans des prédispositions non-syndromiques. Ils sont regroupés dans le deuxième groupe de gènes : voie de signalisation du TGF-béata. Le troisième groupe de gènes est constitué de gènes codant pour des constituants de l'appareil contractile des CML) et de leurs attaches aux membranes, plasmique et nucléaire et impliqué dans la mécanotransduction du signal. Le gène *MYH11* codant pour les différentes isoformes de la chaîne lourde de myosine spécifique des CML a été le premier de ce groupe à être identifié à partir d'une grande famille baptisée famille « Bourgogne » et de l'association d'AAT/DA avec un excès de cas de persistance du canal artériel (PCA). Le quatrième groupe de gènes est constitué de gènes impliqués dans les hypoplasies de la voie gauche et l'association de bicuspidie de la valve aortique (BVA) avec les AAT/DA. Enfin, un dernier groupe est constitué de gènes dont la fonction est encore peu connue ou n'entrant pas dans les 4 groupes précédents. Dans ce mémoire, seuls les 3 premiers groupes sont détaillés, l'ensemble est cependant résumé dans le tableau 1 et leurs rôles avérés ou supposés dans la physiopathologie moléculaire représentés dans la figure 1.

Cette classification établie pour des besoins didactique est toutefois critiquable car certains gènes peuvent être classés dans différentes classes du fait de l'imbrication des mécanismes sous-jacents. Par exemple, le gène *FBN1*, responsable du syndrome de Marfan codant pour un composant essentiel des fibres élastiques au sein de la MEC est également impliqué dans la voie de signalisation du TGF-béta. Enfin, l'hétérogénéité des phénotypes (formes cliniques, syndromes) en lien avec ces gènes réponde en partie à l'hétérogénéité moléculaire des différentes formes alléliques et souvent à des mécanismes transcriptionnels, post-traductionnels intra- ou extracellulaires faisant intervenir la notion de perte ou de gain de fonction lorsque ceux-ci sont, au moins en partie élucidés.

TABLE 1-Principaux gènes de prédisposition aux AAT/DA

Gènes	Protéines	Syndromes associés [OMIM#]	Hérédité	Pathologie Moléculaire, phénotype AAT/DA fréquence estimée	Référence Princeps
Composants de la Matrice Extra-Cellulaire					
<i>FBNI</i>	Fibrilline de type 1	Syndrome de Marfan [154700] et fibrillinopathies [102370, 129600, 614185, 616914, 604308, 184900, 608328]	AD	La fibrilline 1 est une glycoprotéine de la MEC, composant structurel des microfibrilles des fibres élastiques. Les variants de <i>FBNI</i> à l'origine du syndrome de Marfan reconnaissable cliniquement ou non prédisent un effet quantitatif (haploinsuffisance) en lien avec la voie de signalisation du TGF-béta par excès de relargage de cytokines et/ou qualitatif (dominant négatif) sur la trame microfibrillaire. Les AAT/DA concernent quasi-exclusivement les sinus aortiques (dans la phase initiale) et les DA surviennent sur une aorte dilatée. La notion d'AAT/DA syndromique ou non est désormais abolie par la nouvelle classification diagnostique qui aboutit au diagnostic de Marfan lorsqu'un variant (probablement) délétère dans <i>FBNI</i> est identifié. Ceux-ci sont les plus fréquemment rapportés dans les AAT/DA rapportés entre x-y% selon les études faisant de <i>FBNI</i> , le gène majeur des AAT/DA.	1991 (Dietz et al., 1991)

<i>COL3A1</i>	collagen alpha-1(III) chain	SED vasculaire [130050] Polymicrogyrie avec ou sans SED vasculaire [618343]	AD AR	Composant essentiel des faisceaux de fibres collagéniques artérielles et viscérales, en association avec les autres collagènes fibrillaire (type I et V). Les variants à l'origine d'AAT/DA syndromiques (SEDv) prédisent surtout un effet qualitatif par effet dominant négatif intra et/ou extracellulaire. L'haploinsuffisance entraîne un phénotype atténué, avec une pénétrance incomplète et notamment des AAT/DA non-syndromiques. La localisation des AAT/DA concerne toute l'aorte et les DA peuvent survenir sur une aorte non ou peu dilatée. La perte de fonction est responsable à l'état homozygote d'anomalies cérébrales (polymicrogyrie). Les variants (probablement) délétères dans <i>COL3A1</i> sont rares dans les AAT/DA, rencontrés dans environ 0-0,5% des cohortes.	(Kontusaari et al., 1990)
<i>LOX</i>	protein-lysine oxidase 6-	AAT/DA 10 [617168]	AD	AAT/DA concernant surtout les sinus aortiques et l'aorte ascendante, parfois associés à une BVA et des signes marfanoïdes. La pénétrance est incomplète, modulée par la pression artérielle et les autres facteurs de risque. Les variants délétères entraînent une perte de fonction à l'état hétérozygote par un défaut de sécrétion et une diminution de l'activité enzymatique. Les variants (probablement) délétères dans <i>LOX</i> sont dans environ 0-0,5% des cohortes AAT/DA.	(Guo et al., 2016; Lee et al., 2016)

<i>FBN2</i>	fibrilline de type 2	Arachnodactylie et contractures congénitales [121050]	AD	AAT rares et peu évolutifs au niveau des sinus aortiques, DA exceptionnelles dans le syndrome CCA, où les variants délétères sont concentrés dans la région « néonatale » du gène. L'implication de <i>FBN2</i> dans des formes réellement non syndromiques d'AAT/DA est discutée mais des variants (probablement) délétères sont identifiés dans 1-2% des cas selon les études.	(Gupta et al., 2004)
<i>ELN</i>	Elastine	Sténose aortique supra-valvulaire [185500], cutis laxa AD 1[130160]	AD	Allèles Gain de fonction (hypermorphe, dominant négatif) responsables d'AAT/DA syndromiques. Le gène est peu étudié dans les cohortes	(Szabo et al., 2006)
<i>BGN</i>	Biglycan	Meester-Loeys syndrome [300989]; dysplasie spondylo-épimétaphysaire [300106]	Lié à l'X	Le biglycan constitue le noyau protéique de petits protéoglycans jouant un rôle majeur dans la formation et l'homéostasie des fibres collagènes ainsi que la différenciation et la multiplication cellulaire de voisinage par ses interactions avec des facteurs de croissance (notamment TGF-béta). Les modèles de souris KO, déficientes en biglycans étudiés avant l'implication de <i>BGN</i> en pathologie humaine sont associés soit à un phénotype osseux, en lien avec la dysplasie spondyloépimétaphysaire humaine soit avec des AAT/DA sévères et précoces, en lien avec le syndrome de Meester-Loeys humain. Les corrélations génotype/phénotype sont difficiles à établir devant le peu de cas rapportés et d'études publiées chez l'Homme.	(Meester et al., 2017)

<i>MFAP5</i>	Microfibril-Associated GlycoProtein (MAGP2) 2	AAD/DA 9 [616166]	AD	Le gène code pour une glycoprotéine associée aux fibres élastiques et au réseau microfibrillaire, MAGP2. Des études montrent outre un rôle de stabilisation des microfibrilles des interactions avec les cytokines actives TGF-béta, les intégrines et la voie de signalisation NOTCH. Une seule étude publiée, seulement 2 variants délétères identifiés dans une population de 670 patients AAT/DA (0.15%).	(Barbier et al., 2014)
<i>EFEMP2</i>	Fibuline 4	Cutis laxa, autosomal recessive, type IB [614437]	AR	La fibuline 4 est nécessaire à la formation des fibres élastiques. Les variants délétères sont essentiellement des allèles nuls avec une perte de fonction à l'origine d'une réticulation et d'une maturation défectueuse des fibres élastiques. Les AAT/DA sont précoces et sévères dans ce type de cutis laxa. L'hypothèse de variants hypomorphes à l'origine d'AAT/DA non ou sub-syndromiques n'est pas confirmée.	(Huchtagowder et al., 2006)
<i>COL1A1</i>	collagen alpha-1(I) chain	Ostéogénèses imparfaites, Syndrome d'Ehlers-Danlos arthrochalasique type 1, maladie de Caffey [166200, 166210, 259420, 166220, 130060, 619115, 114000]	AD	Elément essentiel du collagène de type I avec <i>COL2A1</i> (2/3-1/3), les variants (probablement) délétères dans <i>COL1A1</i> sont de tous types et répondent en partie à la théorie du suicide collagénique où l'effet-dominant négatif abouti à un défaut de sécrétion plus sévère que l'haploinsuffisance. L'hétérogénéité clinique importante (ostéogénèses imparfaites, SED...) réponds à l'hétérogénéité allélique. Les AAT/DA sont fréquents dans les formes syndromiques viables mais parfois subcliniques et non	(Hortop et al., 1986)

				diagnostiquées.	
<i>COL1A2</i>	collagen alpha-2(I) chain	Ostéogénèses imparfaites II, III, IV, Ehlers-Danlos valvulaire cardiaque, arthrochalasique type 2, combiné ostéogénèse imparfaite [166210, 259420, 166220, 225320, 617821, 619120]	AD	cf. <i>COL1A1</i> avec généralement un phénotype associé moins sévère du fait de la proportion (1/3-2/3 α -1/3 α 2) dans le collagène I.	(Hortop et al., 1986)
<i>COL5A1</i>	collagen alpha-1(V) chain	SED classique type 1 [130000]	AD	Elément essentiel du collagène de type V avec <i>COL5A2</i> (2/3-1/3), les variants (probablement) délétères dans <i>COL5A1</i> sont de tous types et répondent en partie à la théorie du suicide collagénique où l'effet-dominant négatif abouti à un défaut de sécrétion plus sévère que l'haploinsuffisance. Les AAT/DA sont plus fréquents dans le SED classique de type 1.	(Borck et al., 2010)
<i>COL5A2</i>	collagen alpha-2(V) chain	SED classique type 2 [130010]	AD	cf. <i>COL5A1</i> , avec toutefois un phénotype SED classique de type 2 plus modéré et fréquemment sub-clinique. Les allèles nuls, associés à une haploinsuffisance et à un phénotype SED classique très atténué pourraient être rarement impliqués dans des AAT/DA non syndromiques. Une étude rapporte l'identification d'un variant d'épissage délétère certain identifié à partir de l'analyse d'exome d'une cohorte de 102 patients AAT/DA « non syndromique » (1%).	(Park et al., 2017)

<i>AEBPI</i>	Adipocyte enhancer-binding protein 1	SED classique-like, 2 [618000]	AR	De description récente, avec moins de 10 patients rapportés avec un phénotype de SED classique-like 2, le gène <i>AEBPI</i> , code pour la protéine AE Binding, (également appelé <i>ACLP</i> pour Aortic Carboxypeptidase-like Protein) pourrait être impliqué dans des formes récessives d'AAT/DA syndromiques ou subcliniques. Les variants rapportés correspondent à des allèles nuls ou hypomorphes compatibles avec le mode d'hérédité et une perte de fonction.	(Blackburn et al., 2018)
<i>THSD4</i>	Thrombospondin type-1 domain-containing protein 4	Apparenté Marfan	AD	Les isoformes de la protéine (THSD4 ou ADAMTS-Like 6) sont nécessaire à la formation et au maintien des microfibrilles riches en fibrilline 1. De description très récente, 5 variants délétères étudiés montrent une perte de fonction, avec un effet mixte : quantitatif et qualitatif. Les AAT/DA en lien avec <i>THSD4</i> seraient plutôt tardifs, parfois associés à de discrets signes marfanoïdes. 22 variants d'intérêt de <i>THSD4</i> ont été identifiés dans une cohorte de 1880 AAT/DA (1,2%).	(Elbitar et al., 2021)
Voie de signalisation du TGF-béta					
<i>TGFBR1</i>	TGF-beta receptor type-1	Syndrome de Loeys-Dietz 1 [132800]	AD	Les variants de <i>TGFBR1</i> observés dans le SLD1 et les AAT/DA non-syndromiques sont très majoritairement des faux-sens localisés au niveau du domaine fonctionnel cytoplasmique « sérine-thréonine kinase » du	(Loeys et al., 2005)

				<p>récepteur. Une signature d'activation de la voie de signalisation du TGF-béata est constamment retrouvée dans les études <i>ex-vivo</i> et les modèles animaux. Ces éléments seraient plutôt en faveur d'un gain de fonction mais de nombreux débats, polémiques et données contradictoires, ne permettent pas de conclure définitivement entre gain de fonction et perte de fonction, « paradoxale ». Les AAT/DA surviennent surtout au niveau des sinus aortiques et les DA sur une aorte peu dilatée. Les variants (probablement) délétères sont retrouvés dans environ 1-2% des cohortes.</p>	
<i>TGFBR2</i>	TGF-beta receptor type-2	Syndrome de Loeys-Dietz 2 [610168]	AD	<p>Les variants dans <i>TGFBR2</i> impliqués dans le SLD2 et les AAT/DA non-syndromiques et leurs conséquences correspondent à leurs homologues dans <i>TGFBRI</i>. Les SLDS1 et SLD2 ne sont pas différenciables cliniquement et les données de littératures aboutissent aux mêmes données contradictoires entre gain de fonction ou perte de fonction « paradoxale ». Les AAT/DA surviennent surtout au niveau des sinus aortiques et les DA sur une aorte peu dilatée. Les variants (probablement) délétères sont retrouvés dans environ 1-2% des cohortes.</p>	(Mizuguchi et al., 2004)
<i>SMAD3</i>	Mothers against decapentaplegic homolog 3	Syndrome de Loeys-Dietz 3 / ostéoarthrite-anévrisme [613795]	AD	<p>Les variants dans <i>SMAD3</i> à l'origine du syndrome ostéoarthrite-anévrisme (ou LDS3) sont répartis tout au long du gène, incluant faux-sens, non-sens, décalage du cadre de lecture et grandes délétions, y compris de</p>	(van de Laar et al., 2011)

				l'ensemble du gène. Les données sont concordantes avec une perte de fonction mais les mêmes signatures d'activation paradoxale de la voie de signalisation TGF-béta sont identifiées <i>ex vivo</i> . Chez la souris, l'inactivation du gène est associée à de multiples phénotypes dont les AAT/DA mais, uniquement décrits chez les homozygotes. Les variants (probablement) délétères sont retrouvés dans environ 1-7% des cohortes	
<i>TGFB2</i>	transforming growth factor beta-2 proprotein	Syndrome de Loeys-Dietz 4 [614816]	AD	Les variants de <i>TGFB2</i> à l'origine du SLD4 sont de différents types, y compris des microdélétions 1q41 englobant le gène et prédisent une perte de fonction de la cytokine TGF-béta2 associée à une signature paradoxale d'activation de la voie de signalisation dans les études <i>ex vivo</i> et dans des modèles de souris. Ils sont rapportés avec une fréquence de 0.4 à 1% dans les AAT/DA.	(Boileau et al., 2012) (Lindsay et al., 2012, p. 2)
<i>TGFB3</i>	transforming growth factor beta-3 proprotein	Syndrome de Loeys-Dietz 5 [615582]	AD	Les variants à l'origine du SLD5 prédisent une perte de fonction de la cytokine TGF-béta3. La pénétrance est incomplète, notamment pour les AAT/DA et le phénotype plus modéré que dans les autres SLD. Les variants de <i>TGFB3</i> sont identifiés dans 1-2,3% des AAT/DA.	(Rienhoff et al., 2013)
<i>SKI</i>	ski oncogène	Syndrome de Shprintzen-Goldberg (SSG) [182212]	AD	Les variants (faux-sens ou en phase) à l'origine du SSG sont à ce jour tous localisés dans des domaines de liaison aux R-SMAD (pSMAD2 et pSMAD3) et SMAD4, localisés dans l'exon 1 de <i>SKI</i> , un répresseur de la voie de signalisation TGF-béta. Initialement	(Doyle et al., 2012)

				décrits comme résultants en la perte de cette régulation négative et une activation de la voie TGF-béta, ils aboutissent en fait à une stabilisation de SKI et l'absence de sa dégradation au sein des complexes R-pSMAD/SMAD4 en réponse aux stimuli TGF-béta, entraînant de fait une atténuation de la voie de signalisation. Ces découvertes récentes remettent en cause l'activation paradoxale dans les aortopathies.	
<i>SMAD2</i>	Mothers against decapentaplegic homolog 2	En attente	AD	Des variants (probablement) délétères de SMAD2 sont rares et rapportés dans environ 0,5% des AAT/DA. Il s'agit essentiellement de faux-sens (1 seul non-sens) associés à une perte de fonction. Les patients présentent en dehors des AAT/DA un phénotype marfanoïde/LDS	(Micha et al., 2015)
<i>SMAD4</i>	Mothers against decapentaplegic homolog 4	Syndrome de Myhre [139210], polypose juvénile intestinale [175050], télangiectasies hémorragiques (Rendu-Osler) [174900]	AD	Les variants non-sens ou faux-sens (domaine MH1) associés à une perte de fonction dans <i>SMAD4</i> sont impliqués dans une forme particulière de la maladie de Rendu-Osler, associée à une polypose juvénile intestinale où les AAT/DA sont particulièrement fréquents. L'étude d'un variant faux-sens très rare rapporté dans une seule famille d'AAT/DA avec de très discrets signes de SLD sans autre élément montre que ce variant aboutie à une dégradation accélérée de SMAD4 résultant en une diminution de l'expression de protéines contractiles des CMLs en réponse à un stimulus TGF-béta. Ces données préliminaires ne sont pas encore	(Duan et al., 2019)

				confirmées.		
<i>LTBP3</i>	latent-transforming growth factor beta-binding protein 3	syndrome brachyolmie- imparfaite [601216], géléophysique 3 [617809]	amélogénèse dysplasie	AR/AD	Identifiés dans moins de 1% d'une population d'AAT/DA « non syndromiques » et sans variants dans les autres gènes, des variants bialléliques de <i>LTBP3</i> sont en fait retrouvés chez des patients présentant une forme atténuée de syndrome Brachyolmie - amélogénèse imparfaite, autosomique récessif. Les AAT/DA sont probablement une complication tardive de ce syndrome rare mais peu diagnostiqué. L'implication de <i>LTBP3</i> dans les aortopathies en rapport avec un excès de signalisation TGF-béta, suggérée par la nature de ce gène dans la seule étude disponible, n'est pas confirmée.	(Guo et al., 2018)
Appareil contractile des cellules musculaires lisses et mécanotransduction du signal						
<i>MYH11</i>	myosin-11	AAT/DA#4 [132900] Myopathies viscérales		AD AR/AD	De rares variants avec un effet dominant négatif démontré ou prédit dans la chaîne lourde spécifique de la myosine 11, spécifique des CML, sont responsables de l'association AAT/DA et PCA. Des variants associés à une perte de fonction à l'état homozygote ou hétérozygote composite sont responsables de myopathies viscérales récessives, sans AAT/DA, chez l'Homme comme dans les	(Zhu et al., 2006)

				modèles de souris. La duplication du gène (allèle hypermorphe) est associée à un risque accru d'AAT/DA par gain de fonction probable. En ce qui concerne l'association AAT/DA-PCA et les allèles dominant-négatifs rencontrés, les corrélations génotype/phénotype, le gain de fonction ou la perte de fonction ne sont pas établis. L'activation de la voie du TGF-béta suggérée dans une seule étude, n'est pas confirmée et probablement pas la cause des AAT/DA.	
<i>ACTA2</i>	actin, aortic smooth muscle	AAT/DA#6 [611788], Maladie de Moya Moya 5 (614042], syndrome de défaillance multisystémique du muscle lisse [613834]	AD	<i>ACTA2</i> est un gène majeur de prédisposition autosomique dominante aux AAT/DA, impliqué dans 1,5%-21% des populations étudiées (fonction du recrutement). Les AAT/DA sont associés à d'autres atteintes cardio-vasculaires précoces à type de maladie coronarienne, d'accidents ischémiques et de maladie de Moya Moya, parfois dès la petite enfance et chez le nouveau-né, notamment en rapport avec des faux-sens récurrents touchant le résidu Arginine en position 179. Le spectre des variants rencontrés à ce jour est très majoritairement composé de faux-sens touchant des résidus conservés, avec quelques variants d'épissage et de petits remaniements en phase, prédisant un effet dominant-négatif et une altération de la contraction des CML et/ou de la formation des filaments fins de l'unité contractile (perte de fonction). L'activation de la voie du TGF-béta suggérée dans une seule étude n'est pas confirmée et probablement pas la cause des AAT/DA.	(Guo et al., 2007)

<i>MYLK</i>	myosin light chain kinase, smooth muscle	AAT/DA#7 [613780], syndrome mégavessie-microcolon-hypostaltisme intestinal [249210]	AD/AR	Les rares variants délétères dans <i>MYLK</i> , sont soit des non-sens, décalage du cadre de lecture, grandes délétions, associés à des allèles nuls, soit des faux-sens localisé dans un domaine fonctionnel retentissant sur l'activité kinase (à démontrer pour être considérés comme délétères), associés à une perte de fonction. Des particularités concernant les AAT/DA chez les patients avec un variant délétère sont identifiées avec : prédilection pour l'aorte ascendante et épargnant les sinus aortiques, des DA survenant sur une aorte peu dilatée, une pénétrance incomplète. Les variants (probablement) délétères sont rencontrés dans 0,5-1% des cohortes.	(Wang et al., 2010)
<i>PRKGI</i>	cGMP-dependent protein kinase 1	AAT/DA#8 [615436]	AD	Un seul variant faux-sens gain de fonction c.530G>A ou p.(Arg177Gln) identifié dans 3 familles avec des AAT/DA sévères et précoces (pénétrance complète dès 18 ans) associés à d'autres atteintes artérielles. D'autres variants rapportés comme probablement délétères mais en fait, de signification inconnue. La fréquence du faux-sens dans la seule cohorte rapportée est de 0.3%.	(Guo et al., 2013)
<i>FLNA</i>	filamin-A	Dysplasie valvulaire cardiaque liée à l'X [314400], syndrome congénital de l'intestin court [300048], dysplasie frontométaphysaire 1 [305620], hétérotopies périvertriculaires 1 [300048], pseudoobstruction intestinale	Lié à l'X	Les variants délétères dans <i>FLNA</i> sont responsables d'un spectre très large de maladies liées à l'X) regroupées sous le terme de Filaminopathies: anomalies du développement syndromiques, maladies osseuses constitutionnelles, myopathies	(Chen et al., 2018)

		(neuronal) [309350], syndrome de Melnick-Needles [314400], syndromes otopalatodigital types I [300048] et II, dysplasie osseuse terminale		viscérales, dysplasies des valves cardiaques et hétérotopies périventriculaires avec ou sans manifestations neurologiques et associées à une dysplasie conjonctive (autrefois dénommée SED avec hétérotomie périventriculaires. Les variants responsables de cette dernière forme (hétérotopies périventriculaires), sont des allèles perte de fonction et des AAT/DA sont rencontrés dans une proportion significative de patients (18%). A ce jour, ils ne sont pas rapportés dans les autres filaminopathies ou dans des AAT/DA isolés.	
<i>TES</i> , <i>TLNI</i> , <i>ZYX</i>	Testine, Talin 1, Zyxine		AD	Deux études récentes et préliminaires, montrent l'implication des gènes codant pour les protéines du complexe d'adhésion focale (reliant la membrane et la MEC au cytosquelette et à l'acto-myosine) dans les AAT non syndromiques et les dissections coronaires. Les variants rapportés sont des faux-sens. Seul le variant (p.Tyr251His) dans le gène <i>TES</i> a été étudié en détails par des études fonctionnelles en faveur d'un allèle hypomorphe et d'une perte de fonction.	(Li et al., 2021) (Turley et al., 2019)
<i>ARIHI</i>	E3 ubiquitin-protein ligase ARIH1	En attente	AD	Une seule étude préliminaire montre l'implication du complexe LINC (reliant le cytosquelette et l'acto-myosine à la membrane nucléaire) à partir de l'identification d'un variant non-sens et de deux faux-sens correspondants à un allèle nul et 2 allèles hypomorphes (perte de fonction) dans <i>ARIHI</i> qui code pour une enzyme	(Tan et al., 2018)

				dégradant la protéine SUN2 (élément du complexe LINC). Les AAT/DA sont associés à des anévrismes intracrâniens. La fréquence de ces variants dans la cohorte était de 1,4%	
Bicuspidie de la Valve Aortique (BVA) et Hypoplasies de la voie gauche					
<i>NOTCH1</i>	neurogenic notch protein 1 locus homolog	Maladie de la valve aortique 1 [109130], Syndrome d'Adams-Oliver 5 [616028]	AD	Le gène code pour un des 4 récepteurs membranaires de la voie de signalisation Notch. Les rares variants probablement délétères sont des faux-sens associés à une perte de fonction. Celle-ci est associée à une BAV avec calcification sévère et précoce de la valve aortique, à des cardiopathies congénitales à type d'hypoplasie de la voie gauche et plus rarement à des AAT/DA transmis en dominance avec une pénétrance incomplète. La présence de facteurs modificateurs et/ou d'un oligogénisme est fortement suggérée. La physiopathologie moléculaire est encore peu connue.	(Garg et al., 2005)
<i>ROBO4</i>	Round about homolog 4	Maladie de la valve aortique 8 [618496]	AD	D'identification récente, avec peu de données disponibles, 1 variant d'épissage, en phase et un faux-sens délétères dans <i>ROBO4</i> ont été rapportés dans 2 familles avec sténose de la valve aortique, BVA et AAT. Les données fonctionnelles suggèrent un mécanisme distinct interférant dans la fonction de modulation de la transition endothélium > mésenchyme associée à une dysfonction des	(Gould et al., 2019)

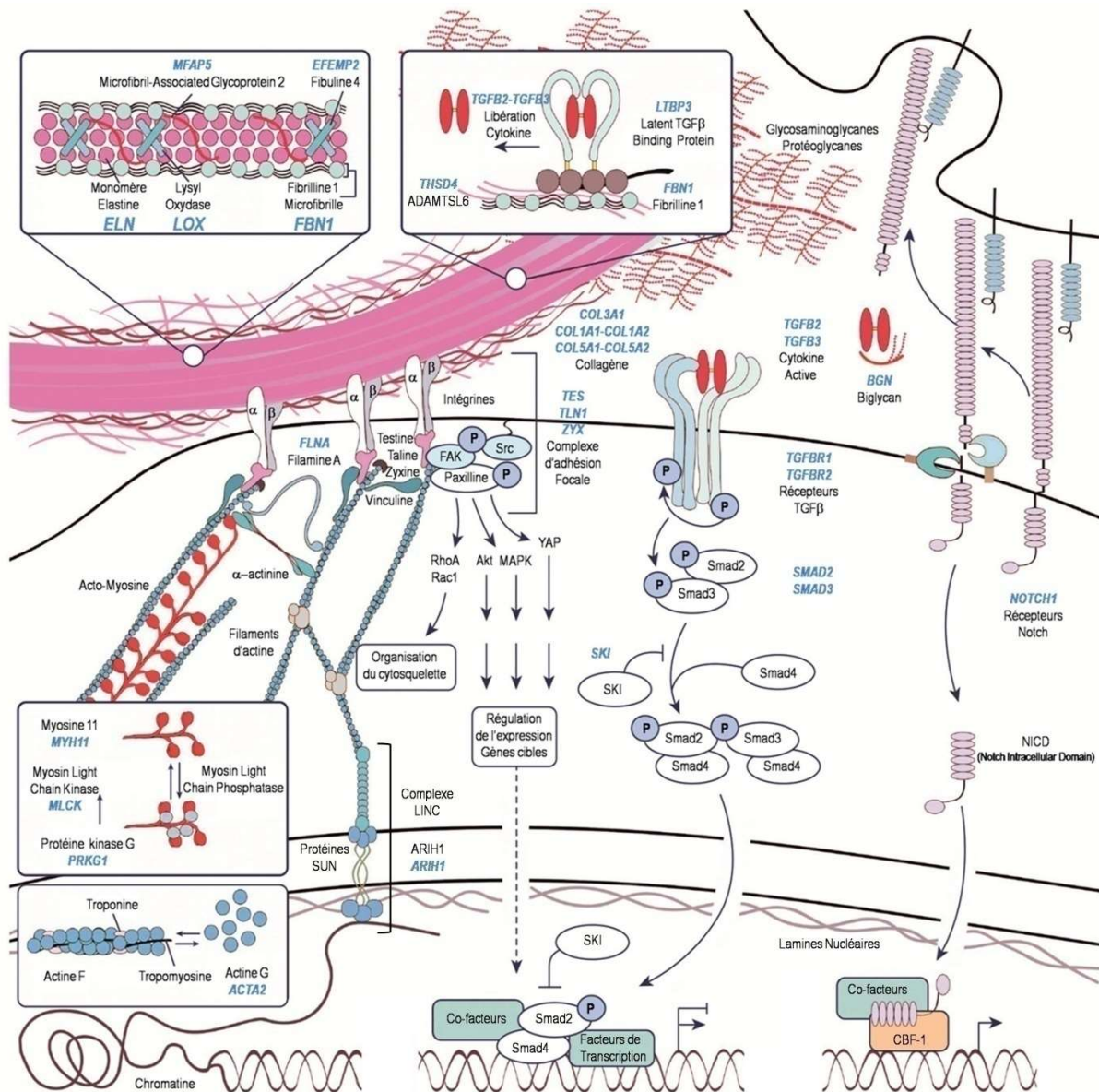
				cellules endothéliales e à une perturbation du flux sanguin.	
<i>MAT2A</i>	Methionine adenosyl transferase II, alpha	En attente	AD	Une association suggérée dans une étude avec un enrichissement de variants rares dans <i>MAT2A</i> dans une cohorte AAT/DA et une famille AAT/BVA dans laquelle un variant faux-sens de signification inconnue dans <i>MAT2A</i> coségrège en dominance avec une pénétrance incomplète (44%).	(Guo et al., 2015)
<i>SMAD6</i>	Mothers against decapentaplegic homolog 6	Maladie de la valve aortique 2 [614823] Synostose Radio-ulnaire [179300]	AD	Préalablement impliqué dans la BVA et des cardiopathies de la voie gauche (coarctation, sténose aortique), les rares variants (probablement) délétères dans <i>SMAD6</i> identifiés dans des familles AAT/DA-BVA (mais aussi des cardiopathies de la voie gauche) sont un non-sens, des faux-sens et des grandes délétions (impliquant également l'exon 1 de <i>SMAD3</i>) associés à une perte de fonction. La fonction du gène est de réguler négativement la signalisation BMP-TGF-béta, il est fortement exprimé dans l'endothélium du cœur et de l'aorte et impliqué dans la mise en place des bourrelets endocardiques.	(Gillis et al., 2017) (Luyckx, MacCarrick, et al., 2019)
Gènes dont la fonction est encore peu connue ou n'entrant pas dans les 4 groupes précédents					

<i>SLC2A10</i>	solute carrier family 2, facilitated glucose transporter member 10	Syndrome de tortuosité artérielle [208050]	AR	Le gène code pour un membre de la famille des facilitateurs du transport du glucose. Les variants (probablement) délétères sont variables (non-sens, décalage du cadre de lecture, faux-sens...), associés à des allèles nuls ou hypomorphes et à une perte de fonction. Les patients présentent une atteinte de l'aorte et des gros vaisseaux avec : allongement, tortuosité, anévrismes AAT/DA mais aussi des accidents ischémiques, une dysmorphie, des anomalies cutanées SED-like et squelettiques marfanoïdes ou SLD-like. Les accidents vasculaires peuvent être sévères dès l'enfance. La physiopathologie est mal comprise même si des travaux suggèrent un effet sur la voie de signalisation du TGF-béta, la synthèse des composants de la MEC et les fonctions de la mitochondrie.	(Coucke et al., 2006)
<i>HCN4</i>	potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4	Syndrome du sinus malade 2 [613123], syndrome de Brugada 8 [163800]	AD	Le gène code pour un canal potassium, les variants (probablement) délétères associés à une perte de fonction sont responsable du syndrome du sinus malade de type 2 caractérisé par une fibrillation auriculaire avec bradyarythmie. Les patients présentent fréquemment une particulière fréquence d'AAT/DA dont le mécanisme n'est pas identifié.	(Vermeer et al., 2016)
<i>FOXE3</i>	Forkhead box-protein E3 (FOXE3)	AAT/DA#11 [617439]; Dysgénésie du segment antérieur de l'œil 2 [610236]	AD	Le gène code pour un facteur de transcription dont les variants dont les variants perte de fonction sont responsables de dysgénésies variables du segment antérieur de l'œil (aphakie, ectopie des cristallins notamment)	(Kuang et al., 2016)

				<p>récessives autosomiques. Quelques rares variants faux-sens (probablement) délétères, localisés dans un domaine de liaison à l'ADN distinct des domaines impliqués dans les anomalies oculaires ont été identifiés et coségrégent dans des familles AAT/DA en dominance avec une pénétrance réduite. Les données fonctionnelles et histopathologiques préliminaires sont en faveur d'un effet des variants sur une augmentation de l'apoptose et des CMLs de l'aorte ascendante (dérivées des crêtes neurales) de leur raréfaction et de leur dédifférenciation en réponse à la mécanotransduction du signal.</p>	
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Figure 1- Gènes, composants, voies de signalisation et mécanotransduction du signal

Représentation schématique des différents gènes impliqués dans les AAT/DA héréditaires (indiqués en bleu) et de leurs partenaires impliqués dans les différentes voies de signalisation et la mécanotransduction du signal (adapté et modifié à partir de Creamer et al (Creamer et al., 2021)).



Chapitre 1 : Composants de la MEC

L'aorte est une artère dite élastique, composée d'une média riche en lames élastiques concentriques et parallèles (entre 50 et 70) reliées par des anastomoses et séparées les unes des autres par un tissu conjonctif hébergeant des CML (unité lamellaire). L'organisation de ces unités lamellaires confère à l'aorte ses propriétés mécaniques qui, outre son rôle premier de contenir le sang, amortie l'éjection cardiaque et l'énergie de la pression systolique en restituant une partie en phase diastolique. Ces propriétés mécaniques sont décomposées en 3 éléments : pré-étirement des fibres élastiques (« elastin pre-stretch ») ; rigidité de la trame collagénique (« collagen strain stiffening ») et contractilité des CML (« SMC contractility ») (Concannon &McGarry, 2021). Elles permettent notamment et progressivement de transformer un flux sanguin alternatif en flux continu dans le lit artériel d'aval. Les différents composants de la MEC et leur organisation sont responsable d'une partie de ces propriétés et un défaut génétique dans un de ces composants, perturbant cette organisation peut aisément expliquer la survenue d'AAT/DA.

Composants des fibres élastiques : « elastin pre-stretch »

Fibrilline de type 1 : syndrome de Marfan, la maladie modèle

La maladie modèle pour les prédispositions génétiques aux AAT/DA est de loin le syndrome de Marfan, qui représenterait environ 5% des cas de dissection aortique tous âges confondus et 50% des cas avant l'âge de 40 ans (Pape et al., 2015; Januzzi et al., 2004). Il s'agit d'une maladie modèle car : 1- c'est l'une des maladies mendéliennes les plus fréquentes (Groth et al., 2015); 2- des critères diagnostiques consensuels permettent d'uniformiser les pratiques (Loeys et al., 2010); 3- le suivi longitudinal et la prévention du risque aortique sont efficaces (Pyeritz, 2019); 4- l'implication de la voie

de signalisation du TGF-béata dans la physiopathologie moléculaire a soulevée l'espoir de disposer d'un traitement étiopathogénique des AAT/DA avec toutefois quelques déconvenues liées essentiellement aux mécanismes moléculaires sous-jacents (Lindeman & Matsumura, 2019; Dianna M. Milewicz & Ramirez, 2019; van Andel et al., 2020).

Le syndrome de Marfan survient de par le monde sans prédilection pour sexe ou l'ethnie avec une prévalence de cas diagnostiqués en population aux alentours de 1 pour 15 000 (Gray et al., 1994; Groth et al., 2015) et évaluée à environ 1/5000-1/10 000 en prenant en compte les cas non dépistés. Le diagnostic est classiquement établi à partir des critères révisés de Gand (tableau 2) (Loeys et al., 2010), qui permettent l'évaluation objective et pluridisciplinaire de critères majeurs : aortique, oculaire et génétique associé à l'évaluation d'un score systémique en 20 points regroupant des items cliniques, radiologiques ou échocardiographiques. Aucun de ces critères n'est spécifique de la maladie lorsqu'ils sont évalués indépendamment mais leur combinaison en association avec l'identification d'un variant (probablement) délétères dans *FBN1* permet d'établir le diagnostic (tableau 2). Une variabilité phénotypique intra ou interfamiliale considérable (expressivité) est communément observée et la pénétrance est considérée comme (quasi-)complète à l'âge adulte. Les cas sporadiques issus de parents non atteints qui représentaient 1/4 des cas lors des premiers développements des analyses génétiques à visée diagnostique, sont actuellement rencontrés dans au moins 50% des patients analysés (Chesneau et al., 2021; Faivre et al., 2007). Cette évolution de la proportion des variants de *FBN1* hérités / *de novo* à l'origine de syndrome de Marfan chez les patients n'est à priori pas en rapport avec un changement du taux de survenue des mutations dans le gène mais plutôt, conséquence de l'impact du dépistage de la maladie par les systèmes de santé. Par ailleurs, nous avons pu montrer un taux de mosaïcisme parental de l'ordre de 5% dans ces situations (Chesneau et al., 2021). Malgré l'évolution des critères diagnostiques, il existe fréquemment des patients présentant une forme incomplète, chez qui le diagnostic ne peut être porté cliniquement avec certitude sans caractérisation moléculaire du défaut génétique. Ceci est

particulièrement vrai chez l'Enfant, surtout lorsqu'il s'agit de cas sporadiques. En ce sens, les analyses génétiques à visée diagnostique sont d'un grand intérêt, car elles permettent de compléter la recherche des critères révisés de Gand, de confirmer un diagnostic suspecté, de réaliser le dépistage familial rapidement afin d'introduire la prévention médico-chirurgicale du risque aortique le plus précocement possible (Ladouceur et al., 2007; Mueller et al., 2014).

Tableau 2 : critères diagnostiques révisés de Gand (Loeys et al., 2010) :

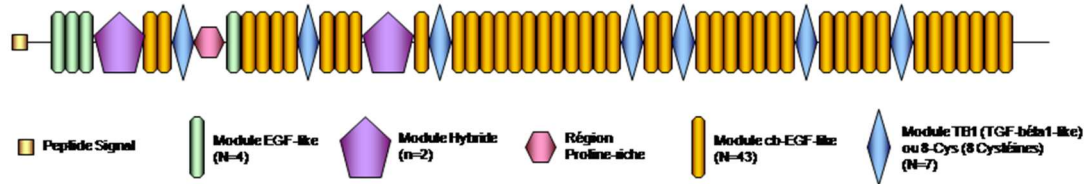
CRITERES MAJEURS	SCORE SYSTEMIQUE	
AAT/DA impliquant les sinus aortiques	Signes du pouce et/ou du Poignet	3/1
Ectopie des cristallins	<i>Pectus carinatum/excavatum</i> ou thorax asymétrique	2/1
Variant délétère ou probablement délétère dans <i>FBN1</i> (classe 4 ou 5, selon l'ACMG)	Déformation en valgus de l'arrière pied/pieds plats	2/1
Score systémique > 7	Pneumothorax spontanés récidivant	2
En l'absence d'histoire familiale,	Ectasie durale (IRM/TDM)	2

<p>le diagnostic est établi lors de l'association :</p> <p>Du critère majeur aortique avec l'un des éléments suivants : critère majeur oculaire ou variant classe 4-5 dans <i>FBN1</i> ou score systémique ≥ 7</p> <p>Du critère majeur oculaire avec l'un des éléments suivants : critère majeur aortique ou variant 4-5 dans <i>FBN1</i> ou score systémique ≥ 7</p> <p>Score systémique ≥ 7</p>	Protrusion acétabulaire (Rx/TDM)	2
	Ratio envergure /taille > 1.05 (dolichosténomélie, en l'absence de scoliose)	1
	Scoliose ou cyphose dorsolombaire	1
	Extension réduite des coudes <170° (<i>flexum</i>)	1
<p>En présence d'une histoire familiale (apparenté 1^{er} degré atteint), un des critères suivants suffit pour établir le diagnostic :</p> <p>Critère majeur aortique</p> <p>Critère majeur oculaire</p> <p>Score systémique ≥ 7</p>	Dysmorphie faciale	1
	Vergetures atrophiques inexplicées	1
	Myopie > 3 dioptries	1
	Prolapsus valvulaire mitral	1
	Score Total	/20

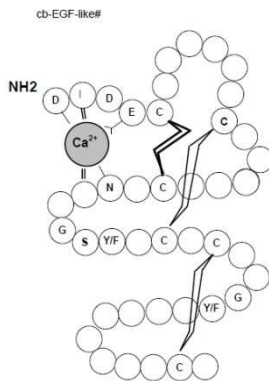
Le syndrome de Marfan est causé par des variants touchant la fonction du gène *FBN1*, qui s'étend sur plus de 237kb sur le chromosome 15 (en 15q21), avec un transcrit de 11 626 pb. Il s'agit d'un grand gène contenant 65 exons codants pour la fibrilline de type 1, protéine de 350kDa et constituant majeur des microfibrilles calcium-binding (10-12nm) qui associées à l'élastine constituent les fibres élastiques du tissu conjonctif (Godwin et al., 2019; Sakai et al., 1986). Le précurseur de la fibrilline de type 1 est composé de 2871 acides aminés et présente une structure remarquable de domaines structuraux répétés en tandems, composant des modules riches en cystéine dont la majorité correspondent aux motifs consensus calcium-binding et /ou Epidermal Growth Factor-like (modules cbEGF-like ou EGF-like) ou TGF-béta-binding (modules TB ou 8 Cys) (figure 2) (Handford et al., 1991; Khau Van Kien et al., 2010). Cette structure multimodulaire est également caractéristique des autres fibrillines (de type 2 et 3, essentiellement exprimées au stade embryonnaire) ainsi que des protéines latentes TGF-béta-binding (LTBP), qui forment la « famille des gènes fibrilline-LTBP » (Robinson et al., 2006). De multiples travaux montrent que du fait de ces propriétés, la fibrilline joue un rôle important au sein de la matrice extracellulaire dans la régulation de la biodisponibilité tissulaire des cytokines du TGF-béta et par voie de conséquence sur cette voie de signalisation (figure 3) (Chaudhry et al., 2007; Isogai et al., 2003; ten Dijke & Arthur, 2007).

Figure 2- La fibrilline de type 1

a- représentation schématique de la structure multimodulaire de la fibrilline de type 1



b- représentation schématique de la structure multimodulaire de la fibrilline de type 1



A ce jour, plus de 3000 mutations dans le gène *FBN1* sont répertoriées dans la base de données locus-spécifique UMD-FBN1, disponible sur Internet (<http://www.umd.be>) (Collod-Bérout et al., 2003). Les mutations sont peu récurrentes (environ 18%), majoritairement privées et réparties tout au long du gène, sans point chaud (en dehors de l'exceptionnelle forme dite « néonatale », où les mutations *de novo* surviennent dans la région des exons 24-32). Les grands réarrangements sont rares et la majorité des mutations (environ 2/3) sont des mutations faux-sens, substituant un acide aminé impliqué dans la formation de pont disulfure (cystéines), dans la liaison au calcium ou dans la maintenance de la structure des modules consensus cb-EGF-like (figure 2b). Dans ces situations, l'effet que l'on peut prédire est un effet dominant négatif (allèles antimorphes): antagonisme entre le produit de l'allèle sauvage et de l'allèle muté

retentissant sur la quantité de sécrétion de fibrilline de type 1 (défaut quantitatif) et/ou sur l'organisation et la maintenance des microfibrilles (défaut qualitatif). Le reste des mutations correspond à des variants non-sens, décalant le cadre de lecture ou d'épissage, prédictives de la formation d'un peptide tronqué, qui lorsqu'il est majoritairement dégradé par le Non-sens Mediated Decay (NMD) résulte en des allèles nuls ou hypomorphes (haploinsuffisance si 50% du produit) : défaut quantitatif de production de fibrilline de type 1. Ces deux mécanismes interagissent avec les deux rôles physiologiques principaux de la fibrilline de type 1 (structure des fibres élastiques et biodisponibilité tissulaire du TGF-béta (figure 3)) et représentent une des clés des corrélations génotype-phénotype qui longtemps limitées, commencent à être établies grâce à l'enrichissement des bases de données internationales (Arnaud et al., 2021; Faivre et al., 2007). Dans ces corrélations, les allèles antimorphes sont associés à un excès très significatif d'atteintes oculaires à type d'ectopie/luxation des cristallins lorsque les allèles nuls et hypomorphes sont associés aux AAT/DA et aux atteintes squelettiques. Cependant, ces corrélations restent pauvres à l'échelle individuelle et la très grande variabilité des phénotypes observés entre patients apparentés ou non pour une même mutation illustre l'importance de facteurs modificateurs, environnementaux et/ou génétiques. Par conséquent, il n'est pas possible de prédire un phénotype pour une mutation donnée, ni d'en établir un pronostic précis. De plus, les mutations du gène *FBN1* peuvent aussi rendre compte d'autres manifestations, parfois très différentes du syndrome de Marfan, regroupées sous le terme de « fibrillinopathies de type 1 », mais cette notion tends à disparaître avec la nouvelle classification diagnostique : Ectopie du cristallin familiale (MIM#129600), AAT/DA familiaux non-syndromiques (voir ci-dessous, MIM#132900), forme dominante du syndrome de Weill-Marchesani [MIM#608328], phénotype MASS [MIM#604308], dysplasie acromicrique [MIM#102370], dysplasie géléophysique 2 [MIM#61485], Stiff-skin syndrome [MIM#184900] ou même atteintes squelettiques isolées (Milewicz et al., 1995). Ceci est notamment le cas lorsqu'un variant délétère est identifié dans *FBN1* dans une famille avec AAT/DA impliquant les sinus aortiques « non-syndromique » sans autre élément

squelettique, oculaire, cutané, pulmonaire ou dural, puisque le diagnostic désormais porté est celui d'un syndrome de Marfan.

Pour toutes ces raisons, les tests génétiques dans le syndrome de Marfan représentent un réel challenge, pour un bénéfice parfois limité. Toutefois, les analyses génétiques sont particulièrement indiqués dans deux situations : 1- chez les sujets chez qui l'apport du critère familial/génétique (tableau 2) est susceptible de compléter le score clinique et d'aboutir au diagnostic (particulièrement chez l'Enfant et dans les formes sporadiques), permettant ainsi de mettre en place la prévention (voir ci-dessus) ; 2- chez les sujets réunissant les critères préalablement à l'analyse, afin d'une part de confirmer le diagnostic et d'autre part de pouvoir disposer d'un test permettant de conduire rapidement et simplement l'enquête familiale. Dans notre expérience, la recherche d'une mutation délétère du gène *FBN1* identifiée à partir d'un cas index chez son apparenté, permet de clarifier son statut dans plus de 50% des cas et ce dépistage génétique se réalise de plus en plus chez des patients non examinés, notamment par échocardiographie et déclarés « asymptomatiques ». L'identification de la mutation est de plus un préalable indispensable à toute demande de diagnostic prénatal ou préimplantatoire, en dehors de toute autre considération, notamment du fait de l'hétérogénéité génétique. Toutefois, compte tenu du pronostic actuel de la maladie, les demandes sont rares.

Enfin, les perspectives de thérapie ciblée, basée sur le blocage de la voie de signalisation du TGF-béta pour traiter préventivement les AAT/DA ou autres, soulignent le besoin de disposer d'un diagnostic précoce, rigoureux et précis, grandement facilité par les tests génétiques (qui permettent d'obtenir un critère diagnostic indépendamment de l'âge et du phénotype). En effet, il est probable que seuls certains patients atteints de syndrome de Marfan ou de prédispositions génétiques apparentées puissent réellement bénéficier de ces thérapies ciblées sur la signalopathie ou d'autres mécanismes (cf. infra, voie de signalisation du TGF-béta) (Al-Abcha et al., 2020; Van Driest et al., 2020; van Andel et al., 2020)).

Autres gènes codants pour des composants des fibres

élastiques :

***ELN*, Elastine et AAT/DA :**

Le gène *ELN*, qui code pour la tropoélastine maturée en élastine, protéine majeure de la trame élastique est depuis longtemps impliqué en pathologie artérielle. Il est un des éléments clé du syndrome de gènes contigus correspondant à la microdélétion récurrente en 7q11.23 (locus WBS) répondant au syndrome de Williams-Beuren [MIM#194050], responsable du phénotype artériel, notamment de sténoses aortiques supra-avalvulaires, pulmonaires, rénales..., et d'hypertension artérielle (Ewart et al., 1993). De manière intéressante, l'haploinsuffisance d'*ELN* aboutie à une augmentation paradoxale du nombre de lames élastiques et de la population de CML dans la média aortique à l'origine de la pathologie sténosante (Li et al., 1998). La duplication et la triplification de la région WBS, à l'origine d'un syndrome neurodéveloppemental « en miroir » du syndrome de Williams-Beuren [MIM#609757], correspond à un gain de fonction hypermorphie d'*ELN*. 46% des patients atteints de duplication 7q11.23 présentent un AAT/DA âge dépendant et 15% une persistance du canal artériel (cf. chap.3)(Morris et al., 2015; Parrott et al., 2015). Les variants délétères intragéniques d'*ELN* sont quant à eux impliqués dans les formes familiales de sténose aortiques supra-avalvulaires [MIM#185500] avec des allèles perte de fonction et de *cutis laxa* dominante (ADCL1) [MIM#123700] où des manifestations aortiques à type de sténose supra-valvulaire (allèles perte de fonction) ou d'AAT/DA (allèles gains de fonction, effet dominant négatif) peuvent être rencontrées (Graul-Neumann et al., 2008; Szabo et al., 2006).

***FBN2*, Syndrome CCA et AAT/DA :**

Les variants délétères de la région centrale du gène *FBN2* (exons 23-34 associés à la région dite « néonatale ») sont associés au syndrome de Beals-Hecht ou CCA (Congenital Contracture Arachnodactyly), apparenté au syndrome de Marfan dont il partage de nombreuses caractéristiques cliniques communes (i.e. grande taille, scoliose, arachnodactylie...) [MIM#121050]. Ce recouvrement phénotypique est tel que, Beals et Hecht (Beals & Hecht, 1971) ont dans leur première description du syndrome CCA suggérés que la description en 1896 des atteintes de la petite Gabrielle par Bernard Jean Antonin Marfan, à l'origine du syndrome éponyme, correspondait en fait à un syndrome CCA et non à un syndrome de Marfan (Marfan, 1896). Opposé au syndrome de Marfan, en ce qui concerne les atteintes aortiques quelques observations de patients atteints d'AAT, généralement modérés ont pointées l'intérêt d'une surveillance aortique (Gupta et al., 2004; Siddiqui & Panesar, 2019). Le gène *FBN2* code pour la fibrilline de type 2, une glycoprotéine de 2912 amino-acides très proche de la fibrilline de type 1, dont elle partage la structure multimodulaire (figure 2) sauf en ce qui concerne le domaine riche en Proline, remplacé par un domaine riche en Glycine dans la fibrilline de type 2. Comme la fibrilline de type 1, les modifications post-traductionnelles aboutissent à des homodimères intracellulaires, fortement exprimée au cours du développement embryonnaire précoce, notamment dans l'aorte et qui co-localisent plus tardivement au cours du développement avec la fibrilline de type 1 au sein des microfibrilles élastiques (Trask et al., 1999; Zhang et al., 1995). Une série récente de 174 patients présentant un CCA diagnostiqué cliniquement et analysés pour le gène *FBN2*, montre la présence d'une dilatation de la racine aortique chez 21/93 (22,6%) patients chez qui les données étaient disponibles (Meerschaut et al., 2020). Sur cette population, un variant (probablement) délétère de *FBN2* a pu être identifié chez seulement 44 patients (25%) dont 4 seulement présentait un AAT (9%). Les auteurs concluent que les AAT/DA bien que possibles et rare dans ce contexte syndromique ne font pas partie du spectre clinique. Toutefois, le séquençage de panels de gènes impliqués dans les aortopathies et incluant le gène *FBN2* chez des patients avec AAT/DA non syndromiques identifie des

variants (probablement) délétères à une fréquence comparable à celle d'autres gènes, soulevant la question de variants associés à une prédisposition non syndromique (Arnaud et al., 2019; Overwater et al., 2018).

***MFAP5* et AAT/DA**

C'est par une stratégie de séquençage d'exome entier à partir de l'ADN de 4 patients issus d'une famille avec AAT/DA non-syndromiques transmis en dominance que l'équipe du Pr Boileau a identifié pour la première fois un variant non-sens dans le gène *MFAP5* (Barbier et al., 2014). Le séquençage des 10 exons et bordures introniques de *MFAP5* dans une collection d'ADN issue de 670 patients AAT/DA (225 familiaux, 178 simplex issus de la population française et 267 issus d'une population américaine) a identifié un seul variant faux-sens à proximité du site putatif du site de clivage du peptide signal dans une famille américaine. La contribution de ce gène aux prédispositions aux AAT/DA semble donc très faible (0,15%) et cette découverte n'est pas encore répliquée dans la littérature. Le gène *MFAP5* code pour une glycoprotéine associée aux fibres élastiques et au réseau microfibrillaire, MAGP2 (microfibrillar associated glycoprotein 2)(Combs et al., 2013). Les études fonctionnelles *ex vivo* et *in vitro* de ces deux variants montrent une absence de retentissement sur la transcription et une réduction d'environ 50% de protéine MAGP2 exprimée dans la MEC, vraisemblablement en altérant sa maturation post-traductionnelle intra et/ou extracellulaire. Les auteurs concluent à une perte de fonction.

***THSD4*, fibrillinogénèse et AAT/DA :**

A partir de 106 patients issus de 35 familles atteintes d'AAT/DA dans lesquelles les analyses génétiques n'avait pas identifié de variant délétère (sur une cohorte totale de ,

Elbitar et al ont identifié par séquençage d'exome entier des variants d'intérêt dans le gène *THSD4* (*THROMBOSPONDIN TYPE-1 DOMAIN-CONTAINING PROTEIN 4*) (Elbitar et al., 2021). Ce gène code pour deux isoformes de 1018 et 658 résidus amino acides d'une protéine nécessaire à l'assemblage des microfibrilles par liaison covalentes avec la fibrilline de type 1 appelées respectivement ADAMTSL6 α et ADAMTSL6 β (Tsutsui et al., 2010). La deuxième isoforme a la potentialité de restaurer l'assemblage des microfibrilles et le relargage excessif de cytokines du TGF-béata dans un modèle de souris Marfan (Saito et al., 2011). En étendant l'analyse de ce gène candidat à une banque d'ADN issus de 1114 patients AAT/DA français syndromiques ou non et 731 patients américains (dont 346 cas familiaux), Elbitar et al., ont identifié 22 variants d'intérêt dans *THSD4* (1,2% de la population étudiée). 5 de ces 22 variants étaient identifiés dans des familles, permettant une analyse de coségrégation et des études fonctionnelles : un variant non-sens (c.740del ; p.(Leu247*)), un variant décalant le cadre de lecture (c.1402del ; p.(Ala468Glnfs*45), une duplication en phase (c.137_145dup ; p.(Asp46_Gly48dup) et trois faux-sens (p.(Tyr321Asn) ; p.(Gly753Asp) ; p.(Arg7841Trp)). Les AAT/DA chez ces patients surviennent plutôt tardivement et les manifestations cliniques associées comportent de discrets signes marfanoïdes (*pectus excavatum*, dolichosténomélie, scoliose, palais haut et arché, pneumothorax). Les études de transcrits avec ou sans inhibiteurs du Non-sens Mediated Decay (NMD), montrent que l'effet fonctionnel des variants avec arrêt prématuré de la traduction est quantitatif, par haploinsuffisance. Pour les 3 faux-sens, les études de transfection et d'immunocytochimie montrent une désorganisation du réseau microfibrillaire associé à la fibrilline de type 1 (effet qualitatif) et une diminution ou une augmentation de la quantité de fibrilline de type 1 (effet quantitatif) secrété par les cellules en culture. L'analyse histologique et immunohistochimique d'un fragment de pièce opératoire de chirurgie de remplacement aortique chez un patient porteur du variant c.1402del montre l'aspect de dégénérescence kystique de la média et une augmentation des marqueurs de la voie de signalisation du TGF-béata. Ces aspects étant non spécifiques, retrouvés dans d'autres aortopathies génétiques.

Composants de la trame collagénique : « collagen strain stiffening »

***COL3A1* et syndrome d'Ehlers-Danlos de type vasculaire (SEDv, MIM#130050)**

Les syndromes d'Ehlers-Danlos (SED) représentent un groupe hétérogène d'anomalies héréditaires du tissu conjonctif caractérisées par une hyperextensibilité cutanée, une hyperlaxité articulaire et une fragilité tissulaire. Les critères diagnostiques établis selon la classification de New-York (Malfait et al., 2017), distingue treize entités principales (types : classique, classique-like, cardiaque-valvulaire, vasculaire, hypermobile, arthrochaliasique, dermatoparexis, cyphoscoliotique, cornée fragile, spondylo-dysplasique, musculocontractural, myopathique et périodontique) et remplace la classification précédente (de « Villefranche », (Beighton et al., 1998)) afin de prendre en compte les nouvelles descriptions, les découvertes biochimiques et génétiques issues en grande partie de l'essor du séquençage de deuxième génération. Les AAT/DA sont classiquement associés au SED vasculaire (anciennement type IV) et rarement décrits dans les autres types de SED. Transmis selon une hérédité autosomique dominante, le SED vasculaire est causé par des variants touchant la fonction du gène *COL3A1* (gène du procollagène de type III). La prévalence de ce syndrome rare n'est pas connue et globalement estimée entre 1/50 000 et 1/150 000 (Byers, 1995; Byers, 1993). Toutefois, le diagnostic est particulièrement difficile à établir du fait d'une variabilité phénotypique importante, y compris entre sujets atteints d'une même famille et il est fréquent que les enquêtes familiales et génétiques révèlent la présence de patients paucisymptomatiques avec des manifestations subcliniques, porteurs de mutation non ambiguë, incluant des manifestations artérielles en apparence isolées telles que les AAT/DA (Khau Van Kien & Demaria, 2009). Afin de faciliter les démarches diagnostiques, les critères de New-York définissent les indications des tests génétiques qui, seuls en pratique peuvent permettre un diagnostic de certitude, notamment en

présence d'une histoire familiale de rupture ou de dissection artérielle chez des patients de moins de 40 ans (Malfait et al., 2017).

Le SED vasculaire a un pronostic sévère avec comme première cause de décès, des ruptures/dissections artérielles, notamment aortiques. Les séries de patients atteints de SEDv (cas index et apparentés atteints avec un diagnostic confirmé par les tests de laboratoire) évaluent une médiane de survie de 51 ans avec toutefois des écarts très importants (10-80 ans) (Byers et al., 2017; Oderich et al., 2005; Pepin et al., 2000). Les complications artérielles récidivantes chez les patients sont à type de dissections spontanées ou plus rarement d'anévrismes disséquant (66%), de ruptures spontanées (64%), fistules artérioveineuses (15%) et d'anévrismes fusiformes (14%). Elles peuvent être partiellement prévenues par un traitement préventif (céliprolol) qui se révèle efficace en terme de survie et de nombre de complications (Frank et al., 2019; Ong et al., 2010). Classiquement, les artères musculaires, de moyen calibre sont les plus fréquemment touchées. Ainsi, les AAT/DA ne sont pas réellement caractéristiques du SED vasculaire qui restent l'apanage du syndrome de Marfan et des syndromes apparentés. Cependant, ils peuvent survenir y compris au niveau de l'aorte ascendante et sont souvent fatals. L'implication de variants nuls ou hypomorphes de *COL3A1* dans les AAT/DA, impliqués dans des formes subcliniques de syndromes d'Ehlers-Danlos est toutefois probablement plus complexe (cf. infra)(Shalhub et al., 2014).

Le gène *COL3A1* est composé de 51 exons (les exons 4 et 5 sont fusionnés), distribués sur plus de 38kb sur le bras long du chromosome 2, en 2q24.3-q31. Il code pour la chaîne pro α -1(III) du collagène, une protéine de 1466 acides aminés parmi lesquels 1029 sont situés dans le domaine triple hélice, caractérisé par une succession de triplets répétés Gly-X(1/3)Pro-Y. L'organisation génomique de la famille des gènes des collagènes fibrillaires est étroitement liée à l'évolution des espèces et provient de la duplication d'un gène ancestral. Par conséquent, ces gènes ont des homologies de séquence importantes, surtout au niveau du domaine triple-hélice qui comporte 42 exons débutant invariablement par un codon glycine et dont l'alternance d'exons, composé d'un multiple de 18 ou de 33 codons est remarquablement superposable. Du

fait de cette structure singulière, tous les exons de ce domaine sont en phase avec le cadre de lecture et le motif d'acides aminés Gly-X(1/3Pro)Y indispensable à la formation de la triple hélice. Des modifications post-traductionnelles complexes du procollagène de type III aboutissent au clivage des propeptides puis à la formation d'unités d'homotrimères de collagène III, qui forment avec d'autres unités collagènes des structures microfibrillaires de plus en plus complexes jusqu'aux faisceaux de fibres périodiques de collagène. Plusieurs conséquences importantes peuvent être extraites de ces particularités : 1)- Parce que le collagène de type III est un homotrimère, la synthèse d'une quantité égale de chaîne normale et mutée chez les patients résulterait en la production d'un ratio 7 unités triple-hélice anormales pour 1 unité triple-hélice normale (effet dominant négatif). 2)- du fait des importantes homologies de séquences avec des séquences génomiques correspondantes longtemps restées indéterminées, les tests génétiques ont été longtemps limités aux analyses de transcrits (ADN complémentaire) ainsi qu'aux analyses biochimiques des collagènes (électrophorèse en SDS-PAGE) expliquant en partie un biais méthodologique dans le spectre de mutations identifiées. Il s'agit de variants faux-sens prédictifs de la substitution de résidus Glycine obligatoires du domaine triple hélice dans environ 2/3 des cas et dans la très grande majorité des cas restants, de mutations d'épissage prédictives de la synthèse d'une protéine raccourcie, en phase avec le domaine triple hélice (voir plus haut) (Dalglish, 1998). Ce type de variants s'accompagne d'un pronostic sévère sur la maturation post-traductionnelle du collagène de type III (voir infra, autres collagènes, concept du « suicide collagène »), visible sur l'architecture des faisceaux de fibres collagènes en microscopie électronique (effet dominant négatif, qualitatif)(Ong et al., 2012) et sur le plan clinique d'une pénétrance quasi-complète . Avec les progrès technologiques, les données concernant les variants non-sens, ou décalant le cadre de lecture, de grandes délétions, prédictifs d'allèles nuls et d'une haploinsuffisance (effet quantitatif) ou de variants faux-sens non glycine, hypomorphes, se sont accumulées, permettant d'établir quelques corrélations génotype/phénotype (Frank et al., 2015; Kuivaniemi & Tromp, 2019; Plancke et al., 2009). Ces variants s'accompagnent chez les patients d'une pénétrance réduite, évaluée à 50%, d'une espérance de vie augmentée de 10 à 15 ans par rapport aux variants

classiques, de davantage d'AAT/DA et d'une absence de complications digestives par rapport aux variants prédisant dominant-négatifs (Leistritz et al., 2011; Shalhub et al., 2014). Ce type de variant est également retrouvé dans des populations AATDA non syndromiques (Overwater et al., 2018).

Autres collagènes et AAT/DA :

Les autres gènes codants pour des collagènes impliqués dans les AATDA sont essentiellement les gènes *COL1A*, *COL1A2*, *COL5A1* et plus accessoirement *COL5A2*.

Osteogénèses imparfaites, SED classique, cardiaque-valvulaire et vasculaire :

Les AAT/DA font partie des complications cardio-vasculaires des ostéogénèses imparfaites (OI), un groupe de maladies rares associées à une fragilité de l'os, majoritairement causé par des variants touchant la fonction des gènes codants pour le procollagène de type I (*COL1A1* et *COL1A2*) (McNeeley et al., 2012; Radunovic et al., 2011). Une revue récente, montre que 10-30% des patients OI présentent un AAT, avec une dilatation de la racine aortique dépistée fréquemment dès l'enfance, peu évolutive et des DA très rares (Balasubramanian et al., 2019). Chez de rares patients, des variants particuliers de *COL1A1* et/ou *COL1A2* entraînent un phénotype différent, notamment un SED de type classique-like, cardiaque-valvulaire et même vasculaire, associé ou non avec de discrets signes d'OI et des AAT/DA (Malfait et al., 2013, 2017). De manière moins fréquente, les AAT/DA font également partie des complications cardiovasculaires rares (en dehors du prolapsus valvulaire mitral et de la fragilité capillaire) rencontrées dans le SED de type classique en rapport avec des variants (probablement) délétères dans le gène *COL5A1* et exceptionnellement avec le gène *COL5A2* (environ 12% des patients selon Ritelli et al. (Ritelli et al., 2020)).

Les gènes *COL1A1*, *COL1A2*, *COL5A1* et *COL5A2* codent respectivement pour les chaînes pro- α 1(I) et pro- α 2(I) du collagène de type I et de type V qui, au cours d'un processus de maturation post-traductionnelle complexe, s'associent grâce leurs domaines triple-

hélice (Gly-X-Y), en hétérotrimères, avec un rapport 2:1 entre chaînes $\alpha 1$ et $\alpha 2$ (Prockop & Kivirikko, 1995). Au niveau de la média des artères et notamment de l'aorte (où ils constituent 80-90% du collagène total), les hétérotrimères de collagènes de type I, s'associent avec les homotrimères du collagène de type III et les hétérotrimères du collagène de type V, pour former des structures fibrillaires extrêmement régulières qui s'associent progressivement en fibres, puis en faisceaux périodiques de fibres collagène, constituant la trame collagénique en lien avec les propriétés élastiques et la résistance mécanique de l'aorte. Dans cette organisation extrêmement précise, le concept du « suicide collagène » établie que la présence de chaînes pro- α irrégulières car mutées (défaut qualitatif, faux-sens ou en phase) aboutie à un effet dominant-négatif intracellulaire intense et à la dégradation précoce d'éléments hétérotrimères (collagènes I et V) ou homodimères (collagène III) triple-hélice, résultant au final en un défaut de sécrétion global et un retentissement plus sévère sur la trame collagénique qu'un défaut quantitatif initial (haploinsuffisance) (Prockop, 1984). Enfin, les nombreuses voies de signalisation (dont la voie de signalisation TGF- β) et facteurs de régulation de la transcription impliqués dans la mécanotransduction, le remodelage artériel et la synthèse de la trame collagène (en partie autorégulée) (Etich et al., 2020; Roche & Czubryt, 2014), sous-tendent avec les éléments précédents que les gènes codant pour les constituants essentiels de la trame collagénique sont candidats pour les prédispositions mendéliennes aux AAT/DA. Leur implication, notamment dans les formes non-syndromiques (en dehors du collagène de type III) semble cependant peu fréquente avec toutefois quelques variant (probablement)délétères et de signification inconnue en excès identifiés dans les quelques cohortes rapportées où ces gènes ont été analysés (Fang et al., 2017; Ziganshin et al., 2015).

LOX, Lysyl-oxydase et AAT/DA :

Le gène *LOX* code pour la Lysyl-oxidase (ou protéine-lysine 6-oxydase), une enzyme cuivre qui catalyse la désamination oxydative des résidus lysine et hydroxylysine dans les collagènes et l'élastine, première étape de leur réticulation dans la MEC (Hämäläinen

et al., 1991). Son implication dans les AATDA suggérée depuis une vingtaine d'année par un modèle de souris (Mäki et al., 2002) est récente (2016), répliquée de manière indépendante, avec une contribution significative dans les prédispositions autosomiques dominantes aux AATDA (Guo et al., 2016; Lee et al., 2016). A partir d'une grande famille d'AAT/DA non syndromique, Guo et al, ont identifié par séquençage d'exome entier un variant faux-sens dans *LOX* (p.Ser280Arg) localisé dans le domaine catalytique de l'enzyme, candidat par ses caractéristiques pour expliquer la prédisposition (Guo et al., 2016). Ils ont ensuite recherché par séquençage d'exome ou Sanger la présence d'autres variants dans *LOX* chez 410 patients indépendants d'AAT/DA familiaux et identifiés 2 variants non-sens : c.125G>A (p.Trp42*) et c.604G>T (Gly202*), et 3 variants faux-sens : c.743C>T (p.Thr248Ile); c.800A>C (p.Gln267Pro) et c.1044T>A (p.Ser348Arg) également localisés dans le domaine catalytique très conservé de l'enzyme, soit 1,2% des familles AATDA étudiées. Les études fonctionnelles, de coségrégation familiales et l'analyse des pedigrees indiquent : une diminution modérée de l'activité de la lysyl-oxydase (perte de fonction); une pénétrance incomplète (p.Trp42*) et (p.Gln267Pro) ; la présence d'une bicuspidie de la valve aortique chez 3 des 18 patients présentant un des 5 variants (18%) ainsi que quelques signes marfanoïdes, squelettiques ou cutanés (*pectus excavatum*, scoliose, dolichosténomélie, vergetures précoces inexplicées... L'aspect histopathologique d'échantillons d'aorte issus de pièce opératoire de 2 patients (p.Gly202*) et (p.Ser280Arg) montre des aspects non spécifiques de dégénérescence kystique de la média associés avec une augmentation du dépôt de la trame collagénique (rouge Sirius). De manière quasi concomitante, Lee et al ont rapporté l'étude exhaustive d'une mutation faux-sens c.893C>T (p.Met298Arg) identifiée à partir du séquençage génome entier d'un patient avec un diagnostic clinique de syndrome de Marfan (non confirmé en génétique moléculaire) et coségrégeant avec les AATDA transmis en dominance dans sa famille (Lee et al., 2016). Les données cliniques issues de cette famille, confirment la présence de signes marfanoïdes chez les patients. Pour confirmer le retentissement fonctionnel de ce variant, les auteurs ont utilisé la technologie CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/clustered regularly interspaced short palindromic repeats-associated protein 9 nuclease) pour

générer un modèle souris avec le faux-sens équivalent ($Lox^{+/M292R}$). La caractérisation de ce modèle de souris montre : 1- chez les homozygotes, une mortalité post-natale précoce (quelques heures) en rapport avec des complications hémorragiques d'AATDA et une expression tissulaire normale de *Lox* s'accompagnant d'une perte de l'activité enzymatique 2- chez les hétérozygotes : une survie prolongée de 6 mois avec, un épaissement la paroi aortique en rapport avec une fragmentation des lames élastiques de la média aortique associée à une rigidité aortique augmentée et un allongement de la longueur de l'aorte de 10% par rapport aux souris témoins. Par la suite, les études complémentaires menées sur ce modèle de souris (Lee et al., 2019) ont montrées les données suivantes : les souris hétérozygotes ($Lox^{+/M292R}$).développent un AAT/DA au niveau de l'aorte ascendante lors de stress hémodynamiques (traitement par angiotensine II résultant en une hypertension artérielle induite) ; la rétention par le réticulum endoplasmique et la dégradation du pro-peptide muté via la voie du protéasome/autophagie, entraînant un défaut de sécrétion mais n'interagissant pas avec la protéine sauvage, normalement maturée et exprimée mais en quantité réduite.

L'ensemble de ces données est compatible avec une perte de fonction à l'origine des AAT/DA causés par *LOX*.

Depuis ces 2 études princeps, peu de familles sont rapportées dans la littérature. Des variant délétères de *LOX* sont identifiés dans des populations AATDA avec une fréquence d'environ 1-2% (Arnaud et al., 2019; Beil et al., 2021).

BGN, syndrome de Meester-Loeys et dysplasie spondylo-épimétaphysaire:

En analysant en NGS, 11 patients avec des critères cliniques de syndrome de Marfan (non confirmés sur le plan moléculaire) pour 368 gènes codants pour des composants de la MEC ou impliqués dans la voie de signalisation du TGF-béta, Meester et al (Meester

et al., 2017) ont identifiés 2 variants (probablement) délétères dans *BGN* :c.5G>A (p.Trp2*) et c.908A>C (p.Gln303Pro) altérant potentiellement l'épissage car localisée au niveau de l'avant-dernière base de l'exon 7 (sans étude de transcrits réalisée). Ils ont ensuite séquencé par méthode SANGER les 8 exons et bordures introniques de *BGN*, l'ADN de 360 patients et 155 patientes atteints d'AAT/DA syndromiques ou non et identifié un variant faux-sens localisé au niveau du site donneur d'épissage de l'exon 2 : c.238G>A (p.Gly80Ser) ainsi qu'indirectement du fait de l'échec d'amplification de certains amplicons, deux grandes délétions intragéniques par microarrays (puces SNPs) impliquant les exons 2 à 8 de *BGN*. L'étude des transcrits réalisée à partir de cultures de fibroblastes cutanés avec et sans inhibiteur du NMD (puromycine) montre que le variant c.238G>A (p.Gly80Ser) entraîne une anomalie complexe de l'épissage avec 8% de transcrits normaux et 3 transcrits aberrants comportant des séquences introniques de 45 (en phase), 11 et 368 (hors phase) paires de bases. Ces 3 dernières anomalies ayant été identifiées chez des patients avec un diagnostic initial de syndrome de Loeys-Dietz, de syndrome de Melnick-Needles et de filaminopathie, respectivement. L'analyse du phénotype des patients présentant un variant délétère de *BGN* dans ces 5 familles montre : 1- des AAT/DA particulièrement précoces chez les patients (un patient avec AAT dépisté à l'âge d'un an, un patient avec une DA à l'âge de 15 ans), une atteinte aortique touchant la racine aortique ou l'aorte ascendante, des anévrismes intracrâniens chez un patient, une insuffisance modérée de la valve mitrale ou aortique. Chez les patientes, le phénotype aortique était plus inconstant avec absence de dilatation aortique ou AAT/DA plus tardifs. 2- des atteintes extra-aortiques : marfanoïdes (déformation du *pectus*, hyperlaxité, contractures, vergetures) ou rencontrées dans le syndrome de Loeys-Dietz (luette bifide, hypertélorisme, instabilité de la colonne cervicale); une dysplasie squelettique avec luxation de hanche, platyspondylie, dysplasie épiphysaire et des phalanges. L'analyse histopathologique et immunocytochimique de fragments d'aorte de deux patients opérés (c.238G>A et délétion des exons 2-8) montre : 1- une absence ou une réduction drastique de l'expression de biglycan (immunocytochimie), 2- une atteinte très modérée ou absente des fibres élastiques comparée à celle observée chez un témoin atteint d'un syndrome

de Loeys-Dietz (variant p.Asp263His dans *TGFB3*) (coloration Verhoeff- Van Gieson) et une forte réduction de la trame collagénique (Trichrome Masson), 3- une augmentation de noyaux marqués pour la protéine pSMAD2, en faveur d'une hyperactivation de la voie de signalisation du TGF-béta.

Le gène *BGN* est localisé dans la région sous-télomérique du bras long du chromosome X chez l'Homme, en Xq28) ainsi que chez la souris. Il code pour la protéine biglycan qui appartient à la classe I de la famille des small leucine-rich proteoglycans (SLTPs). Complété de chaîne(s) glycosaminoglycane(s), ces petits protéoglycans fortement exprimés dans la MEC (notamment l'os, le cartilage et l'aorte), sont impliqués dans de multiples processus. Ils participent avec d'autres protéoglycans (décorine, fibromoduline, lumican) à la formation des fibres collagènes, à leur régulation et à leur maintien au sein de la matrice extracellulaire. Ils participent également à la différenciation et à la prolifération cellulaire par leurs interactions avec divers facteurs de croissance (TGF-béta, notamment) (Halper, 2014).

L'étude d'un premier modèle de souris déficientes en biglycan (*bgn*-knockout (KO)-129Sv/C57BL6) a en premier lieu objectivé un phénotype osseux caractérisé par une dysplasie sévère avec retard de croissance, des anomalies de formation de l'os et une ostéopénie sévère au cours de la croissance, complètement exprimée chez les souris mâles, inconstante et plus discrète chez les femelles (Xu et al., 1998). Les AAT/DA n'ont pas été rapportés dans ce modèle très étudié de souris KO et le phénotype s'est complété d'autres atteintes (anomalies dentaires et musculaires, ostéoarthrite). Plus tard dans une autre lignée consanguine de souris déficientes en biglycan (*bgn*-KO-BALB/cA), Heegaard et al., ont rapporté une mortalité précoce des souris mâles par dissection ou rupture aortique, suggérant bien avant la confirmation par Meester et al., le rôle attendu de *BGN* dans les aortopathies génétiques humaines (Heegaard et al., 2007). De la même manière, chez l'Homme, la maladie osseuse constitutionnelle en rapport avec des variants faux-sens (c.439A>G, p.(Lys147Glu) et c.776G>T, p.(Gly259Val) délétères de *BGN* (dysplasie spondyloépiméphysaire liée à l'X) a été identifiée avant l'atteinte aortique, sans notion d'atteinte aortique chez les rares patients rapportés (Cho et al.,

2016). S'agit-il de phénotypes différents, en lien avec des variants altérant la fonction de *BGN* de manière différente (domaines fonctionnels ?) ou d'une même maladie humaine ? De nombreuses questions demeurent en suspens et en attente d'études complémentaires.

***AEBP1* et SED classique-like :**

De description récente et avec moins de 10 patients rapportés avec un phénotype de SED (SED classique-like 2, MIM#618000), le gène *AEBP1*, qui code pour la protéine AE Binding, (également appelé *ACLP* pour Aortic Carboxypeptidase-like Protein) pourrait être impliqué dans des formes récessives d'AAT/DA syndromiques ou subcliniques (Alazami et al., 2016; Blackburn et al., 2018; Hebebrand et al., 2019; Ritelli et al., 2019; Syx et al., 2019). Les variants rapportés sont essentiellement des non-sens ou décalant le cadre de lecture, à l'état homozygote ou hétérozygote composite, avec une perte de fonction et un retentissement sur la trame collagénique objectivée dans les études publiées. La protéine ACLP est composée de différents domaines d'interaction avec différentes protéines de la MEC, dont le domaine central discoidin-like de liaison aux collagènes, avec une affinité particulière pour les collagènes I, III et V (Blackburn et al., 2018). Ses fonctions supposées concernent l'organisation et remodelage de la MEC (surtout trame collagénique) ainsi que la prolifération, la différenciation des cellules musculaires lisses et myofibroblastiques aortiques en modulant la voie de signalisation du TGF-béta (Layne et al., 1998; Tumelty et al., 2014). Bien que candidat, son rôle dans les aortopathies génétiques n'est cependant pas démontré à ce jour.

***EFEMP2* (Fibuline 4), *cutis laxa* et AAT/DA :**

Les *cutis laxa* constituent un groupe hétérogène de dysplasies conjonctives héréditaires ou acquises, caractérisées par un excès de peau, ridée, qui pends et ayant perdue son élasticité, associées ou non à différentes atteintes extra cutanées. En dehors de la forme dominante en rapport avec des variants gain de fonction du gène de l'élastine, la forme

récessive de type IB, en rapport avec des variants homozygotes ou hétérozygotes composites dans le gène *EFEMP2* est particulièrement associée à des formes sévères d'AAT/DA. Le gène code pour la protéine EFEMP2 pour « EGF-containing fibulin-like extracellular matrix protein-2 », plus communément appelée Fibuline-4, très fortement exprimée dès la période embryonnaire dans l'aorte où son rôle supposé principal est celui d'un échafaudage pendant la maturation du collagène extracellulaire (Papke et al., 2015) mais, l'étude de différents variants faux-sens impliqués en pathologie humaine montre un rôle plus global sur un ensemble de protéines de la trame collagénique ou élastique (LTBPs, fibrilline...), notamment la réticulation (« cross-linking ») induite par l'activité lysyl-oxydase (Sasaki et al., 2016). Enfin, un rôle sur la prolifération et la différenciation des cellules musculaires lisses est également proposé (Huang et al., 2010). Les patients atteints présentent en dehors de l'atteinte cutanée, des signes marfanoïdes (*pectus excavatum*, arachnodactylie), un emphysème pulmonaire précoce, des hernies inguinales, parfois diaphragmatiques et des atteintes cardiovasculaires sévères, à type d'AAT/DA, de tortuosité et de sténoses vasculaires responsables de décès précoces, souvent chez l'enfant et parfois détectable en période prénatale (Huchtagowder et al., 2006; Letard et al., 2018). L'analyse ex-vivo de fibroblastes cutanés ou de tissu aortique de patients, montre des stigmates d'activation de la voie de signalisation du TGF-béta (Renard et al., 2010). A ce jour, l'hypothèse de variants hypomorphes d'*EFEMP2* à l'origine d'AAT/DA non syndromiques ou associés à une *cutis laxa* sub-clinique ne semble pas confirmée mais, le gène est peu étudié.

Chapitre 2 : Voie de signalisation du TGF-béta

En 1993, Boileau et al. (C. Boileau et al., 1993) ont décrit une famille française avec de nombreux patients présentant des critères squelettiques et cardiovasculaires de Marfan, réunissant les critères diagnostiques alors utilisés (classification de Berlin, (Beighton et al., 1988)) et dont le défaut génétique n'était pas localisé sur le chromosome 15 (locus *FBNI*) ou sur le chromosome 5 (locus *FBN2*), soulevant l'hypothèse d'une hétérogénéité génétique dans le syndrome de Marfan. Plus tard, l'équipe a localisé le gène impliqué dans cette famille au niveau du bras court du chromosome 3, en 3p24.2-p25 confirmant l'hypothèse d'un syndrome de Marfan de type 2 (ou Boileau-Jondeau) (*MFS2* (Collod et al., 1994)). Ces découvertes ont entraîné des débats et des polémiques (hétérogénéité génétique ou nouveau syndrome?) dans la communauté scientifique et médicale du syndrome de Marfan (Gilchrist, 1994). Dix ans plus tard, à partir d'un jeune patient japonais présentant un syndrome de Marfan (selon la classification diagnostique de Gand 1996, alors utilisée (De Paepe et al., 1996)) et un réarrangement chromosomique complexe de la région 3p24.1 (centromérique par rapport au locus *MFS2*), interrompant le gène *TGFBR2* (gène du récepteur II du TGF-béta), un variant d'épissage et trois variants faux-sens dans ce gène ont été identifiées à partir de 5 familles avec un syndrome de Marfan, dont la famille française liée au locus *MFS2* (Mizuguchi et al., 2004). Les données concernant la position conservée de ces variants dans le domaine sérine-thréonine kinase du récepteur, la coségrégation des mutations avec les atteintes cliniques dans les pedigrees correspondants, les tests *in vitro* en faveur d'une perte de fonction sur la voie de signalisation, démontraient que les mutations du gène *TGFBR2* pouvaient être à l'origine d'un syndrome de Marfan. Quelques mois après cette découverte, des mutations dans les gènes *TGFBR1* et *TGFBR2* étaient également impliquées dans un nouveau syndrome avec AAT/DA et des caractéristiques marfanoïdes (Loeys et al., 2005). Le syndrome de Loeys-Dietz (SLD) du nom des auteurs, initialement caractérisé par une triade : anévrismes/tortuosité artérielle diffuse, hypertélorisme et anomalie du palais ou de la lèvre (fente ou équivalent), s'est rapidement révélé plus hétérogène sur le plan clinique que cette seule triade (Loeys et al.,

2006; Mizuguchi & Matsumoto, 2007). Les auteurs mettaient en évidence par des expériences biochimiques et histologiques sur des fragments de tissu aortique de patients atteints, une signature d'activation paradoxale de la voie de signalisation du TGF-béata en faveur d'un gain de fonction (en lien avec les découvertes alors réalisées dans le syndrome de Marfan, cf. supra). Cette activation est paradoxale car les variants identifiés dans les sous-unités des récepteurs impliquées dans les SLD aboutissent à une perte de fonction dans certains modèles cellulaires *in vitro*, mais sont majoritairement en rapport avec des variants faux-sens prédisant un effet-dominant négatif retentissant sur le trafic intracellulaire et l'adressage à la membrane. Ces points soulèvent le « paradoxe d'une activation de la voie de signalisation du TGF-béata » dans la pathogénèse des AAT/DA (Figure 3) avec, de nombreuses controverses et interrogations.

Progressivement, différents gènes codant pour des éléments clés (*TGB2*, *TGFB3*, *SMAD2*, *SMAD3*, *SKI*) de cette voie de signalisation ont été impliqués dans des formes syndromiques ou non d'AAT/DA, la notion de syndrome de Marfan de type 2 a été balayée pour être remplacée par celle de « syndromes apparentés au syndrome de Marfan », avec les différents types de SLD. Le rôle précis de la dérégulation de la voie de signalisation du TGF-béata dans les aortopathies (activation ou perte, protectrice ou aggravatrice, spécifique ou voie finale commune) reste débattu et encore au moins en partie obscurs. Deux modèles s'affrontent : dans le premier modèle, une phase initiale d'inhibition de la voie de signalisation aboutie à une diminution de l'expression des protéines cibles (appareil contractile, MEC) résultant en une phase de réponse par stimulation de l'angiotensine II et de la voie de signalisation du TGF-béata, dans le deuxième modèle l'impact des variants délétères sur la voie de signalisation serait différent selon l'origine embryonnaire des CMLs aortiques (crêtes neurales ou premier champs cardiaque et mésoderme ou deuxième champs cardiaque)(MacFarlane et al., 2019; Michel et al., 2018; Sawada et al., 2018). La compréhension des mécanismes sous-jacents est cruciale pour disposer de thérapies ciblées efficaces permettant de prévenir les AAT/DA et leurs complications.

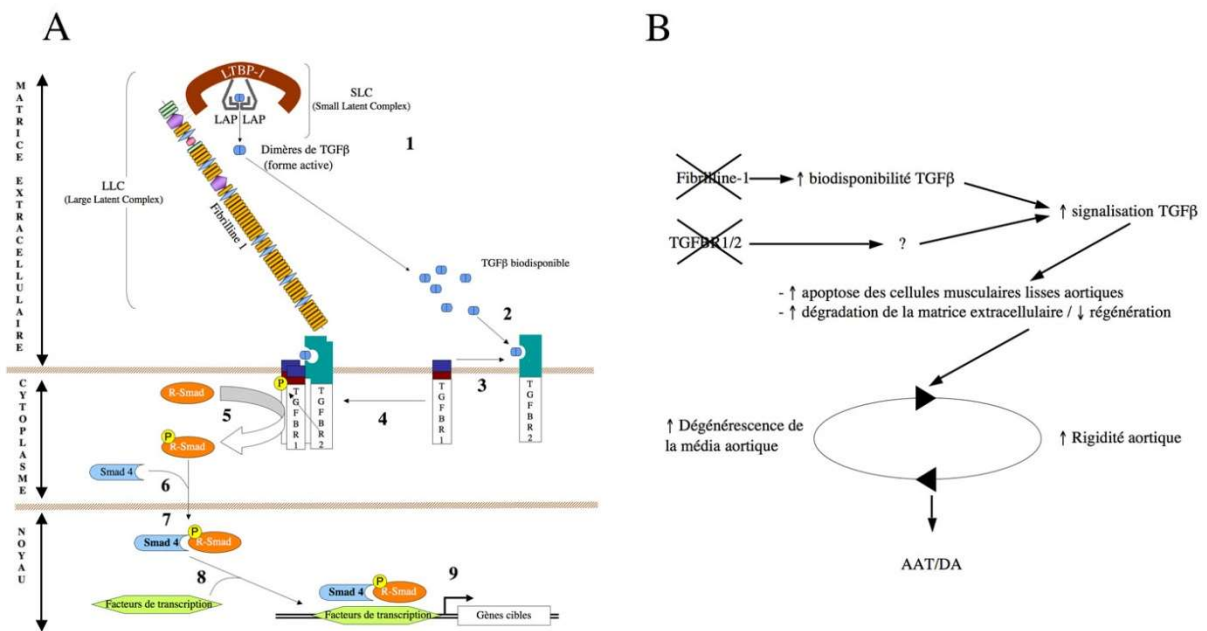
Figure 3 : « Paradoxe de la voie de signalisation du TGF-béta »

Figure 3 : A - Transduction du signal TGF-béta : (1)- Des complexes latents de petite taille (SLC pour Small Latent Complex) composés d'homodimères de cytokines TGF-béta inactifs associés avec les peptides LAP (Latency-associated peptide) et la protéine latent TGF-béta-binding-1 (LTBP1) interagissent avec l'extrémité N-terminale de la fibrilline de type 1 pour former des complexes latents de grande taille (LLC pour Large Latent Complex). Les homodimères de TGF-béta (TGFB1, TGFB2 et TGFB3) peuvent être libérés du complexe LLC et (2), constituer le ligand de la sous-unité II du récepteur du TGF-béta (TGFBR2) qui (3) en association avec une sous-unité I (TGFBR1) recrute d'autres sous-unité I et II pour former un complexe récepteur tetrahétérodimérique transmembranaire. Celui-ci, une fois constitué aboutit à la phosphorylation des sous-unités I (4). Une fois activé, le domaine sérine/thréonine kinase des sous-unités I phosphoryle une protéine « receptor-regulated Smad » (R-Smad, SMAD2 et SMAD3, dans le cas du TGF-béta) (5), qui en association avec la protéine Smad-4 (6) est transloquée dans le noyau (7). Dans le noyau, ce complexe protéique Smad s'associe à des facteurs et cofacteurs de transcription pour former un complexe (8) se liant sur des éléments promoteurs de gènes cibles, régulant ainsi leur transcription (9). B - Un modèle potentiel pour les AAT/DA dans le contexte du syndrome de Marfan et des syndromes apparentés: une mutation dans le gène de la fibrilline de type 1 ou des gènes *TGFBR1/TGFBR2* conduit à une dérégulation de la signalisation TGF-béta et à une altération de l'expression des gènes cibles qui elle-même entraîne une accélération de la dégénérescence de la média et de la rigidité aortique, conduisant à l'apparition d'AAT/DA.

TGFR1/TGFR2, SLD1/SLD2 : Gain de fonction ou perte de fonction ?

Du fait de données discordantes sur l'effet des variants de *TGFR1* et *TGFR2* à l'origine des SLD1 et SLD2 mais aussi d'AAT/DA familiaux non-syndromiques, il est difficile de conclure définitivement entre perte de fonction et gain de fonction. De nombreux travaux complémentaires sont nécessaires pour espérer aboutir à un consensus et au développement d'une thérapie ciblée efficace. A ce jour, l'espoir du blocage de la voie de signalisation par les ARA2 (angiotensin-II receptor type 1 antagonists, losartan).comme traitement générique des aortopathies génétiques, confronté aux premiers résultats des études menées essentiellement dans le syndrome de Marfan (mais également d'autres comme les LDS1 et LDS2), reste peu concluant (Daugherty et al., 2017; Lindeman & Matsumura, 2019).

Des variant (probablement) délétères dans *TGFR1* et *TGFR2* sont identifiés dans des populations AATDA avec une fréquence d'environ 1-2% (Arnaud et al., 2019; Beil et al., 2021; Overwater et al., 2018).

SMAD3, Syndrome de Loeys-Dietz de type 3 ou « ostéoarthrite-anévrismes » :

C'est par une stratégie conventionnelle de localisation par la méthode des LOD-scores, menée à partir de trois familles avec AAT/DA syndromiques dominants que van de Laar et al., ont localisé puis identifié trois variants touchant la fonction du gène *SMAD3* (van de Laar et al., 2011). Les données ont depuis été répliquées dans d'autres familles, par d'autres équipes, y compris la nôtre, faisant du gène *SMAD3* un des gènes les plus fréquemment rencontrés dans les AAT/DA syndromiques ou non (Chesneau et al., 2020; Hostetler et al., 2019; Regalado et al., 2011). Sur le plan du phénotype, les patients présentent de manière variable des atteintes cardio-vasculaires souvent précoces (AAT/DA, anévrismes des branches de l'aorte et des gros vaisseaux, tortuosité

artérielle), des caractéristiques faciales modérées (anomalie du palais et de la luvette, hypertélorisme), des atteintes squelettiques marfanoïdes (scoliose, arachnodactylie, dolichosténomélie, *pectus excavatum* ou *carinatum*, pieds plats...), cutanées (consistance veloutée de la peau avec ecchymoses extensives, vergetures et varices) et surtout une ostéoarthrite précoce (touchant davantage les os du carpe/tarse, du métacarpe/tarse que les phalanges et associée à une dégénérescence précoce des disques intervertébraux, des ménisques et des cas d'ostéochondrite disséquante), le distinguant des autres syndromes associés aux AAT/DA. Pour cette raison, le terme de syndrome « anévrisme-ostéoarthrite » a été initialement proposé par les auteurs (van de Laar et al., 2011, 2012). Progressivement, ce terme est remplacé par celui de SLD3, ce qui a causé de multiples polémiques (MacCarrick et al., 2014; Schepers et al., 2018) et le spectre des atteintes s'est étendue à des manifestations neurologiques à type de neuropathie périphérique sensitivomotrice ressemblant à la maladie de Charcot-Marie de type 2, à des atteintes auto-immunes (acrocyanose, Sjögren, polyarthrite rhumatoïde) ainsi qu'à des manifestations allergiques fréquentes (Aubart et al., 2014).

Sur le plan moléculaire, le gène *SMAD3*, composé de 9 exons code pour la protéine MAD3 pour « Mothers against decapentaplegic homolog 3 » appartenant à la famille des effecteurs intracellulaires de la voie de signalisation canonique TGF-béta activés par récepteur (R)-SMAD dépendant (figure 3). Le spectre des variants rencontrés dans le SLD3/syndrome anévrisme-ostéoarthrite est très large avec des variants faux-sens répartis tout au long du gène et des domaines fonctionnels de la protéine (60%), de variants non-sens ou décalant le cadre de lecture (30%), des variants d'épissage (7%) et des grands réarrangements à type de délétion complète ou partielle du gène (3%). Les données génétiques sont en faveur d'une perte de fonction (Schepers et al., 2018). Les études menées ex-vivo sur des fragments de pièce opératoire d'aorte de patients et de cultures cellulaires dérivées, confirment le « paradoxe de l'activation de la voie de signalisation du TGF-béta » avec, comme dans les autres SLD : une accumulation nucléaire de protéine SMAD2 phosphorylée (pSMAD2) dans les CML et une augmentation de l'expression de gènes TGF-béta-dépendants (facteurs de croissance de

la MEC), signature d'une activation paradoxale de la voie de signalisation. Plusieurs modèles de souris déficientes en Smad3 (-/-) ont été rapportées dans la littérature avec une survie des souris homozygotes KO de quelques mois, des atteintes du cartilage proche de l'ostéoarthrite humaine, des infections en rapport avec une déficience immunitaire et des cancers colorectaux (Li et al., 2009; Yang et al., 1999; Zhu et al., 1998). Le phénotype aortique de ces souris déficientes en Smad3 (-/-) a été étudié *a posteriori* de la description du syndrome anévrisme-ostéoarthrite et confirme l'atteinte à type d'AAT/DA mais uniquement chez les souris homozygotes (van der Pluijm et al., 2016; Ye et al., 2013).

Des variant (probablement) délétères dans *SMAD3* sont identifiés dans des populations AAT/DA avec une fréquence d'environ 1-7% (Arnaud et al., 2019; Beil et al., 2021; Overwater et al., 2018).

TGFB2, Syndrome de Loey-Dietz de type 4

La découverte de variants délétères dans le gène *TGFB2*, a été rapportée simultanément par deux équipes indépendantes (Boileau et al., 2012 ; Lindsay et al., 2012). Dans la première étude, une stratégie conventionnelle de localisation par la méthode des LOD-scores puis de séquençage de gènes candidat à partir d'une famille française et d'une famille américaine a permis d'identifier un variant décalant le cadre de lecture et un variant non-sens dans *TGFB2*. Les auteurs ont ensuite analysé une population de 276 patients avec AAT/DA familiaux et identifié un variant non-sens et une petite duplication décalant le cadre de lecture chez 2 patients (2/276, 0.7%). Dans la deuxième étude, c'est à partir de deux patients présentant un syndrome microdélétionnel 1q41 englobant le gène *TGFB2* et diverses atteintes dont un AAT/DA que les auteurs ont étendu l'analyse à une cohorte de 86 patients AAT/DA (dont 29 cas familiaux) et identifié 6 variants (probablement) délétères dans *TGFB2* (3 faux-sens, 1 non-sens, 2 délétions intragéniques, 6/86, 7%). La présence de variants prédictifs d'une haploinsuffisance dans les deux études était en faveur d'une perte de fonction. La signature d'une activation paradoxale de la voie de signalisation du TGF-béta

(comprenant accumulation nucléaire de protéine SMAD2 et SMAD3 phosphorylées (pSMAD2, pSMAD3) dans les CML (immunocytochimie) et une augmentation de l'expression de gènes TGF-béta-dépendants (facteurs de croissance de la MEC, collagènes) sur des échantillons de pièce opératoire d'aorte de patients était objectivée dans les deux études. Dans la première étude, une surexpression paradoxale significative de *TGFB2* à la fois sur les transcrits (qPCR) et la protéine (Western-blot) était relevée sur les échantillons d'aorte mais pas sur les cultures (myo)fibroblastiques dérivées de fragments d'aorte ou de biopsie cutanée de patients (Boileau et al., 2012). Sur le plan du phénotype, les patients rapportés présentent en dehors des AAT/DA, une tortuosité artérielle, des caractéristiques crânio-faciales (hypertélorisme, anomalies de la lèvre) et un *habitus marfanoïde* modérés et recouvrant en partie les phénotypes Marfan et SLD. Ce phénotype est désormais classé SLD4 avec les mêmes polémiques que pour le SLD3 (MacCarrick et al., 2014; Schepers et al., 2018). Les modèles murins hétérozygotes, reproduisent le phénotype SLD4 humain mais la surexpression paradoxale de *TGFB2* décrite sur l'aorte des patients n'est pas retrouvée (Ishtiaq Ahmed et al., 2014; Lindsay et al., 2012).

Des variant (probablement) délétères dans *TGFB2* sont identifiés dans des populations AAT/DA avec une fréquence d'environ 0.4-1% (Arnaud et al., 2019; Overwater et al., 2018).

***TGFB3*, Syndrome de Loey-Dietz de type 5**

C'est à partir de l'analyse du séquençage d'exome entier en trio d'une enfant présentant une anomalie de développement sans AAT/DA (retard de croissance, musculature peu développée avec retard d'acquisition neuro-motrice sans déficience intellectuelle, une arthrogrypose distale et des caractéristiques morphologiques combinant des éléments marfanoïdes et de SLD) et de ses parents, que Rienhoff et al. ont identifié un variant faux-sens (c.1226G>A; p.(Cys409Tyr) *de novo* dans *TGFB3* (Rienhoff et al., 2013). Quelques mois plus tard, Mayas et al., rapportent un autre variant faux-sens (c.899G>A, ou p.(Arg300Gln)) *de novo* dans *TGFB3* chez une enfant présentant une discrète

dilatation de la racine aortique (Z score à +2.1DS), un prolapsus et une insuffisance modérée de la valve mitrale, avec des caractéristiques communes (luette bifide, hypertélorisme, *pectus excavatum*, faible masse musculaire) et d'autres différentes (discrète avance staturale, hyperlaxité articulaire sans arthrogrypose) (Matyas et al., 2014). Ces deux descriptions ont été rapidement suivies d'une grande étude menée à partir d'une grande famille avec AAT/DA syndromique (signes de SLD et de Marfan) et de 470 patients présentant un AAT/DA syndromique ou non (dont 120 analysés par exome) rapportant 11 variants (probablement) délétères dans *TGFB3* transmis en dominance avec pénétrance incomplète (4 faux-sens dont un retrouvé 4x, 1 non-sens, 1 épissage, 2 délétions d'une seule base avec décalage du cadre de lecture, 2,3% de la cohorte), les données cliniques et familiales (43 patients) ainsi que l'étude *ex-vivo* d'échantillon opératoire d'aorte d'un patient (Bertoli-Avella et al., 2015). Sur le plan clinique, les atteintes cardiovasculaires comportaient, AAT/DA souvent tardifs, inconstants (pénétrance incomplète) et touchant souvent l'aorte descendante, rarement l'aorte abdominale et ses branches, des anomalies modérées de la valve mitrale et une absence de tortuosité artérielle. Des caractéristiques faciales de SLD (hypertélorisme, fente palatine et luette bifide) ou de Marfan (dolichocéphalie, palais haut et arché, rétrognathie) étaient fréquentes mais inconstantes (pénétrance incomplète), associées à un *habitus marfanoïde* (avance staturale, déformation du *pectus*, arachnodactylie) également inconstant. L'étude immunohistochimique de l'aorte d'un patient retrouvait une signature d'activation paradoxale de la voie de signalisation du TGF- β (augmentation de pSMAD2, pERK et TGFB1 par rapport au témoin). Sur le plan moléculaire, le spectre des variants rencontrés prédit une haploinsuffisance (non-sens et apparition prématurée d'un codon stop, soumis au NMD) ou une perte de fonction (faux sens localisés dans des domaines fonctionnels de la cytokine) et le syndrome en rapport avec *TGFB3* classé SLD5 par les auteurs (Schepers et al., 2018).

SKI, syndrome de Shprintzen-Goldberg (SSG)

Décrit en 1982 par Shprintzen et Goldberg (Shprintzen & Goldberg, 1982), le SSG associe *habitus marfanoïde* (arachnodactylie, déformation du *pectus*, scoliose, pieds plats avec déformation du pieds), camptodactylie, craniosynostose (inconstante), dysmorphie faciale (hypertélorisme, exophtalmie, orientation en bas et en dehors des fentes palpébrales, hypoplasie mandibulaire et malaire, palais haut et arché), hypotonie néonatale et déficience intellectuelle, des atteintes cardio-vasculaires (prolapsus de la valve mitrale, AAT/DA), des signes cutanés (peau fine et translucide), des hernies de la paroi abdominale (inguinale, ombilicale). Le recouvrement phénotypique avec le syndrome de Marfan et les SLD est important (Loeys et al., 2005). C'est assez logiquement que des variants touchant la fonction du gène *SKI*, ont été rapportés en 2012 à partir de 18 et 10 patients SSG par deux équipes indépendantes, fournissant ainsi les bases moléculaires à l'origine du SSG (Carmignac et al., 2012; Doyle et al., 2012). Ces variants : faux-sens, petites délétions en phase, qui prédisent un effet dominant-négatif, étaient tous localisés dans un point chaud de l'exon 1 de *SKI* correspondant à des domaines fonctionnels conservés, impliqués dans la liaison aux protéines R-SMAD (SMAD2 et SMAD3). L'étude de la ségrégation familiale de ces variants montrait une origine *de novo*, une coségrégation dominante et un cas de mosaïcisme somato-germinal. De la même manière que dans les autres aortopathies, une signature d'activation de la voie de signalisation du TGF-béta dans les cultures de fibroblastes cutanés de deux patients était mise en évidence dans la deuxième étude, en faveur d'une perte de fonction de l'activité de répression de la voie de signalisation (Doyle et al., 2012). Cependant, dans le syndrome récurrent et fréquent de microdélétion 1p36 (incluant *SKI*), modèle humain de l'haploinsuffisance de *SKI*, les patients ne présentent pas de SGS (Battaglia et al., 2008). Le gène *SKI* et le gène *SKIL* (*SKI*-like) codent pour des facteurs de transcription ubiquitaires et régulant négativement la voie de signalisation du TGF-béta (Luo, 2004). Les isoformes produites par ces deux gènes, s'associent en dimères au niveau d'un domaine « coiled-coil »/Leucine zipper C-terminal correspondant aux exons 3' et comportent au niveau de leurs parties N-terminale de courts domaines de liaison R-

SMAD (x2, le deuxième localisé dans un domaine DHD (Dachshund Homolgy Domain)) et SMAD4 (SAND domain pour Sp100, AIRE-1, NucP41/75, DEAF-1) traduits à partir des exons 1 et correspondant au point-chaud SSG (Schepers et al., 2015). Par des expériences multiples (édition du génome/Knock-In ; biologie structurale, biochimie, analyse de cultures de fibroblastes de patients SGS), modélisant les mutations de *SKI* rencontrées dans le SGS dans différents systèmes *in vitro*, *ex vivo* et *in silico*, Gori et al., montrent que celles-ci abolissent l'interaction avec les facteurs phosphorylés R-SMAD (pSMAD2 et pSMAD3) et SMAD4 entraînant l'absence de dégradation de *SKI* en réponse au signal TGF-béta/Activin (Gori et al., 2021). Cette stabilisation de *SKI* au sein du complexe pSMAD2/pSMAD3-SMAD4-SKI-SKIL aboutie à une diminution de la réponse transcriptionnelle TGF-béta et de l'expression des gènes cibles, répondant difficilement, et remettant clairement en cause le dogme du paradoxe de l'activation de la voie de signalisation du TGF-béta comme l'explication étiopathogénique des AAT/DA.

SMAD2 et AAT/DA

A partir d'une démarche gène candidat, Micha et al., ont analysé le gène *SMAD2* dans une cohorte de 365 patients avec AAT/DA âgés de moins de 65 ans et testés négatifs pour les principaux gènes de prédisposition (*FBN1*, *TGFBR1*, *TGFBR2*, *ACTA2* et *MYH11*) puis étendu l'analyse de ce gène à une cohorte de 219 patients avec AAT/DA familiaux dont les données d'exome étaient disponibles (Micha et al., 2015). Ils ont identifié 2 variants faux-sens (c.1346T>C, p.(Leu449Ser) et c.1369G>A, p.(Gly457Arg)) dans la première cohorte (2/365 ; 0,54%) et un faux-sens (c.1163A>G, p.(Gln388Arg)) dans la deuxième cohorte (1/219 ; 0,45%). Ces faux-sens, sont localisés à des positions invariables, dans le domaine hautement conservé MH2 de *SMAD2* (orthologues et paralogues). Le variant p.(Leu449Ser) était identifié chez une patiente présentant de multiples anévrismes probablement disséquant des artères vertébrales et carotides avec une histoire familiale d'AAT/DA (mère et oncle maternel décédés de complication d'AAT/DA et grand-père maternel décédé subitement à 44ans) ainsi qu'une arachnodactylie et des pieds plats bilatéraux, sans autres signes de Marfan ou de SLD. Le variant p.(Gly457Arg) survenait

de novo chez une patiente avec un AAT de la racine aortique (Z score 3,6) et quelques caractéristiques marfanoïdes ou de SLD (arachnodactylie, scoliose modérée et thorax asymétrique, pieds plats et hernie inguinale opérée). Le variant p.(Gln388Arg) était identifié chez une patiente avec un AAT (5cm au niveau de l'aorte ascendante sans atteinte des sinus aortiques ou d'autre portion de l'aorte et de ses branches), de discrets signes marfanoïdes et une ostéoarthrose sévère des hanches et des genoux. Sur le plan familial, une sœur avec un AAT (4,5cm de l'aorte ascendante sans autre atteinte aortique ou artériel), avec douleurs articulaires chroniques, discopathie dégénérative et prothèse totale de genoux bilatérale, au niveau des parents, une mère décédée subitement à 56 ans et un père de 86 ans avec des antécédents d'infarctus du myocarde à 40 ans. Par la suite 10 autres variants faux-sens probablement pathogènes et un variant non-sens ont été rapportés dans la littérature avec une faible fréquence chez des patients présentant AAT/DA et des signes marfanoïdes ou SLD-like modérés (Cannaerts et al., 2019; Granadillo et al., 2018; Schepers et al., 2018; W. Zhang et al., 2017). Les données disponibles sont peu détaillées mais *SMAD2*, est probablement responsable, en faible proportion de prédispositions mendéliennes dominantes aux AAT/DA.

***LTBP3* et AAT/DA**

En étudiant les données de séquençage d'exome dans une population de 271 patients avec une histoire familiale d'AAT/DA, testés négatifs pour les principaux gènes, Guo et al., ont rapporté deux familles (0,7% de la cohorte) dans lesquelles des variants (1 non-sens et 2 décalage du cadre de lecture) bi-alléliques, homozygotes ou hétérozygotes composites coségrégèrent avec les atteintes (D.-C. Guo et al., 2018, p. 3). Ces atteintes comportaient outre les AAT/DA, des atteintes dentaires (amélogénèse imparfaite, dysplasie de l'émail, oligodontie) et squelettiques (petite taille, ostéoporose), déjà associées à des allèles perte de fonction homozygotes dans *LTBP3* et le syndrome de brachyolmie-amélogénèse imparfaite (Noor et al., 2009; Verloes et al., 1996). Les auteurs ont ensuite analysé les séquences d'exome de 338 patients avec DA non-syndromique survenue avant 56 ans, sans histoire familiale, testés négatifs pour les

principaux gènes AAT/DA et identifié 9 variants hétérozygotes rares dans *LTBP3* (p.Ala25del x2, p.Pro45Argfs*25, p.Pro517Leu, p.Asp820Asn, p.Tyr1020Cys, p.Ser1200Asn (x2, dont un patient avec variant probablement délétère dans *COL3A1*), p.Glu1257Asp), sans toutefois apporter d'argument formel pour leur implication. En reprenant le phénotype de souris KO *Ltbp3*^{-/-} jusque-là uniquement associé aux anomalies dentaires et squelettiques (Dabovic et al., 2002; Morkmued et al., 2017), les auteurs montrent que celles-ci ont un diamètre de la racine aortique augmenté et une augmentation de nombre des lames élastiques dans la média aortique comparé aux souris « sauvages ». Du fait de la fonction du gène, qui code pour la protéine Latent TGF-béta Binding Protéin-3 (LTBP-3), impliqué dans le complexe LLC séquestrant les cytokines TGF-béta (figure 3), les auteurs évoquent une fonction double : excès de relargage de cytokines à l'origine d'une activation TGF-béta et atteinte des microfibrilles riches en fibrilline de la trame élastique. A ce jour, ces données n'ont pas été répliquées et si les AAT/DA semblent faire partie des complications tardives du syndrome rare de brachyolmie-amélogénèse imparfaite autosomique récessif en rapport avec *LTBP3*, l'implication de gène dans les AAT/DA est incertaine.

***SMAD4*, Rendu-Osler et Polypose**

Des variants délétères de *SMAD4*, à l'origine d'une perte de fonction (non-sens ou faux-sens dans le domaine MH1) et d'une diminution de la voie de signalisation du TGF-béta sont impliqués dans une forme particulière de la maladie de Rendu-Osler, associée à une polypose juvénile (Carr et al., 2012; Gallione et al., 2006). Les AAT/DA sont des complications très fréquentes dans cette forme particulière et rare de Rendu-Osler associée à une polypose juvénile (jusqu'à 40% des patients) (Heald et al., 2015; Wain et al., 2014). Par ailleurs, un variant SNP fonctionnel fréquent (rs12455792) localisé dans la région régulatrice 5'UTR du gène *SMAD4* est associé au risque d'AAT/DA dans des études cas-témoins (Odd Ratio ajusté :1.58, 1.09-2.3 pour intervalle de confiance de 95%), avec des études fonctionnelles montrant une expression de *SMAD4* modulée par le génotype de ce SNP et corrélée avec l'apoptose des CMLs, la dégradation de la MEC

ainsi qu'à des marqueurs de l'inflammation dans la paroi aortique (Wang et al., 2017, 2018,). Enfin, l'équipe de Dianna Milewicz rapporte l'étude d'un variant faux-sens : c.290G>T p.(Arg97Leu) identifié à partir du séquençage d'exome d'une population de 223 patients (0,4%) avec AAT/DA familiaux (Duan et al., 2019). Les données cliniques et familiales montrent : la présence du variant coségréant en dominance avec 3 cas d'AAT/DA (cas index, père, frère), l'absence de signes de Rendu-Osler ou de polypose juvénile chez les 3 patients, de discrets signes de SLD (tortuosité de l'aorte descendante et abdominale, lulette avec raphé médian, scoliose, hyperlaxité) chez le cas index et une bicuspidie de la valve aortique avec DA de type A chez son frère. Les études fonctionnelles, dans un modèle cellulaire montrent que ce variant p.(Arg97Leu) localisé dans le domaine MH1 de SMAD4, entraîne une accélération de sa dégradation et une diminution de l'expression de protéines contractiles des CMLs (ACTA2 et CNN1), marqueurs de différenciation en réponse au TGF-béta. De rares variants dans *SMAD4* pourrait donc être à l'origine d'une prédisposition dominante aux AAT/DA, sans Rendu-Osler/Polypose Juvénile ou syndrome de Myhre.

Chapitre 3 : Appareil contractile des cellules musculaires lisses, « SMC contractility »

L'identification de variants délétères constitutionnels du gène *MYH11* dans l'association AAT/DA et PCA, a fourni le premier exemple de l'implication des protéines contractiles produites spécifiquement par les cellules musculaires lisses dans les maladies héréditaires mendéliennes, ouvrant la voie à de multiples découvertes et à des perspectives de recherche immenses. Pour cette raison, ce chapitre sera plus détaillé dans ce mémoire.

MYH11, AAT/DA et PCA

Association AAT/DA et PCA, altération de la compliance aortique en IRM fonctionnelle

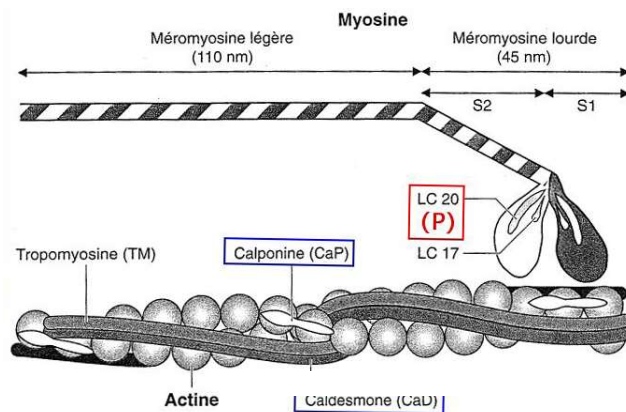
Avec une fréquence estimée d'environ 1/2700 naissances (Hoffman & Kaplan, 2002; Morris et al., 2018), la persistance du canal artériel (PCA) est une anomalie congénitale cardiovasculaire fréquente, majoritairement en lien avec la prématurité. La PCA est généralement considérée comme sporadique et bénigne. Elle récidive cependant dans 5% des fratries, suggérant la présence d'une composante génétique, mais peu de facteurs sont identifiés dans un contexte non syndromique. En dehors de la prématurité, la fréquence de la PCA est moins connue. Une PCA peut survenir isolément ou dans un contexte polymalformatif.

Bien avant la description des syndromes de Loeys-Dietz où l'association avec la PCA est également rencontrée mais moins fréquente (cf. chapitre 2), nous avons remarqué à partir de l'étude attentive de 40 sujets issus d'une grande famille française : la famille « Bourgogne » (composée de plus de 200 sujets sur 6 générations) que cette PCA pouvait coségréger selon une hérédité autosomique dominante avec les AAT/DA au sein de familles, en l'absence de tout syndrome identifiable indiquant une prédisposition génétique particulière (Glancy et al., 2001; Khau Van Kien et al., 2004). Ceci a pu être confirmé au cours d'un protocole de dépistage comportant examens d'imagerie (Echo-Doppler transthoracique, IRM aortique conventionnelle et fonctionnelle (Lalande et al., 2002)) et une analyse génétique, permettant de localiser par la méthode de LOD-scores l'association AAT/DA et PCA sur le bras court du chromosome 16 (Khau Van Kien et al., 2005). Sur ce sous-pedigree de 3 générations, il y avait 4 cas de DA (1 de type A et 3 de type B), 4 cas d'AAT de la racine aortique et 11 cas de PCA (dont 6 détectés par Echo-Doppler). Il existait également d'autres évènements vasculaires, notamment 5 cas d'accidents vasculaires cérébraux (dont 2 en rapport avec un anévrisme/dissection carotidien) et 3 cas de décès subits inexplicables chez des sujets de moins de 45 ans. Tous

les sujets, même asymptomatiques, porteurs de l'haplotype associé à la maladie avaient une rigidité aortique marquée lors des mesures en IRM fonctionnelle. Chez les sujets âgés de moins de 35 ans, l'évaluation de la compliance aortique permettait de distinguer une valeur seuil de 1,3 mm²/mmHg permettant de séparer les sujets porteurs ou non de l'affection. Ces données ont par la suite été confirmées par l'identification de variants délétères dans le gène *MYH11* comme responsable de l'association AAT/DA et PCA avec des paramètres d'altération précoce des propriétés mécaniques de l'aorte dans cette famille (Lalande et al., 2008; Zhu et al., 2006).

Le gène *MYH11*, la myosine 11 et l'unité contractile

Le gène *MYH11* comporte 43 exons répartis sur environ 150kb en 16p13.12-p13.13, à partir desquels sont transcrits par épissage alternatif quatre isoformes de chaîne lourde de myosine-11 spécifique du muscle lisse (SM1A, SM1B, SM2A, SM2B). La myosine-11 est une protéine hexamérique appartenant à la classe II de la superfamille des myosines qui comprend 12 classes (Masters et al., 2017). Elle se compose d'une paire d'homodimères majoritairement et différenciellement exprimés (SM1-MHC ou SM2-MHC) ou plus rarement d'hétérodimères (SM1-MHC et SM2-MHC) de chaînes lourdes (SM-MHC) et de deux paires de chaînes légères, l'une régulatrice initiant la contraction calcium-dépendante (phosphorylée par la myosin light-chain kinase, MYLK) et l'autre essentielle (20kDa et 17kDa) (figure 4).

Figure 4: Représentation schématique des filaments fins et épais de la CML

Représentation schématique des filaments fin et épais de la cellule musculaire lisse (LC 20: chaîne légère régulatrice 20kDa ; LC17: chaîne légère essentielle 17kDa). La myosine spécifique de la cellule musculaire lisse est classiquement composée de 3 domaines (comme les autres myosines de classe II) :

- un domaine globulaire: le segment S1 ou tête, qui contient les sites de fixation de l'ATP (activité ATPase) et de fixation à l'actine permettant la contraction musculaire
- le segment S2 ou fragment de jonction ou col, permettant la flexibilité
- la méromyosine légère ou queue de myosine, où la dimérisation des deux chaînes lourdes s'effectue selon un enroulement alpha-hélicoïdal avec un motif « coiled-coil », permettant la rigidité de la queue de myosine et la transmission de la force de contraction. Les 28 derniers résidus aminoacides de partie C-terminale permettent l'assemblage au sein des filaments épais (Ikebe et al., 2001).

Par digestion ménagée à la trypsine on obtient les fragments S1+S2=méromyosine lourde et la méromyosine légère.

Contrairement aux muscles squelettique et cardiaque, l'unité contractile des CML n'est pas organisée en sarcomères réguliers mais s'ancre à la membrane par la filamine A au niveau du complexe d'adhésion focale (intégrines, protéines support de l'adhésion cellulaire (chapitre 5)) et dans le cytoplasme aux corps denses. Le réseau de myofilaments est extrêmement malléable dans les CMLs, avec un assemblage et un désassemblage (évanescence), finement régulés en réponse aux besoins et stimuli

mécaniques des CML par des mécanismes encore peu connus, mais clés pour la compréhension en pathologie humaine (Seow, 2005; Wang et al., 2021). Cette malléabilité est assurée au moins en partie par l'assemblage des isoformes SM1 et SM2 au sein de la queue de myosine-11, siège de la plupart des variants délétères à l'origine d'AAT/DA et PCA (Rovner et al., 2002).

Les variants touchant la fonction du gène *MYH11* : Gain de fonction ou perte de fonction ?

Effet dominant négatif : AAT/DA-PCA

Dans la famille « Bourgogne » (Khou Van Kien et al., 2004), deux variants localisés dans la queue de myosine (NM_002474.3(SM1A) :c.4578+1G>T résultant en un saut en phase de l'exon 33 et c.5273G>A ou p.(Arg1758Gln)), coségrégants en Cis avec les atteintes cliniques et subcliniques (altération de la compliance aortique) ont été identifiés, dans la famille décrite par Glancy et al. (Glancy et al., 2001), une délétion en phase de 72 nucléotide (c.3722_3793del ou p.Arg1241_Leu1264del) également localisée dans la queue de myosine. Le variant faux-sens p.(Arg1758Gln) apparaît désormais comme probablement bénin (ClinVar) et probablement en simple déséquilibre de liaison avec le variant d'épissage dans la famille Bourgogne. Les données immuno-histochimiques de la paroi aortique de deux patients opérés de la famille Bourgogne, les prédictions *in silico* de formation des domaines coiled-coil de la queue de myosine-11, les expériences *in vitro* (protéines recombinantes exprimées dans des CML Rb-1, montrant une colocalisation et une coimmunoprécipitation) montraient clairement un effet-dominant négatif des variants identifiés (Zhu et al., 2006). Par la suite, l'équipe de Dianna Milewicz en séquençant le gène *MYH11* dans une population de 93 familles avec AAT/DA et 3 familles avec AAT/DA et PCA a mis en évidence trois variants faux-sens (probablement) délétères dans 2 des 3 familles avec l'association AAT/DA et PCA (Pannu et al., 2007). Le premier faux-sens : p.Arg712Gln est localisé à la jonction de la tête et du col de la

myosine-11. La coségrégation du variant, l'absence de ce variant dans une population témoin, la conservation du résidu chez les paralogues et les orthologues, l'effet de variants documentés à cette position conservée dans les gènes orthologues, la modélisation *in silico* étaient en faveur de son impact sur la fonction. Ce variant est désormais rapporté à plusieurs reprises dans la base de donnée ClinVar comme probablement délétère (sans étude fonctionnelle démontrant clairement son impact). Les deux autres faux-sens : p.Leu1264Pro et p.Arg1275Leu coségréants en-cis dans la deuxième famille sont situés dans le domaine coiled-coil de la queue de myosine-11. L'analyse *in silico* montrait que seul le premier faux-sens était susceptible d'interférer avec la formation du domaine coiled-coil. Les analyses histologiques et immunohistochimiques de la paroi aortique de pièces opératoires d'un patient de chaque famille montraient en plus des aspects classiques de dégénérescence kystique de la média (observés dans la famille Bourgogne), des plages d'hyperplasie de cellules musculaires lisses, notamment au niveau des *vasa-vasorum* de l'adventice aortique réduisant considérablement la lumière de ceux-ci avec des aspects de dysplasie fibro-musculaire. Le profil d'expression obtenu à partir de ces échantillons ou de cultures de myocytes aortiques dérivés, semblait distinct du syndrome de Marfan et des syndromes apparentés : absence de stigmates d'hypersignalisation de TGF-béta mais surexpression d'IGF-1 (Insulin-like Growth Factor 1), d'angiotensine II et stigmates d'inflammation vasculaire macrophagique. Ces données étaient contredites un peu plus tard par une autre équipe (Renard et al., 2013) qui en séquençant les gènes *ACTA2* (voir plus loin) et *MYH11* ont identifiés le variant c.4578+1G>A dans une des deux familles avec AAT/DA étudiée. De manière intéressante, ce variant touche la même position que dans la famille Bourgogne mais, avec une substitution différente. Les auteurs concluent qu'il est prédit pour entraîner un saut d'exon sans toutefois l'étudier. Par contre, à partir d'échantillons d'aorte d'un patient porteur de ce variant ils montrent en immunohistochimie un aspect arrondi et un marquage augmenté pour l' α -actine des CML (interprété comme une prolifération des CML) ainsi que pour pSmad2 et CTGF (Connective Tissue Growth Factor), comparativement à une aorte témoin interprété comme signature d'une

hyperactivation de la voie de signalisation du TGF-béta, suggérant que des défauts moléculaires différents peuvent rejoindre cette voie finale commune...

Depuis, l'association AAT/DA-PCA, en rapport avec des variants prédisant un effet dominant-négatif (faux-sens dans la queue de myosine-11 essentiellement), a été confirmée dans d'autres publications (Ardhanari & Swaminathan, 2020; Imai et al., 2015; Larson et al., 2020). Un variant (c.4578+1delG) touchant le même site donneur d'épissage de l'intron 33 que dans la famille Bourgogne et l'étude de Renard et al. et résultant en un saut de l'exon 33 en phase a été identifié dans une famille avec PCA dominante à partir d'une analyse d'exome dans une cohorte de nouveaux-nés avec cardiopathie congénitale (LaHaye et al., 2016). Nous avons identifié un autre variant (c.4599+3A>G) résultant également en un saut de l'exon 33 dans une famille AAT/DA-PCA, indiquant qu'il s'agit d'un défaut récurrent dans cette association (voir section « Titres et travaux », Principales communications affichées, Chesneau et al. ESHG 2020, article en cours de soumission).

Allèles hypermorphes : duplication 16p13.11, associée à un risque augmenté d'AAT/DA

Très peu de variants du nombre de copies (CNV) ont été identifiés en association avec les AAT/DA. Néanmoins, un CNV récurrent, la microduplication 16p13.11 englobant le gène *MYH11*, fréquente en population générale, généralement considéré comme un facteur de risque aux troubles neurodéveloppementaux avec une pénétrance réduite (8,4%) est associé avec un risque augmenté de 10x d'AAT/DA (Allach El Khattabi et al., 2020; Kuang et al., 2011). A ce jour, cette association n'est pas retrouvée avec la microdélétion 16p13.11, réciproque suggérant que les allèles hypermorphes de *MYH11*, prédisposent aux AAT/DA, mais pas l'haploinsuffisance.

Allèles perte fonction: Myopathies viscérales récessives et dominantes

Plusieurs études montrent que les allèles nuls (notamment délétion 16p113.11) ou associés à une perte de fonction de *MYH11*, sont associés avec un phénotype récessif de myopathie viscérale : microcolon-mégavessie-hypopéristaltisme (Gauthier et al., 2015; Moreno et al., 2016; Wang et al., 2019; Yetman & Starr, 2018). Un variant décalant le cadre de lecture en fin de gène, prédisant une protéine allongée de quelques acides aminés et échappant au NMD, susceptible d'empêcher la dimérisation des queues de myosine par un effet dominant négatif et une perte de fonction (figure 4)(Ikebe et al., 2001) est également rapporté avec une forme dominante de myopathie viscérale (Gilbert et al., 2020).

Modèles murins :

Les modèles de souris $KO^{Myh11-}/Myh11-$, déficientes en myosine-11 présentent un phénotype de myopathie viscérale avec une PCA et une survie prolongée, sans atteinte rapportée notamment AAT/DA chez les souris hétérozygotes $+/Myh11-$ (Morano et al., 2000). Pour les KO spécifiques de différentes isoformes SM2 ou SMB : un phénotype de myopathie viscérale est également observé avec une mortalité précoce chez les souris homozygotes $Myh11SM2-/Myh11SM2-$, une survie prolongée et apparemment normale pour les hétérozygotes sans phénotype AAT/DA (Chi et al., 2008) ; une absence de phénotype pour les souris $KO-Myh11-SMB$ $Myh11SMB-/Myh11SMB-$ mais une diminution de la force de contraction et de la vitesse de raccourcissement des CML (Babu et al., 2001). Un modèle Knock-In de souris introduisant un faux-sens (p.Arg247Cys) dans la tête de myosine-11 a été particulièrement étudié, avec une absence de phénotype AAT/DA-PCA chez les homozygotes ou hétérozygotes en condition normale, une réduction des paramètres d'élasticité de l'aorte et la survenue d'AAT/DA lors d'hypertension artérielle induite par régime hypersodique ou angiotensine II (Bellini et al., 2017; Kuang et al., 2012). Il n'y a pas de description de modèles de souris Knock-In pour l'effet-dominant négatif dans la queue de myosine-11.

Corrélations génotype/phénotype :

Les myopathies viscérales, en rapport avec *MYH11*, sont associées à des allèles pertes de fonction (notamment haploinsuffisance par délétion du gène entier) à l'état homozygote, hétérozygote composite ou dans un cas par un effet dominant négatif prédisant l'impossibilité d'assemblage de la queue de myosine 11, à l'état hétérozygote.

Les allèles hypermorphes, associés à un gain de fonction sont associés à un risque significativement accru d'AAT/DA.

En ce qui concerne l'association AAT/DA-PCA, les corrélations sont bien plus complexes à établir. Les variants rapportés, étayés par des études fonctionnelles établissant leur caractère délétère certain sont rares. Elles sont quasiment limitées aux variants d'épissage récurrents induisant un saut de l'exon 33 résultant en une délétion en phase de 72 acides aminés et un effet dominant négatif dans la queue de myosine-11. Dans ce contexte, une perte de fonction associée à d'autres facteurs modificateurs (digénisme, épistasie, deuxième évènement somatique...) a été suggéré (Harakalova et al., 2013) mais un gain de fonction (relaxation, suractivation de la phosphorylation, malléabilité de l'unité contractile...) est également possible. A l'image d'une fermeture-éclair défailante, celle-ci peut être cassée (*i.e.* perte de fonction) ou bloquée (*i.e.* gain de fonction).

En conclusion, l'implication de *MYH11* en pathologie humaine a ouvert la voie à de multiples découvertes et à d'importantes perspectives de recherche.

ACTA2

Le gène *ACTA2* localisé en 10q23.31 et composé de 9 exons, code pour l' α -actine2, spécifique des CML vasculaires. En 2007, l'équipe de Dianna Milewicz, rapporte l'étude d'une famille d'une grande famille avec l'association AAT/DA et livedo réticulaire dominante (suggérée comme une entité particulière depuis les années 1970, (Bixler & Antley, 1976)), permettant d'identifier le gène *ACTA2* comme un gène majeur d'AAT/DA héréditaire (Guo et al., 2007). Dans cette famille, le défaut génétique localisé

en10q23q24 par la méthode des LOD-scores, les auteurs ont identifiés un variant faux-sens (p.Arg149Cys) dans *ACTA2* coségrégeant en dominance avec les atteintes. Ils ont ensuite étudié ce gène dans une cohorte de 97 familles avec AAT/DA non-syndromiques et identifié 14 familles avec 9 variants faux-sens coségrégeant avec les atteintes, dont 4 avec le variant p.(Arg149Cys) et 3 avec un faux-sens touchant le même résidu Arginine258 (p.Arg258His et p.Arg258Cys), soit 14,4% dans cette cohorte. Aucun variant n'était identifié dans une population de 192 témoins sains, en lien avec une très forte conservation des séquences d'*ACTA2*. L'analyse de ces familles montrait : une pénétrance réduite des AAT/DA (0.48), peu dépendante de l'âge avec des DA de type B chez 3 patients de moins de 20 ans mais une médiane de survie de 67 ans ; la présence de PCA dans 2 familles et de BVA chez 3 patients, ainsi que la présence d'anomalie de l'iris (*iris flocculi*) dans 2 familles. Les analyses *in silico*, modélisant l'impact de ces faux-sens sur la structure tridimensionnelle ; les analyses immunocytochimiques à partir de CML dérivées de pièces opératoires d'aorte de 2 patients montrant une atteinte des filaments fins d'actine (Figure 4) étaient en faveur du rôle délétère de ces faux-sens. L'analyse histologique des fragments d'aorte de ces deux patients, montrait des aspects comparables à ceux observés pour les patients atteints d'AAT/DA en rapport avec *MYH11* : aspects classiques de dégénérescence kystique de la média (observés dans la famille Bourgogne), des plages d'hyperplasie de CML, au niveau des *vasa-vasorum* de l'adventice aortique réduisant la lumière de ceux-ci avec des aspects de dysplasie fibromusculaire. Les auteurs ont ensuite rapporté une étude complémentaire, précisant le phénotype et rapportant d'autres variants faux-sens identifiés dans 6 familles supplémentaires et la présence d'autres complications cardiovasculaires inhabituellement précoces à type de maladie coronarienne, d'accidents vasculaires cérébraux ischémiques et de maladie de Moya Moya (Guo et al., 2009). L'implication d'*ACTA2* dans les AAT/DA et les atteintes associées a rapidement été confirmée par d'autres équipes avec une fréquence de 1,5 à 21% selon le recrutement des populations étudiées (Disabella et al., 2011; Hoffjan et al., 2011; Morisaki et al., 2009; Renard et al., 2013). Le spectre des variants rencontrés à ce jour est très majoritairement composé de faux-sens touchant des résidus conservés, avec quelques variants d'épissage et de petits

remaniements en phase, prédisant un effet dominant-négatif et une altération de la contraction des CML et/ou de la formation des filaments fins de l'unité contractile (perte de fonction). Les atteintes sont particulièrement sévères pour les faux-sens récurrents touchant l'Arginine en position 179, avec des manifestations parfois sévères dès la période néonatale débouchant sur des recommandations particulières de prévention et de suivi (Regalado et al., 2018). Enfin, des recommandations de prévention (comprenant le traitement à vie par bêtabloquants à dose efficace) et de suivi spécifiques aux AAT/DA et autres complications cardio-vasculaires en rapport avec *ACTA2* ont été établies récemment (van de Laar et al., 2019).

MYLK

Le gène *MYLK*, transcription alternative complexe:

Le gène *MYLK* localisé en 3q21.1 est composé de 31 exons répartis sur 250kb, codants alternativement pour différentes isoformes de myosin light-chain kinase (MLCK) et la télokinase (également appelée kinase-related protein) avec une organisation complexe mais conservée (Herring et al., 2006). Les transcrits correspondants sont différenciellement exprimés de manière spécifique selon le type de cellule, avec une localisation cellulaire distincte et à partir de différents promoteurs. Les isoformes MLCK phosphorylent les chaînes légères régulatrices de différentes myosines de type II : une seule isoforme MLCK, dite courte pour le muscle lisse viscéral et vasculaire (transcrite à partir des exons 15 à 31 et d'un promoteur dans l'intron 14), 7 isoformes MLCK, dites longues pour les myosines non-musculaires ubiquitaires (différenciées de l'isoforme courte par l'épissage alternatif des 14 premiers exons de *MYLK*) et une seule isoforme ubiquitaire de télokinase (transcrite à partir des 3 derniers exons du gène et d'un promoteur localisé dans l'intron 28). Cette phosphorylation calcium-dépendante (augmentation du calcium intracellulaire via les canaux cationiques) des chaînes légères régulatrices est médiée par la calmoduline (CaM) (interaction avec MLCK au niveau du domaine CaM-binding correspondant aux exons 28 et 29 et domaine kinase de *MYLK*) et initie la contraction myosine-actine (domaines Ig1 et Ig2 de fixation à l'actine, la partie

correspondant à la téléokine contient le domaine de liaison à la myosine). En dehors de ce rôle, la téléokine stabilise les filaments de myosine, inhibe par compétition l'activité MLCK et active la myosin light chain phosphatase dans la phase de décontraction.

MYLK et AAT/DA:

C'est par une démarche gènes-candidats que l'équipe de Dianna Milewicz a identifié le gène *MYLK* comme responsable d'AAT/DA familiaux dominants (Li Wang et al., 2010). L'étude par séquençage des gènes *MYLK*, *CALM1* (codant pour la calmoduline CaM) et codant pour les chaînes légères de myosine-11 (*MYL6*, *Myl6B*, *MYL9*) dans une population de 94 cas-index de familles AAT/DA, a permis d'identifier un seul variant faux-sens non rapporté dans *MYLK* (c.5275T>C, p.Ser1759Pro), localisé dans le domaine CaM (liaison à la calmoduline) et coségrégeant avec 3 cas d'AAT/DA dans une famille de 8 personnes analysées. L'analyse des autres gènes étant négative. Les auteurs ont ensuite séquencé l'ADN de 99 autres cas-index de familles AAT/DA pour les exons de *MYLK* et identifié un variant non-sens au début du domaine kinase (c.4438C>T, p.Arg1480*) coségrégeant avec 7 cas d'AAT/DA et 2 cas de mort subite inexplicée dans une famille de 13 personnes analysées ainsi qu'un autre faux-sens (c.5260G>A, p.Ala1754Thr) également localisé dans le domaine CaM mais sans possibilité d'étude familiale. Le variant non-sens était compatible avec une perte de fonction de *MYLK* (non-sens soumis au NMD > haploinsuffisance ou protéine tronquée sans une grande partie du domaine kinase). Pour les 2 faux-sens dans le domaine CaM, les expériences *in silico*, *in vitro* confirmaient cette perte de fonction pour le variant p.Ser1759Pro (liaison à la calmoduline abolie, activité kinase réduite d'un facteur 6), avec moins d'évidence pour le deuxième faux-sens (liaison diminuée à la calmoduline, activité kinase réduite d'un facteur 4).

Ces données préliminaires ont depuis été confirmées par d'autres équipes (Hannuksela et al., 2016; Luyckx, Kumar, et al., 2019) ; La compilation de 60 patients issus de 7 familles, avec 5 allèles nuls et 2 faux-sens associés à une perte de fonction kinase

démontrée montre une pénétrance globale de 38% des AAT/DA qui sont caractérisés par une majorité (20/23, 87%) de DA plutôt de type A (17/20, 85%) avec des AAT de l'aorte ascendante (n'impliquant pas les sinus aortiques) peu marqués, des DA survenant sur une aorte peu dilatée et des dissection de type B sans nécessairement d'AAT au niveau de la racine aortique (Wallace et al., 2019).

***PRKG1*, un variant faux-sens gain de fonction**

Le gène *PRKG1* localisé en 10q11.2 et composé de 18 exons répartis sur plus de 220kb, code pour les deux isoformes α et β de la protéine kinase cGMP dépendante de type I (PKG-1) (cytosolique ou soluble par opposition au type II, membranaire), transcrite par épissage alternatif des deux premiers exons (souvent dénommés 1α et 1β) (Sellak et al., 2013). Ces deux isoformes s'assemblent en homodimères, le type α étant prépondérant dans les CMLs artérielles. La PKG-1 joue plusieurs rôles importants dans les CML, notamment dans la relaxation du complexe acto-myosine en abaissant le taux de calcium intracellulaire et en activant la myosine light chain phosphatase. L'activité kinase de la PKG-1 est en rapport avec un changement de conformation entraîné par la fixation de cGMP (cyclic guanosine 3',5'-monophosphate) au niveau de 2 sites de liaison (cGMP binding-domains A et B).

C'est à partir d'une analyse d'exome menée dans des familles d'AAT/DA, sans défaut moléculaire identifié jusqu'alors, que l'équipe de Dianna Milewicz a identifié un variant faux-sens non décrit (NM_001098512.2(PKG-1 α):c.530G>A ou p.(Arg177Gln)) dans *PRKG1* coségrégeant en dominance avec les AAT/DA dans une famille déjà rapportée (Guo et al., 2013; Tran-Fadulu et al., 2006). L'analyse des données d'exome et le séquençage direct du gène *PRKG1* de, respectivement 55 cas index avec AAT/DA familiaux et dans une cohorte complémentaire de 307 cas-index AAT/DA a permis d'identifier 3 autres familles présentant le même variant (2 par analyse d'exome et 1 par séquençage direct soit 0.3%). Ce variant coségrégeait avec les AAT/DA dans ces 3

familles. Les données cliniques des 31 patients présentant le variant (ou déduit de par le pedigree) montrait une atteinte aortique sévère, avec une pénétrance complète dès l'âge de 18 ans des AAT/DA avec 63% de DA (type A>B) et 37% d'AAT de la racine aortique, avec une atteinte équivalente chez les femmes et chez les hommes. Parmi les autres évènements vasculaires, étaient relevés dans les pedigrees : dilatation de l'aorte descendante, de l'aorte abdominale et d'autres gros troncs, dans une famille, deux cas d'anévrisme coronaires et pour 3 patients une aorte abdominale tortueuse. Il n'existait aucun argument pour une forme syndromique. La localisation du faux-sens dans le domaine cGMP-A, l'étude *in silico* et la modélisation 3D montrant que le résidu Arginine est directement au contact de cGMP, les études *in vitro* et *ex vivo* montraient une activation de PKG-1 et donc un gain de fonction résultant en une diminution de la phosphorylation MLCK. Ces données préliminaires, ont pu être complétées par : l'addition de deux autres familles AAT/DA avec le même variant permettant d'enrichir le phénotype AAT/DA (Shalhub et al., 2019) ; un modèle de souris Knock-In pour ce faux sens reproduisant la maladie aortique humaine et un traitement par un antioxydant (analogue vitamine B12) efficace (Schwaerzer et al., 2019) et l'étude fine des conséquences de ce faux-sens sur la conformation de PKG1 montrant que l'effet dominant-négatif ne permet pas la conformation de la forme inactive de PKG1, à l'origine du gain de fonction (Chan et al., 2020).

FLNA : Filaminopathies

Le gène *FLNA*, localisé en Xq28 et composé de 48 exons, code pour les isoformes de la filamine A, qui s'assemblent en homodimères par leur extrémité C-terminale, réticule les filaments d'actine en réseaux tridimensionnels et les fixe à la membrane au niveau du complexe d'adhésion focale. La filamine A interagit également avec de multiples protéines partenaires (>90) impliquées dans la mécanotransduction du signal (Razinia et al., 2012). La filamine A est exprimée de manière ubiquitaire ce qui explique vraisemblablement que les variants délétères de *FLNA* sont responsables de multiples affections hétérogènes sur le plan clinique, liées à l'X et parfois létales pour le sexe masculin (dysplasie fronto-métaphysaire, syndrome de Melnick-Needles, syndromes

oto-palato-digital, pseudo-obstruction intestinale, dysplasie valvulaire cardiaque, syndrome de l'intestin court, hétérotopies périventriculaires...) et des atteintes d'organes (cerveau, squelette, peau, cœur, vaisseaux...). Malgré une très forte hétérogénéité clinique et allélique, des corrélations génotypes/phénotypes ont été établies, notamment selon la perte de fonction ou le gain de fonction associée aux variants délétères et leur localisation dans les différents domaines de *FLNA* (Robertson, 2005; Wade et al., 2020). En ce qui concerne les variants perte de fonction associés aux hétérotopies périventriculaires (anciennement dénommé syndrome d'Ehlers-Danlos-hétérotopies périventriculaires), souvent létaux pour le sexe masculin, des signes de dysplasie conjonctives « SED-like » avec des AAT/DA et des PCA sont fréquemment rapportés (de Wit et al., 2011; Reinstein et al., 2013). Dans une étude récente, les AAT/DA seraient rencontrés chez 18% des patients avec des DA survenant précocement avec des diamètres de la racine aortique peu augmentés (Chen et al., 2018).

***TES, TLN1, ZYX* : complexe d'adhésion focale**

Une étude très récente et unique à ce jour (Y. Li et al., 2021), menée par une équipe chinoise, rapporte l'identification de variants délétères dans les gènes *TES* (testine), *TLN1* (taline 1) et *ZYX* (zyxine) à l'origine d'AAT. Ces gènes codent pour des éléments du complexe d'adhésion focale permettant de relier le cytosquelette et le complexe actomyosine à la membrane et à la MEC et impliqués dans de multiples aspects de la mécanotransduction du signal. Préalablement à cette étude, des variants faux-sens rares dans *TLN1* avaient été rapportés chez des patients atteints de dissection spontanée des artères coronaires dont une famille (Turley et al., 2019). Le pedigree de cette famille comportait 3 cas de dissection des artères coronaires (avec une dysplasie fibromusculaire des artères rénales, iliaques associée chez une femme) et deux apparentés indemnes de problèmes vasculaires, compatibles avec un mode de transmission dominant et une pénétrance incomplète. Les auteurs ne rapportent pas de phénotype AAT/DA et précisent l'absence de signes de dysplasie conjonctive. Ils ont entrepris un séquençage de l'exome « en quintet » à partir des 5 patients disponibles et identifié après filtration des données un variant faux-sens rare (rapporté à une

fréquence de 0,053% dans la base de données GnomAD) dans le gène *TLN1* (NM_006289.3:c.6037G>A p.(Ala2013Thr)). La présence du variant chez les 3 patients atteints, la position du résidu Alanine2103 hautement conservée dans un domaine de liaison β -intégrine (IBS2) de la taline 1, l'expression très forte dans l'aorte, l'enrichissement de 10 variants faux-sens rares dans *TLN1* identifiés chez 11 patients par exome ou séquençage direct à partir d'ADN de 675 patients atteints (cas sporadiques), sans étude fonctionnelle cependant suggérait un lien avec l'atteinte vasculaire.

Concernant l'étude de Li et al. (Li et al., 2021), les auteurs ont réalisé une étude cas-témoin en comparant l'analyse des séquences de l'exome entier d'une cohorte «iTAA» 551 patients atteints d'AAT «isolés» (adressés à l'Hôpital de Beijing pour chirurgie de remplacement aortique, sans histoire familiale, ni arguments pour une forme syndromique) et d'une cohorte témoins de 1092 sujets (issus de la population chinoise, sans notion d'AAT/DA, ni autre précision qu'un âge moyen de 37ans). Après une première filtration des données de séquences, ils ont identifié dans la cohorte «iTAA», 17 variants connus et 10 variants délétères dans les gènes connus de prédisposition aux AAT/DA (5,08%, *FBN1* : 24, *LOX* : 1, *ACTA2* :1, *MFAP5* :1) et sélectionné pour des études complémentaires 2 variants faux-sens dans le domaine conservé LIM1 de *TES* (domaine d'interaction protéine/protéine, composé de 7 cystéines et d'1 histidine) identifié uniquement chez 4 patients AAT (NM_015641.4:c.751T>C chez 3 patients et c.838C>T ou, p.Tyr251His x3 et p.Tyr280His). Pour démontrer que le variant p.Tyr251His touchait la fonction de *TES*, ils ont généré un modèle Knock-In de souris *Tes*^{Y249H} (la position 249 chez la Souris correspond à la position 251 chez l'Homme) par édition du génome CRISPR/Cas9. L'étude des souris *Tes*^{Y249H}, KO *Tes*^{-/-}, comparées aux témoins sauvages *Tes*^{+/+}, montrait : 1- une survie prolongée chez les homozygotes avec un diamètre de l'aorte augmentée *Tes*^{-/-} > *Tes*^{Y249H} > *Tes*^{+/+}, 2- une paroi aortique morphologiquement normale en histologie pour les 3 types de souris, 3- une pression artérielle diminuée *Tes*^{-/-} > *Tes*^{Y249H} > *Tes*^{+/+} (sans différence observée du rythme cardiaque), 4- une expression diminuée de *Tes* dans le tissu aortique *Tes*^{-/-} > *Tes*^{Y249H} > *Tes*^{+/+} (RT-PCR et Western blot), 5- une diminution significative de l'expression des gènes impliqués dans la contraction

des CMLs (*Myh11*, *Acta2*, *Tagln*) : *Tes*^{-/-} > *Tes*^{Y249H} > *Tes*^{+/+} corrélée avec l'expression diminuée du facteur de transcription Srf (analyse du transcriptome, RT-PCR et Western-blot), 6- une vasoconstriction diminuée de l'aorte en réponse à l'acétylcholine et nitroprussiate de sodium *Tes*^{-/-} > *Tes*^{Y249H} > *Tes*^{+/+} (anneau vasculaire), 8- une diminution de la contractilité et une migration augmentée des CMLs aortiques en culture *Tes*^{-/-} > *Tes*^{Y249H} > *Tes*^{+/+} (test de contraction du collagène).

Les auteurs ont ensuite reproduit une partie de ces expériences aux gènes *ZYX* et *TLN1* dont les variants faux-sens (de signification inconnue) étaient retrouvés en excès dans une deuxième étude cas-témoins et montré que l'expression de ces gènes est corrélée à celle des protéines de l'appareil contractile et de leur facteur de transcription Srf, au moins en partie indépendamment de *TES*.

Ces deux études génétiques, récentes et préliminaires, le rôle des protéines du complexe d'adhésion focale indiquent un vaste champ de recherche pour la compréhension de la physiopathologie des AAT/DA, susceptible de déboucher sur une prise en charge spécifique et une médecine personnalisée.

***ARIH1* : complexe LINC**

Le complexe LINC (Linker of Nucleoskeleton and Cytoskeleton) est un complexe protéique (Nesprines, SUN et Lamines) reliant la membrane externe et interne du noyau au cytosquelette (dont complexe acto-myosine) aux multiples fonctions : mécanotransduction du signal, interaction avec la chromatine, régulation transcriptionnelle, position du noyau... (Bouزيد et al., 2019).

Dans une publication pluridisciplinaire, associant des équipes travaillant sur la drosophile et sur les aortopathies génétiques, Tan et al. (Tan et al., 2018), rapportent la caractérisation de rares variants dans le gène *ARIH1*, 1 non-sens (NM_005744.5:c.551C>T ou p.(Arg171*)) et deux faux-sens (c.131A>G ou p.(Glu44Gly) et c.43G>C ou p.(Glu15Gln)) identifiés dans les données de séquençage de l'exome de 220 patients AAT/DA familiaux (1,4% de la cohorte). Ce gène, composé de 14 exons

localisés en 15q24.1 code pour la protéine E3 ubiquitin-protein ligase ARIH1, de 557 acides aminés dont une des fonctions démontrée est de dégrader la protéine SUN2, composant du complexe LINC. Les données cliniques et familiales concernant les 3 patients AAT/DA avec les 3 variants délétères dans *ARIH1*, montraient : 1- pour le variant non-sens (p.Arg171*), une origine *de novo*, une maladie anévrismale particulièrement précoce et sévère (avec chirurgie de remplacement de l'aorte ascendante à l'âge de 6 ans, puis DA de type B avec chirurgie multiple de l'arche aortique et de l'aorte thoraco-abdominale, dilatation de la carotide commune et du tronc brachio-céphalique), des atteintes articulaires et cutanées « SED-like », l'absence de myopathie et de neuropathie associée ; 2- pour le faux-sens p.(Glu44Gly), une patiente de 72 ans, sans AAT/DA mais des anévrismes intracrâniens (artères basilaire et carotide interne) et une forte histoire familiale d'AAT/DA et d'anévrismes intracrâniens transmis en dominance (5 apparentés), sans étude de co-ségrégation réalisée 3- pour le faux-sens p.(Glu15Gln), une patiente avec AAT et DA de type A à l'âge de 52 ans avec un frère avec AAT de l'aorte descendante dépisté à 57 ans et présentant le variant, une mère et un frère décédés subitement sans données disponibles à 59 et 52 ans. L'étude fonctionnelle des variants dans un modèle de drosophile était compatible avec une perte de fonction et des allèles, nul pour le non-sens et hypomorphes pour les deux faux-sens. L'étude en microscopie électronique de CML aortiques dérivées des pièces opératoires de 2 patients (p.(Arg171*) et p.(Glu15Gln)) des anomalies morphologiques sévères des noyaux (indentations) et comparables à celles obtenue par Knockdown *ARIH1* (ShRNA) des CMLs témoins.

Ces données préliminaires et à ce jour non répliquées indiquent l'implication du complexe LINC dans la physiopathologie des AAT/DA et de la maladie anévrismale artérielle et renforcent le modèle d'un groupe d'aortopathies génétiques en rapport avec l'appareil contractile et la mécanotransduction du signal dont les bases moléculaires sont différentes d'une signalopathie du TGF-béta.

Deuxième Partie : Analyse de la Recherche, Projet de Recherche et Perspectives

Médecin spécialiste en Génétique Médicale, Praticien Hospitalier temps plein au CHU de Nîmes, mon parcours « Recherche » a toujours découlé de situations cliniques et de rencontres avec des confrères, collègues ou chercheurs, le plus souvent dans le contexte d'une démarche diagnostique importante pour la prise en charge de patients rencontrés en consultation médicale ou dont nous analysons les prélèvements. Ce parcours se déroule sur plusieurs périodes distinctes, liées à mes études, fonctions et affectations successives indiquées dans mon *Curriculum vitae*. Je présenterai donc ce parcours, et citerai les différents travaux selon la numérotation indiquée dans la partie Travaux (pages X-Y) correspondante.

Internat, spécialisation en Génétique Médicale et DEA.

Après des études de premier cycle et de deuxième cycle à l'UFR de Médecine Lyon Sud (Université Lyon I) où j'avais initié un parcours recherche par l'obtention d'un certificat de Maîtrise en Sciences Biologiques et Médicales, j'ai été affecté au CHU de Dijon à l'issue de mon concours d'internat. La spécialité de Génétique Médicale (DES41) n'existait pas encore et je m'orientai initialement vers la Pédiatrie. Après deux semestres, mon Service National Civil en Coopération dans le Service de Pédiatrie de l'Hôpital Principal de Dakar (Communication Orales- n°11) m'a profondément marqué et je me suis orienté en Génétique Médicale. Cette spécialité, venait d'être créée et je fais partie de la première promotion d'Internes inscrits dans ce cursus. Rapidement, l'observation d'un fœtus

polymalformé par carence en vitamine K maternelle en rapport avec de multiples antécédents de chirurgie digestive m'a amené à ma première publication scientifique (réf. n°60, annexes n°10). C'est également au cours d'une consultation de Génétique Médicale avec mon Maître de Stage, le Dr Annie NIVELON-CHEVALLIER que ma Thématique de « Recherche », sur les AAT/DA c'est déterminée. Le motif de cette consultation était en rapport avec le décès en salle opératoire d'une jeune femme et de son fœtus à 8 mois grossesse d'une DA de type B, appartenant à une grande famille (la Famille « Bourgogne », cf. chapitre 3) avec de multiples accidents vasculaires sévères et des décès soudains, survenant à des âges inhabituellement jeunes. Le consultant, oncle de cette jeune femme et fils d'un patient ayant également présenté une DA de type B, s'inquiétait logiquement mais n'était pas entendu de ses médecins. Il n'existait chez lui, aucun signe de Marfan ou de syndrome d'Ehlers-Danlos de type IV (ancien nom du type vasculaire), seules entités connues à cette époque dans les AAT/DA. Le consultant a ensuite été adressé au Pr Jean-Eric Wolf, cardiologue au CHU pour une échographie cardiaque où un anévrisme de la racine aortique était dépisté. Dans un premier temps, l'effectif important de cette famille et la fréquence anormale des évènements cardiovasculaires observés, notamment des cas de persistance du canal artériel, compatibles avec un défaut génétique dominant m'ont amené à faire le lien, à la demande de mon maître de stage, entre les différents interlocuteurs du CHU afin de mettre en place un protocole de dépistage dans cette famille. Il apparaissait nécessaire, compte tenu des atteintes enregistrées dans cette famille, d'explorer la totalité de l'aorte thoracique et c'est dans ce contexte que j'ai rencontré le Pr F. Brunotte, qui travaillait alors sur l'IRM fonctionnelle cardiaque avec une méthode semi-automatisée et un logiciel de reconnaissance des contours développés en Recherche par le Dr A. Lalande, Scientifique dans son service. Des fruits de ces rencontres, est rapidement née l'hypothèse de pouvoir appliquer cette méthode à l'évaluation des propriétés mécaniques de l'aorte au cours du protocole de dépistage que l'on mettait en place (réf n°22, Annexe n°9). Parallèlement, nous avons sollicité d'autres équipes pour leur expertise et avis, notamment le Pr X. Jeunemaitre à Paris et celle du Pr H. Plauchu à Lyon, dont le Dr S. Peyrol qui travaillait sur la Microscopie Electronique de la peau,

« fenêtre » des artères (réf. n°9, publications en rapport avec la thématique de Recherche). Les premières données médicales et généalogiques ont été présentées lors d'un congrès francophone de Génétique (Principales Communications Orales, réf. N°10) et lors d'une réunion informelle, il a été décidé d'organiser la Recherche et de rédiger un protocole de Recherche Clinique. Ce projet après avoir obtenu l'accord du Comité de Protection des Personnes a été retenu lors d'un appel d'offre interne au CHU permettant de financer les premières analyses. C'est ainsi que je me suis mis en disponibilité de mon Internat pour réaliser un DEA de Génétique Humaine à Paris VI, grâce à l'obtention d'une bourse de recherche de la Fondation pour la Recherche Médicale, et travailler sur les échantillons des patients lors d'un stage dans l'unité du Pr X. Jeunemaitre, sous sa direction au Collège de France/Unité INSERM U36 (Pr P. Corvol). Au cours de ce stage, nous avons pu développer différents modèles d'études de liaison (avec l'aide du Dr F. Mathieu), permettant d'exclure les loci *FBN1*, *MFS2* (désormais *TGFBR2*, cf. Chapitre 2) et *COL3A1*. Nous avons également développé une méthode de séquençage de l'ADN complémentaire du gène *COL3A1* à partir de fibroblastes cutanés en culture qui sera transférée en diagnostic au cours de mon semestre interCHU dans le laboratoire de Génétique de l'Hôpital G. Pompidou, dirigé par le Pr X. Jeunemaitre et ensuite dans le laboratoire de Génétique Moléculaire du CHU de Montpellier (réf. 8, 9, 13 et 17, publications en rapport avec la thématique de Recherche).

Ce parcours Recherche, au cours de mon internat c'est réalisé en plus de l'activité hospitalière et des différents stages nécessaires pour accomplir du DES de Génétique Médicale. Ceci est matérialisé par des publications sans rapport avec la Thématique de Recherche (réf. 51 à 60).

AHU, spécialisation en Génétique Médicale.

J'ai réalisé ma mobilité au CHU de Besançon, où j'ai pris les fonctions d'Assistant Hospitalo-Universitaire dans le service de Génétique, Histologie et Embryologie (Pr JL

Bresson), avec une charge d'enseignement et de consultations de Génétique Médicale importante (réf. N°46 et 40, publications sans rapport avec la Thématique de Recherche).

Parallèlement, j'ai pu poursuivre mon parcours recherche au cours de ma Thèse d'Université (Université de Bourgogne). Grâce à une collaboration avec le Centre National de Génotypage pour le génotypage de la « Famille Bourgogne », nous avons pu localiser le gène en 16p12.13, à partir des modèles d'étude de liaison développés au cours du DEA. Au cours de ce travail, nous avons pu montrer le rôle discriminant de la mesure de la compliance artérielle en IRM fonctionnelle chez les patients porteurs de la prédisposition *MYH11* mais qui n'exprimaient, ni AAT/DA, ni PCA. La localisation du gène a permis de ces travaux ayant fait l'objet de ma Thèse d'Université (réf. 20, 21 et 22 publications en rapport avec la thématique de Recherche ; chapitre 3, *MYH11*).

Praticien Hospitalier, CHU Montpellier / INSERM U827

J'ai pris mes fonctions en 2005 de Praticien Hospitalier au CHU de Montpellier dans le laboratoire de Génétique Moléculaire (Pr M. Claustres) avec pour double mission, de co-encadrer l'activité de diagnostic de Référence en Génétique Moléculaire des Dystrophinopathies, et de développer et encadrer une activité de diagnostic en Génétique Moléculaire du Syndrome de Marfan et des Syndromes Apparentés. C'est au cours de cette période, au sein d'un environnement mixte, mêlant recherche et clinique à l'Institut Universitaire de Recherche Clinique que j'ai pu compléter mon parcours :

- Obtention d'un Diplôme d'Etudes Spécialisées Complémentaires en Biologie Moléculaire, me permettant d'être agréé pour l'examen des Caractéristiques Génétiques constitutionnelles et prénatales.
- Validation de ma Thèse d'Université (réf. 20, 21, 22, annexes). Le Dr L. Zhu a poursuivi mes travaux, avec le Pr Jeunemaitre, en identifiant le gène *MYH11* comme responsable

de l'association AAT/DA dans la famille « Bourgogne » (Chapitre 3, réf. 19 publication en rapport avec la Thématique de Recherche, annexes). Cette découverte est majeure dans la Thématique de la Génétique des AAT/DA, ayant ouvert la voie à un nouveau groupe de gènes codants pour l'appareil contractile des CMLs et/ou impliqués dans la mécanotransduction du signal (Chapitre 3). A cette période, l'affaire « Myriad Genetics » et des gènes *BRCA1-BRCA2* avec la jurisprudence concernant les mesures de Protection et d'exploitation des Brevets, ont conduit le Pr Xavier Jeunemaitre à déposer un Brevet au nom de l'INSERM (cf. Travaux), pour lequel je suis co-inventeur.

- **Activité « Dystrophinopathies » (réf. 43 44, 45, 48 et 49) :** il s'agissait d'une activité diagnostique pour laquelle le laboratoire était Référent National (avec le laboratoire de Paris, Cochin), animant le Réseau correspondant au sein de l'Association National des Praticiens de Génétique Moléculaire et l'établissement de bases de données « Locus Spécifique » pour le développement des essais. Au cours de cette activité à la frontière clinique / recherche, j'ai pu au sein de l'équipe et avec l'aide du Dr S. Tuffery acquérir de nouvelles compétences, nécessaires pour actualiser le diagnostic de patients en attente de diagnostic, assurer la veille technologique du laboratoire en développant de nouvelles technologies et en les transférant en diagnostic. Parallèlement, j'ai encadré et co-encadré des étudiants et pu obtenir un financement de l'Association Française Contre les Myopathies pour encadrer le post-doctorat du Dr A. Ishmukhametova (cf. activités d'encadrement).

- **Activité « Marfan et Apparentés » (réf. 7-8, 10-15, 18, 19):** Malgré la fréquence de la maladie, les besoins en diagnostic moléculaires pour le Syndrome de Marfan étaient à cette époque peu couverts par le seul laboratoire Français (Pr C. Boileau) et rapidement de nombreux correspondants nous adressés les prélèvements de patients pour analyses (plus de 600 patients/familles, dont la plupart avec un diagnostic positif). Ces analyses étaient complexes et lourdes car nous ne disposions pas des technologies de séquençage de nouvelle génération actuelles. Nous avons alors mis au point une technique permettant d'amplifier par PCR (ou RT-PCR) puis de séquencer en SANGER l'ensemble des exons et bordures introniques des gènes *FBN1*, *TGFBR1* et *TGFBR2*, selon le

principe : 1 patient – 1 plaque 96 puits, de développer une multitude d’analyses complémentaires (recherche de grands réarrangements en MLPA, d’anomalies d’épissage en RT-PCR, dHPLC puis HRM...) nécessaires pour caractériser les variants identifiés et fiabiliser le diagnostic moléculaire. Parallèlement, l’identification de nouveaux gènes impliqués dans les AAT/DA impliquait un transfert progressif, en diagnostic. Dans ce contexte, il apparaissait nécessaire de pouvoir transférer en Recherche appliquée quelques situations complexes mais d’un grand intérêt pour la Recherche. Parmi les situations, rencontrées je citerai deux exemples : 1- celui de variants faux-sens impliquant un résidu Glycine à une position particulière des modules cbEGF-like de la fibrilline de type 1, que l’on ne pouvait considérer que comme de signification inconnue et donc sans bénéfice pour le diagnostic des patients et de leurs apparentés. Les études complémentaires que nous avons réalisées nous ont permis de caractériser cette position conservée comme « obligatoire » et de résoudre cette situation bloquante (réf. 12). 2- celui d’une patiente atteinte d’un SED particulièrement sévère (décédée à l’âge de 10 ans de complications digestives), nous amenant à décrire pour la première fois une forme récessive de SED en rapport avec *COL3A1*, différente d’un SED vasculaire dominant et associée à des anomalies cérébrales (réf. 13 et 4, annexes).

- Promu responsable d’un groupe de Recherche Clinique « Génétique des AAT/DA » lors de la création de l’Unité INSERM U827 par le Pr M. Claustres : découlant de l’activité Hospitalière où j’avais constitué une petite équipe (dont Mme A. Plancke, Ingénieure), mais également de Projets de Recherche, d’encadrement de stagiaire (cf. Enseignement-Encadrement-Vie scientifique).

Praticien Hospitalier, CHU Nîmes

Pour des raisons à la fois personnelles et professionnelles, j’ai quitté mes fonctions au CHU de Montpellier et le Laboratoire de Génétique Moléculaire pour le laboratoire de Cytologie Clinique et Cytogénétique du CHU de Nîmes (Pr T. Lavabre-Bertrand) où je suis jusqu’à ce jour, le seul Médecin qualifié en Génétique Médicale sur le territoire de

Santé. Ce choix était extrêmement difficile, car il imposait d'arrêter l'activité diagnostique consacrée au Diagnostic Moléculaire du Syndrome de Marfan et des Syndromes Apparentés développée au CHU de Montpellier. Les conséquences étaient importantes pour les nombreux correspondants, les patients et leurs familles. L'activité n'était pas prolongée au CHU de Montpellier. Pour cette raison, ma prise de fonction au CHU de Nîmes pour une activité de Génétique Médicale clinique (Pédiatrie, Diagnostic Anténatal, Conseil Génétique...) était conditionnée à la possibilité de remonter cette activité. Grâce au soutien du Pr Lavabre-Bertrand mais également de du Dr J. Chiesa (responsable de l'UF Génétique Médicale et Cytogénétique) et de la Direction du CHU de Nîmes, j'ai pu recruter Mme A. Plancke, mettre en place et développer cette activité diagnostique sur le Plateau Commun de Biologie Moléculaire du Pôle « Biologies-Pathologie ». Durant cette interruption, d'un peu plus d'un an, les demandes d'analyses familiales (pour les patients dont le défaut moléculaire avait été caractérisé lors de mes activités au CHU de Montpellier), ont pu être relayées au laboratoire du Pr C. Boileau (Paris). Ceci a permis de limiter l'impact sur les patients.

Comme tous les laboratoires hospitaliers ou hospitalo-universitaires, réalisant des analyses diagnostiques de Génétique, l'irruption de la technologie de séquençage « de deuxième génération » (NGS) a été une réelle disruption technologique. De l'analyse des gènes *FBN1*, *TGFBR1* et *TGFBR2* en séquençage conventionnel (SANGER) longue et fastidieuse, nous avons développé successivement des panels ciblés de 15, 29 et désormais 35 gènes analysés en NGS beaucoup plus rapidement et efficacement. A ce jour, nous avons recrutés 775 patients/familles, originaires de toute la France et même d'Outre-Mer et analysés plus de 680 d'entre-elles, avec un diagnostic positif pour de nombreuses familles, utile au dépistage et à la prévention du risque aortique. Concernant la Recherche, nous commençons à exploiter les données recueillies sous la forme de quelques publications scientifiques en collaboration, notamment avec le Centre de Référence « Syndrome de Marfan et Apparentés » de Toulouse (réf. 1-3, 5-7), d'autres travaux et projets sont en cours.

Cette activité de Biologie et de Génétique Moléculaire, ne représente qu'une partie de mes activités au CHU de Nîmes. J'ai également une activité importante : de consultations

médicales, d'expertise clinique pour le diagnostic de maladies rares, d'anomalies du développement et de syndromes malformatifs (réf. 23-25, 27-30, 32-35, 37-39), de coordination (Centre labellisé Maladie Rare, Fédérations Montpellier-Nîmes, mise en place du Plan France Médecine Génomique et des Réunions Concertées Pluridisciplinaires) et participe à la Recherche d'autres équipes en temps qu'Investigateur (ref. 26, 31, 36).

Analyse critique et Perspectives

Après vingt années d'exercice en Génétique Médicale, avec une pratique clinique et biologique étroitement en lien avec des activités de Recherche, plusieurs raisons m'ont amenées à m'engager dans cette candidature à l'Habilitation à Diriger des Recherches.

- L'évolution importante des connaissances et des découvertes issues de l'identification de nouveaux gènes responsables de prédispositions aux AAT/DA, telles que j'ai tenté de les rapporter dans ce mémoire, soulèvent de nombreux questionnements et hypothèses de Recherche.

- La chirurgie aortique programmée, personnalisée à un diamètre donné, selon la nature du défaut génétique impliqué est déjà une réalité. En ce qui concerne le développement de thérapies préventives, ciblées sur le défaut génétique, la Recherche est également une nécessité. En effet, l'espoir initial de disposer d'un traitement préventif par les Antagonistes des Récepteurs de l'Angiotensine 2 (losartan) ciblant la voie de signalisation du TGF-béata dans le syndrome de Marfan, les SLD, voire étendue à l'ensemble des prédispositions aux AAT/DA (car supposée initialement par certains être la voie finale commune), s'avère être un semi-échec. Une mauvaise compréhension de la physiopathologie moléculaire, en est probablement la cause. Cela nécessite indiscutablement le développement de la Recherche, à la fois fondamentale, mais également clinique et appliquée.

- Le génotypage de patients et de familles touchés par des formes syndromiques ou non d'AAT/DA pour les nombreux gènes impliqués (dont certains sont encore peu étudiés et connus), entraîne l'identification de variants de signification inconnue, à l'origine d'une non information pour le diagnostic des patients. Certains sont d'un intérêt majeur pour la recherche. Leur caractérisation par des études fonctionnelles (parfois simples, parfois nécessitant des moyens lourds et complexes) peut permettre une meilleure compréhension de la physiopathologie moléculaire et en retour de fournir une information diagnostique.

- Disposant désormais de nombreuses données issues du diagnostic génétique de patients et de familles, ainsi que du génotypage de nombreux gènes encore peu étudiés. Ces données, complétées d'informations cliniques de bonne qualité ont une masse critique suffisante pour envisager un transfert en Recherche et une valorisation sous la forme de publications scientifique de bon niveau.

Pour se faire, mon rattachement à l'Unité de Recherche-F15 de l'UFR de Médecine de Nîmes, affiliée à l'Institut des Biomolécules Max Mousseron (IBMM, Montpellier) était une première étape pour envisager ce transfert et engager les collaborations nécessaires. Cette affiliation était logique compte tenu, de la thématique de l'Unité, de la proximité avec l'activité hospitalière et du fait que les Praticiens Hospitalo-Universitaires ou non de mon laboratoire y étaient déjà rattachés.

L'encadrement d'Internes de Génétique, au cours de leurs semestres clinique (Dr M. IMBERT-BOUTEILLE, Dr C. CENNI-LEMATTRE) ou moléculaire (Dr B. CHESNEAUX) dans notre laboratoire, particulièrement motivés et investis, a suscité en moi l'intérêt de pouvoir encadrer des étudiants Médecins, Pharmaciens ou Scientifiques en Master 2 et en Thèse, sur cette thématique de Recherche.

Enfin, la transmission des savoirs et des compétences, m'apparaît désormais comme un objectif majeur dans les années à venir. J'ai pu développer au cours de mon parcours une expertise reconnue dans le domaine qu'il m'apparaît nécessaire de transmettre et de relayer.

Annexes

1. Zhu L, **Khau Van Kien P***, Vranckx R*, Lalande A, Boisset N, Mathieu F, Wegman M, Glancy L, Brunotte F, Gasc JM, Bruneval P, Wolf JE, Michel JB, Jeunemaitre X. Mutations in myosin heavy chain 11 cause a syndrome associating thoracic aortic aneurysm/dissection and patent *ductus arteriosus*. *Nat Genet* 2006;38(3):343-349 (* contribution égale). (L).
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Mutations in myosin heavy chain 11 cause a syndrome associating thoracic aortic aneurysm/aortic dissection and patent ductus arteriosus

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We have recently described two kindreds presenting thoracic aortic aneurysm and/or aortic dissection (TAAD) and patent ductus arteriosus (PDA)^{1,2} and mapped the disease locus to 16p12.2-p13.13 (ref. 3). We now demonstrate that the disease is caused by mutations in the *MYH11* gene affecting the C-terminal coiled-coil region of the smooth muscle myosin heavy chain, a specific contractile protein of smooth muscle cells (SMC). All individuals bearing the heterozygous mutations, even if asymptomatic, showed marked aortic stiffness. Examination of pathological aortas showed large areas of medial degeneration with very low SMC content. Abnormal immunological recognition of SM-MHC and the colocalization of wild-type and mutant rod proteins in SMC, in conjunction with differences in their coimmunoprecipitation capacities, strongly suggest a dominant-negative effect. Human *MYH11* gene mutations provide the first example of a direct change in a specific SMC protein leading to an inherited arterial disease.

TAAD, one of the most severe cardiovascular conditions in adults, may occur in the context of inherited connective tissue disorders such as the Marfan (MIM 154700) and Ehlers-Danlos (MIM 130050) syndromes. Familial clustering of common TAAD is, however, more complex and heterogeneous⁴, and three causal loci have been mapped to chromosomes 11q23.2-q24 (*AAT1*)⁵, 5q13-14 (*AAT2*)⁶ and 3p24-25 (*AAT3*)⁷. The *AAT3* locus was recently shown to correspond to the *TGFBR2* gene, mutations in which may cause up to 5% of familial TAAD cases⁸. We recently reported one American¹ and one French²

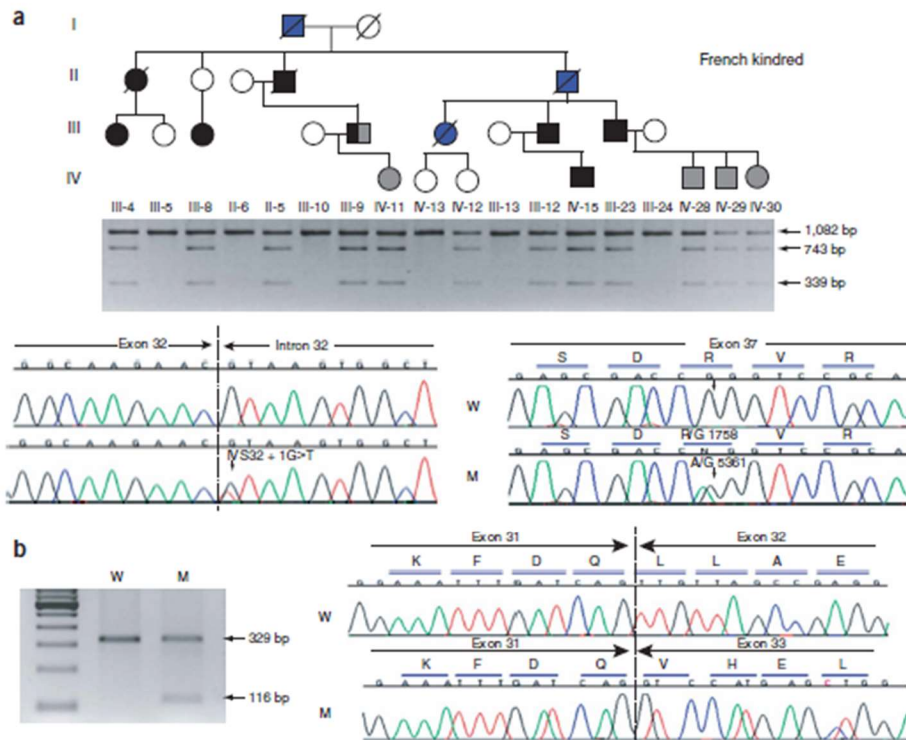
kindred in which TAAD was unexpectedly found in association with PDA. PDA is one of the most common congenital cardiovascular malformations, affecting approximately 1 in 2,000 live births⁹. It is generally regarded as sporadic, but its occurrence in 5% of the siblings of PDA cases suggests there may be a genetic component. No specific gene defect has yet been reported, other than mutations in the gene encoding the transcription factor *TFAP2B* in the rare Char syndrome¹⁰ and a recessive locus at 12q24 in Iranian pedigrees¹¹. Further clinical characterization and genetic analysis of the large French kindred with TAAD and PDA suggested a single pathophysiological and genetic entity² and led to the mapping of the disease locus to a 20-cM interval on chromosome 16p12.2-p13.13 (ref. 3).

The disease locus contains more than 60 known and unconfirmed genes. Genetic analysis of six members of the American kindred confirmed cosegregation with this region but did not allow reduction of the interval (data not shown). The sequencing of several candidate genes, including *NOMO1*, *ABCC6* and *BFA1*, showed no mutation². Among the known genes, *MYH11*, which encodes the smooth muscle myosin heavy chain (SM-MHC), a major specific contractile protein produced in SMC, was located ~1.5 Mb from the peak maximum lod score (4.10 for marker D16S103). Systematic mutation screening showed two heterozygous mutations affecting the same allele in the French kindred (Fig. 1a). The first was a substitution at the splice-donor site of intron 32 (IVS32+1G→T). The second was a missense mutation in exon 37 (G5361→A), resulting in a charged amino acid, arginine, being replaced by an uncharged amino acid, glutamine (R1758Q). Both mutations were identified in all subjects carrying

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Figure 1 *MYH11* mutations in the French kindred. **(a)** Partial structure of the French kindred. Unaffected (open symbols) and affected individuals (closed symbols) are shown according to disease status: TAAD (black) and PDA (gray). Subjects who died of stroke are indicated in blue. Electrophoresis showed the absence (1,082-bp amplification product) or presence (743 bp + 339 bp) of a new *Afl*I restriction site created by the mutation at the splice-donor site of intron 32 (NS32+1G→T). Subject IV-12 has a mutation in this gene but is asymptomatic. The numbering of the subjects refers to the one used in the genealogical tree in annex 1 of our previously published study³. Left: electropherograms from a wild-type (W) and a mutated (M) subject for the heterozygous mutation of the splice-donor site of intron 32 (NS32+1G→T). Right: electropherograms from a wild-type (W) and a mutated (M) subject for the heterozygous mutation of the splice-donor site of intron 32 (NS32+1G→T). This additional mutation was present in all individuals bearing the first mutation. **(b)** Consequence of the splice-donor (NS32+1G→T) mutation: exon 32 was missing. Partial *MYH11* cDNA amplification was performed, using total RNA extracted from cultured fibroblasts of one affected individual (III-12, M) and one control (W). The expected 329-bp wild-type (W) fragment was obtained in both the control and the mutated (M) individual; an additional 116-bp fragment was found in the mutated (M) individual. Direct sequencing of the W and M fragments demonstrates the skipping of exon 32 (213 bp), leading to an in-frame loss of 71 amino acids (L1456_N1526del).

the disease haplotype, but neither was found in 340 normal chromosomes. The transcriptional consequences of the splice site mutation were confirmed by analysis of the mRNA extracted from the cultured fibroblasts of two affected subjects. Amplification and direct sequencing of the corresponding cDNA demonstrated that exon 32 was missing (Fig. 1b), resulting in an in-frame deletion of 71 amino acids (L1456_N1526del) in the C-terminal region of SM-MHC. A similar search for mutations in the American kindred detected a 72-nucleotide deletion within exon 28 of the *MYH11* gene (3810_3881del) (Fig. 2) that was not detected in 340 normal chromosomes. This in-frame deletion corresponds to the loss of 24 amino acids (R1241_L1264del), also in the C-terminal region of SM-MHC. The smooth muscle myosin molecule is composed of two heavy chains (SM-MHC), two essential light chains and two regulatory light chains.

One SM-MHC dimer has two N-terminal globular heads on which the ATP- and actin-binding sites are located, and one coiled-coil rod assembled from two SM-MHC α -helical C-terminal tails¹². The rod region consists of a series of heptad repeats resulting in 28-residue periodicity, with alternate bands of positive and negative charges that are essential for rod assembly and thick filament formation¹³. Thus, the *MYH11* mutations observed in the French and American kindreds may affect its coiled-coil structure and the assembly of myosin thick filaments.

We first investigated the structural consequences of the French mutation for the aortic wall by means of histology and immunohistochemistry in aortic tissue samples obtained surgically from two affected subjects (individuals III-12 and III-23 in the pedigree, Fig. 1a). Both samples showed severe abnormalities with focal areas

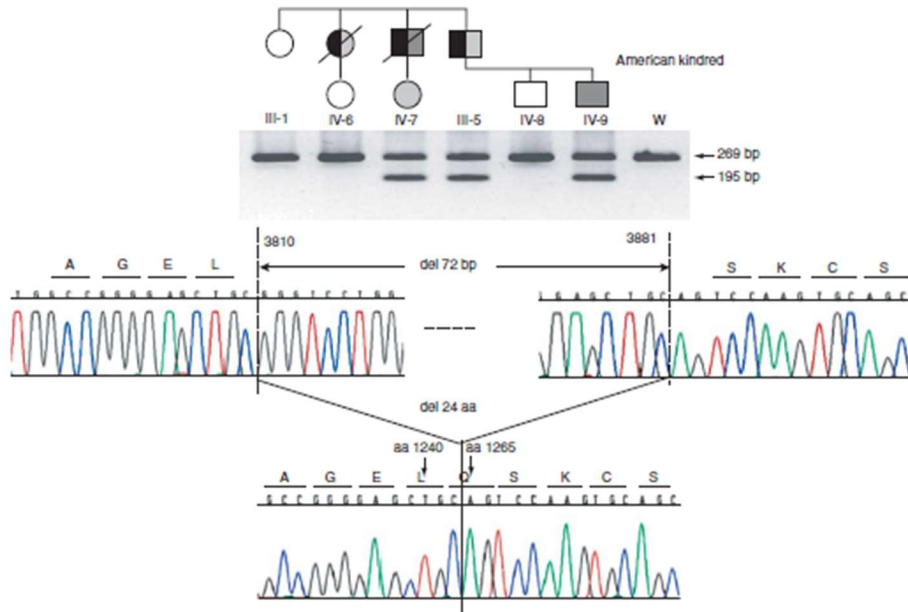


Figure 2 *MYH11* mutation in the American kindred. (a) Structure of the American kindred. Symbols are as for Figure 1b. There is an in-frame deletion at exon 28. Amplification products show a 72-bp deletion in the heterozygous state. Direct sequencing of the wild-type (top) and mutant (bottom) alleles shows a 72-bp deletion (nucleotides 3810–3881), causing the loss of 24 amino acids (amino acids 1241–1264) from the C terminus of the protein.

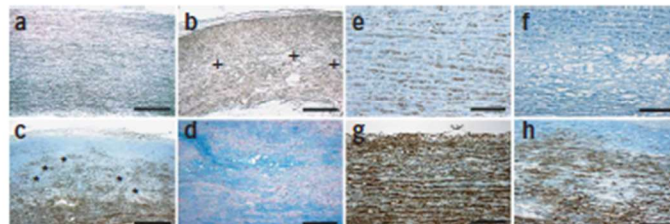
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of SMC loss and disruption and loss of elastic fibers (Fig. 3a–c) and collagens. In these areas, there was an accumulation of Alcian blue–positive mucopolysaccharides (Fig. 3d), a cystic degeneration pattern commonly observed in TAA. In the remaining SMC, smooth muscle α -actin (Fig. 3c), heavy caldesmon and vimentin were produced normally (data not shown). Positive or negative staining of SM-MHC was observed, depending on the tissue preparation (Fig. 3e–h). In contrast to control aortic samples, pathological samples fixed in formalin showed no staining with a monoclonal antibody directed against a peptide epitope of the C-terminal SM-MHC domain, but

we observed positive labeling in frozen preparations. Positive staining was observed with both types of preparation if a polyclonal antibody was used (data not shown), suggesting that the assembly formed by the mutated and wild-type coiled-coil SM-MHC domains was more easily denatured by formalin fixation than its homozygous wild-type counterpart. Immunoblotting of proteins extracted from one pathological aorta confirmed the presence of similar amounts of the wild-type and mutant SM-MHC isoforms (Fig. 4a,b).

The presence of both proteins, combined with changes in immunological recognition, suggested that the *MYH11* mutation induced a

Figure 3 Structural and immunolabeling abnormalities of the aortic tissue. Pathological aortic tissue was obtained from two individuals with TAA of the French pedigree (III-12 and III-23), giving same results. (a,b) Orcein staining of elastic fibers. (a) Normal control. (b) The pathological tissue shows a decrease in number and the rupture of elastic fibers (+). (c) Actin labeling shows a major decrease in the number of SMC, with mucoid degeneration and a cystic pattern (*). (d) Alcian blue staining shows the deposition of mucopolysaccharides in the areas from which the vascular SMC have been lost and in which the extracellular matrix is degraded. (e–h) SM-MHC production in the aortic media. (e) Normal aortic media labeling pattern of vascular smooth muscle cells using SM-MHC monoclonal antibody (Novocastra) against C-terminal SM-MHC on a section from formalin-fixed and paraffin-embedded normal control aorta tissue. (f) Absence of immunostaining of media vascular smooth muscle cells using Novocastra antibody on a section from formalin-fixed and paraffin-embedded aorta tissue from one affected individual. (g–h) Presence of SM-MHC labeling of media vascular smooth muscle cells using SM-MHC monoclonal antibody (Novocastra) on a frozen section of aorta tissue from control (g) and one affected individual (h). Scale bars: 900 μ m (a–d), 225 μ m (e,f) and 450 μ m (g,h).



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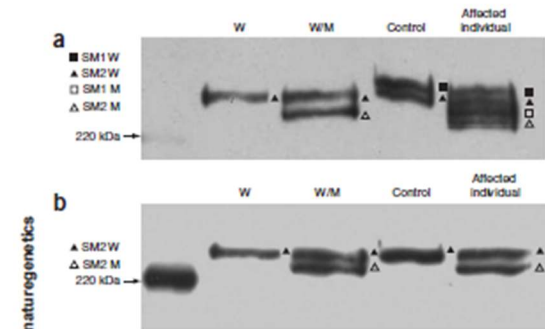


Figure 4 Characterization of SM-MHC in the aortic tissue by immunoblotting. Recombinant wild-type (W) and mutant (M) SM2 isoform proteins extracted from transfected CHO were used as controls. Using 5% SDS-PAGE, we separated 1.5 µg total protein from control aortic tissue and 3 µg total protein from aortic tissue of an affected individual (French kindred, III-12) and then carried out immunoblotting with anti-SM-MHC (a) and anti-SM2 (b). Alternative splicing at the 3' ends of the primary SM-MHC transcript generates two isoforms, SM1 and SM2 (ref. 30), with SM2 4 kDa shorter than SM1. In the control tissue, both isoforms are can be visualized by immunoblotting with anti-SM-MHC (a) whereas only the SM2 isoform is can be visualized by immunoblotting with anti-SM2. In the affected individual's tissue, immunoblotting demonstrated the presence of both SM1 and SM2; furthermore, each isoform contained the wild and mutant proteins, with the mutant almost 8 kDa shorter than the wild-type protein owing to the 71-residue deletion.

conformational change, consistent with changes in the stability of the α -helical coiled-coil structure, as previously shown for heterozygous mutations in the rod region of other myosin-II heavy chains^{14,15}. *In silico* prediction showed that both the French and American mutations affected the probability of coiled-coil formation at the C terminus of SM-MHC (Fig. 5a). We investigated the consequences of the French mutation, corresponding to the deletion of 71 residues in the rod sequence. Recombinant wild-type and mutant proteins were produced in smooth muscle Rb-1 cells. Transient transfection with expression vectors encoding the rod region of SM-MHC showed that the wild-type and mutant rod were colocalized (Fig. 5b). Cyan fluorescent protein (CFP)- or yellow fluorescent protein (YFP)-tagged constructs were used to test for interaction between the wild-type and mutant proteins. The wild-type SM-MHC rod did not precipitate its mutant counterpart, whereas such coimmunoprecipitation

was achieved if the tagged and nontagged mutant constructs were coexpressed or if the tagged and nontagged wild-type constructs were coexpressed (Fig. 5c). This altered interaction between wild-type and mutant SM-MHC, together with the production of both proteins in pathological tissue, provides supplementary evidence that the mutant acts via a dominant-negative mechanism.

The ascending aorta is placed under major mechanical stress during each cardiac cycle. We have shown in the French kindred that individuals bearing the disease haplotype show altered aortic stiffness parameters: notably, aortic compliance, which serves as an early marker of the disease³. Extension of this study to 49 first-degree relatives showed that aortic diameter was generally similar in those with and without the mutation, but that carriers of the mutation had a lower aortic compliance (66% decrease) and a higher pulse wave velocity (73% increase; both $P < 0.001$, Table 1). Further comparison

Figure 5 *In vitro* assay of the interaction between wild-type and mutant SM-MHC rod. (a) Probability of coiled coil formation according to COILS²⁸. The number on the horizontal axis refers to the amino acid position of the SM2 isoform. The arrowheads on the wild-type profile indicate the region of residues deleted in the French kindred (green) and in the American kindred (red). The 71-residue deletion in the French kindred changes the probability of coiled coil formation, as the peak (green arrow) becomes narrower and shallower. However, the 24-residue deletion in the American family creates a new peak (red arrow), indicating a decrease in the probability of coiled coil formation between mutant and mutant. (b) Colocalization of wild-type and mutant SM-MHC rods. With the cyan filter, the Rb-1 cells clearly display CFP-W-Rod expression. In the same field, with the yellow filter, YFP-M-Rod proteins were also detected; Anti-SM-SMC staining indicates that the recombinant rod proteins were recognized by this antibody, as was the endogenous full-length SM-MHC. The wild-type and mutant SM-MHC rod proteins colocalized. (c) Coimmunoprecipitation of wild-type and mutant SM-MHC rod proteins. Anti-GFP was used to immunoprecipitate CFP- or YFP-tagged SM-MHC rod proteins. Coprecipitation of non-tagged SM-MHC rod proteins was possible, depending on the interaction between homologous and heterologous rods. The precipitated proteins and supernatants were subjected to immunoblotting with anti-SM-MHC. The CFP-tagged wild-type rod coprecipitated with the non-tagged wild-type rod (left) as did the YFP-tagged mutant with the non-tagged mutant (right), but the CFP-tagged wild-type rod did not coprecipitate with the non-tagged mutant rod (middle). IP: immunoprecipitation, SN: supernatant.

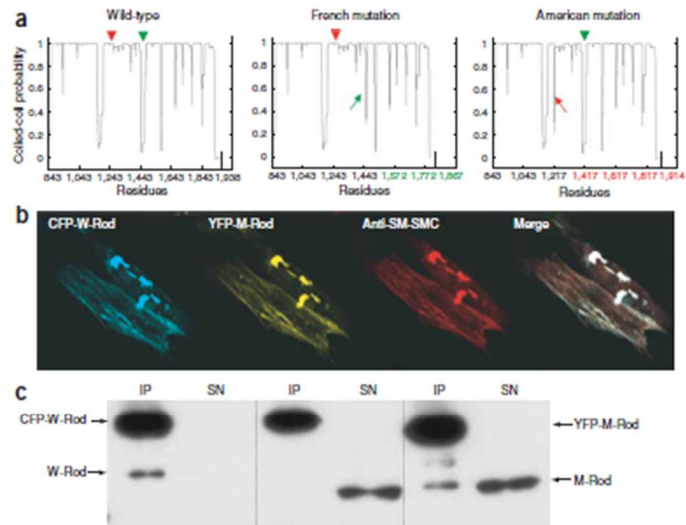


Table 1 Relationships between mutation status and aortic parameters in the French kindred

	M- versus M+			M+ symptomatic versus asymptomatic			
	All M-	All M+	P ^a	Symptomatic	Asymptomatic	P ^b	P ^c
Number	33	16	–	7	9	–	–
Age (years)	32 ± 12	36 ± 13	0.24	46 ± 12	28 ± 9	0.01	0.57
Sex (M/F)	14/19	9/7	0.36	5/2	4/5	0.28	0.11
Aortic diameter (mm)	31.2 ± 2.9	34.5 ± 6.4	0.20	37.3 ± 7.8	32.3 ± 4.5	0.22	0.88
Aortic diameter/BSA (cm m ⁻²)	1.88 ± 0.19	1.98 ± 0.31	0.42	2.16 ± 0.37	1.84 ± 0.14	<0.05	0.53
Aortic compliance (mm ² mmHg ⁻¹)	1.86 ± 0.71	0.63 ± 0.32	<0.001	0.54 ± 0.33	0.70 ± 0.32	0.31	<0.001
Pulse wave velocity (m s ⁻¹)	4.28 ± 1.37	7.39 ± 3.44	<0.001	9.63 ± 4.05	5.66 ± 1.45	0.09	0.02

M+; mutation carrier; M-; without mutation. Symptomatic individuals included five individuals with TAAI, one with PDA, and one with both TAAI and PDA; asymptomatic individuals were without TAAI or PDA. ^aComparison between all M- and M+ individuals; ^bcomparison between M+ symptomatic and M+ asymptomatic individuals; ^ccomparison between M- and M+ asymptomatic individuals.

showed that young asymptomatic mutation carriers already had aortic indices similar to those of symptomatic mutation carriers and significantly different from those of normal relatives of the same age (Supplementary Tables 1 and 2 online). Thus, *MYH11* heterozygous mutation leads to an early and severe decrease in the elasticity of the aortic wall, consistent with the role of SMC in maintaining the mechanical properties of the thoracic aorta³⁶.

Major changes in the structure and composition of the ductus arteriosus occur before and after delivery, and these changes require smooth muscle cells to migrate, proliferate, differentiate and contract¹⁷. Mice deficient in SM-MHC have been generated¹⁸. Homozygous *MYH11*^{-/-} mice have several abnormalities, including a delay in closure of the ductus arteriosus. Despite species differences in closure rate and morphology¹⁹, the presence of PDA in this knockout model and in both the French and American kindreds demonstrates the involvement of SM-MHC in this phenomenon. SM-MHC-deficient mice also present a giant thin-walled bladder and abnormal intestinal movement. We observed no symptoms that would be caused by such anomalies in our subjects. Nor did we observe hematuria, a sign present in a family with pseudoxanthoma elasticum bearing a 900-kb heterozygous deletion including the *MYH11* gene²⁰. These phenotypic differences may be accounted for by different consequences of the two mutations: haploinsufficiency for one and a dominant-negative effect for the other.

Mutations in the cardiac-specific myosin heavy chain genes *MYH6* and *MYH7* are responsible for cardiac disorders such as atrial septal defect and hypertrophic cardiomyopathy^{21,22}. The inherited aortic diseases characterized to date have been associated with defects in structural proteins of the extracellular matrix, such as fibrillin and collagen. The TGF- β signaling pathway has also recently been implicated in aortic aneurysms and dissections^{8,23}. Human *MYH11* gene mutations provide the first example of direct changes in a contractile protein produced specifically in SMC. This new pathogenic mechanism opens up new possibilities for increasing our understanding of as-yet unexplained arterial diseases.

METHODS

Subjects. Forty-nine members of the French 'Bourgogne' family and six members of the American family were analyzed. These two pedigrees have been described elsewhere^{1,3}. Written informed consent for all procedures, including genetic analysis and the protocol for aortic parameter evaluation, was obtained from each participant or from the parents of younger children. The study was approved by the local ethical committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Bourgogne n°2000/15; 00/03/16) and the French Ministry of Health.

Genotyping procedures. Analysis of microsatellite markers of the 16p12.2-p13.13 region was performed for the American kindred, as previously performed for the French kindred³. Direct bidirectional sequencing of the 42 exons of the human *MYH11* gene (encoding smooth muscle myosin heavy chain 11 isoform SM2 (MIM 160745)) was carried out for two affected individuals of each family (French family III-12 and IV-11, American kindred III-5 and IV-7) and two controls, using the ABI BigDye Terminator cycle sequencing kit (Applied Biosystems). The Sequencher 4.0.5 program (Gene Codes) was used to analyze and align the sequences. Once the mutation had been identified, it was analyzed in other members of the family by direct sequencing of exons 32 and 37 for the French kindred and by amplification of exon 28 and direct gel electrophoresis for the American kindred.

RT-PCR. RNA was extracted from skin fibroblasts with the QIAgen RNeasy Mini kit (QIAgen). The first-strand cDNA was synthesized from 1 μ g of total RNA. Primers were designed to bind to exon 31 and exon 33 of human *MYH11* mRNA.

Histology and immunohistochemistry. Aortic tissue samples obtained during surgery were immediately frozen on dry ice. A part of each specimen was fixed in formalin and embedded in paraffin. For histology, cells were stained with hematoxylin and eosin, and the extracellular matrix was stained with orcein, Alcian blue and Sirius red by standard techniques. For immunohistochemistry, antibodies directed against vimentin, smooth muscle α -actin and heavy caldesmon (Dako) were used on paraffin sections after antigen retrieval. Monoclonal antibodies directed against a specific C-terminal portion of SM2 (Abcam) or against the 235-amino acid portion of the C-terminal domain of human SM-MHC (Novocastra) or polyclonal antibodies against SM-MHC (BTI) were tested on formalin-fixed, paraffin-embedded sections or frozen sections from aortic tissues obtained from normal controls and from two affected individuals, with detection by a standard three-step technique.

MYH11 cDNA construction. The full-length wild-type and mutant human SM-MHC isoform SM2 cDNAs were obtained from human dermal fibroblasts. Five consecutive fragments (nucleotides 89–1509, 1383–2741, 2603–4141, 4080–5144 and 5020–6073, called I, II, III, IV and V) were amplified by RT-PCR and subcloned separately into pGEM-T (Promega). The restriction sites (such as *Bam*HI, *A*fII, *A*ccI and *B*glII) within these fragments were used to create cohesive ends, facilitating ligation. Clones were selected after verification by direct sequencing. FV-pGEM-T was used as the scaffold vector, and I was the last fragment inserted into this construct, via the *Spe*I/*Bam*HI sites. For the mutant cDNA, the IV fragment, which lacks exon32, was amplified by RT-PCR from dermal fibroblasts from one affected individual of the French family III-12. Sequences of pGEM-T-SM2-W (wild-type) and pGEM-T-SM2-M (mutant) were confirmed by direct sequencing analysis.

The pGEM-T-SM2-W and pGEM-T-SM2-M inserts were then excised by *Spe*I/*Sac*II digestion and subcloned into pIRES2-EGFP and pIRES2-DsRed2 (BD Clontech) separately, using the *Nhe*I/*Sac*II sites, to generate pIRES2-EGFP-SM2-W and pIRES2-DsRed2-SM2-M, respectively. The cDNAs encoding the MYH11 SM2 wild-type rod domain (nucleotides 2648–6073) and its

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mutant counterpart, preceded by a *Sall* site and a Kozak consensus translation initiation site, were inserted as a *Sall*/*SacI* fragment into pIRES2-DsRed2, pECFP-C1 and pYFP-C1 to give W-Rod, CFP-W-Rod, M-Rod and YFP-M-Rod, respectively.

Cell culture and transient transfection. Dermal fibroblasts were obtained by skin biopsy from two members of the French family (III-12, III-23) and one normal volunteer. Cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin and fungizone (PSA). They were incubated at 37 °C in an atmosphere containing 5% CO₂. Chinese hamster ovary (CHO) cells were grown in Ham's F-12 nutrient mixture with 10% fetal calf serum (SVF) and 1% PSA. Rb-1 cells (continuous rabbit aortic smooth muscle cell line²⁴) were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% SVF, 5 mM HEPES, 2 mM glutamine and 1% PSA. The SM2 protein molecular weight standard was obtained by transfecting CHO cells in six-well plates (400,000 cells per well) with pIRES2-EGFP-SM2-W or pIRES2-DsRed2-SM2-M, individually or together, using FuGENE-6 transfection reagent (Roche) according to the manufacturer's instructions. For immunofluorescence, Rb-1 cells were transfected with CFP-W-Rod and/or YFP-M-Rod. For coimmunoprecipitation Rb-1 cells were transfected with CFP-W-Rod and W-Rod, CFP-W-Rod and M-Rod or YFP-M-Rod and M-Rod, separately. Rb-1 cells were transfected by incubation for 24 h with 'rod-inserted' expression vectors and then were plated in four-well Lab-Tek (ATGC) plates; 48 h after transfection, these Rb-1 cells were fixed for immunofluorescence studies.

Protein extraction. Pathological tissues were obtained from the affected individuals (French family, subject III-12: ascending aortic aneurysm and III-23: type A aortic dissection) during surgery. The control normal aortic sample was obtained from a healthy subject. The intima and adventitia of the aortic samples were removed. The aortic media was snap frozen in liquid nitrogen and then crushed and homogenized in extraction buffer (40 mM pyrophosphate, 1 mM MgCl₂, 1 mM EGTA, 1% protease inhibitor cocktail (Sigma), pH 9.5) using a Polytron. The homogenate was centrifuged at 11,000g (Kontron TS155.5) for 15 min at 4 °C. The recombinant proteins were extracted from CHO or Rb-1 cells 48 h after transfection. They were also extracted in 1% Triton X-100 in PBS (phosphate-buffered saline), pH 7.4. Protein concentration was determined by the Bradford method²⁵.

Immunoblotting. The extracted total aortic proteins were subjected to 5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously²⁶. Recombinant SM2 proteins (wild-type and mutant) were used as internal molecular weight standard. Proteins were transferred from the SDS-PAGE gel to polyvinylidene fluoride (PVDF) membrane (PolyScreen, PerkinElmer). Blots were incubated with monoclonal mouse anti-human SM-MHC (1:1,000, Dako) overnight at 4 °C, followed by incubation with peroxidase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch) for 1 h at 20 °C. Signals were detected by a light-emitting nonradioactive enhanced chemiluminescence system (Western Lightning), and blots were developed using Kodak X-Omat AR film.

Immunoprecipitation. Sheep anti-rabbit IgG M-280-coated Dynabeads (Dyna) were used for immunoprecipitation. Anti-GFP (Torrey Pines Biolabs) antibody was first incubated with the beads for 5 h at 4 °C with gentle shaking. Cells lysates containing the recombinant proteins (CFP-W-Rod and W-Rod, CFP-W-Rod and M-Rod, and YFP-M-Rod and M-Rod) were added to the beads in PBS at a pH of 6.5 (ref. 27). The mixture was incubated overnight at 4 °C with gentle rotation. The supernatants were collected and the beads were washed in PBS pH 6.5, resuspended in sample buffer and boiled. The recovered proteins and the supernatants were separated by SDS-PAGE on 5% acrylamide gels and were immunoblotted using anti-SM-MHC (Dako).

Immunofluorescence. Rb-1 cells were washed with PBS, pH 7.4, 48 h after transfection. They were then fixed and permeabilized by incubation with 4% paraformaldehyde (PFA) and 0.1% Triton X-100. The cells were rinsed and saturated by incubation with PBS-10% normal goat serum (NGS) for 30 min at 20 °C. They were then incubated with a 1:50 dilution of anti-SM-MHC (Dako) in PBS-1% NGS for 1 h at 20 °C. The cells were incubated with a 1:40 dilution

of Alexa 647-conjugated polyclonal anti-mouse (Molecular Probes) for 40 min at 20 °C. The cells were washed and mounted in Mowiol (Sigma-Aldrich). The distribution of wild-type and mutant SM-MHC rod regions in Rb-1 cells was analyzed with a Leica TCS SP II (Leica Microsystems) confocal laser scanning microscope equipped with an argon-krypton laser and configured with a Leica DM IRBE inverted microscope. Triple fluorescence for the cyan, yellow and ultrared channels was imaged sequentially with excitation of the argon-krypton-neon laser at 435, 514 and 647 nm. Images (1024 × 1024 pixels) were obtained with a 63× magnification oil-immersion objective.

Coiled-coil region prediction. The COILS program²⁸ was used to predict the probability of coiled-coil formation in the wild-type and mutant SM-MHC rod region. The sequences of the wild-type (from residue 843) and mutant SM2 isoforms, detected in the French and American kindreds, were entered under the following configurations: matrix MTK, window width 21, weighted.

Determination of aortic parameters. Aortic compliance and pulse wave velocity were studied in 49 subjects of the French kindred including 16 mutation carriers and 33 normal controls. For the aortic compliance study, cine magnetic resonance imaging (MRI) of the bifurcation of the pulmonary trunk was carried out in the transverse plane, using automatic detection of the contour of the aorta over the whole cardiac cycle, as previously described²⁹. For the pulse wave velocity study, the aorta was imaged in the transverse plane at the same site. The data were acquired using an ECG-gated gradient pulse sequence with a velocity-encoding gradient. Image analysis was performed with software designed in our laboratory. Contours of ascending and descending aortas were manually drawn on each velocity-encoded image, providing velocity profiles over time. The two velocity profiles were matched according to a mathematical transformation based on two parameters: a translation corresponding to a time difference and a normalization factor. Contiguous images were acquired with a conventional ECG-gated spin echo sequence. The slices covered the whole aortic arch in the transverse plane from the aortic root to the carotid arteries. A point was indicated by an experienced user at the center of the aorta on each slice. The sum of the lengths of the segments defined by the center points provides the distance between the ascending aorta at the pulmonary trunk and the descending aorta at the same point. Pulse wave velocity was calculated as the ratio of this distance and the difference in the time of arrival of the pulse wave at these levels. Dimensions at the aortic root were measured by transthoracic echocardiography².

Statistics. Quantitative values are expressed as mean ± s.d. Groups were compared by means of nonparametric tests, using Systat 7.0 software (SPSS).

Accession codes. GenBank: *Homo sapiens* chromosome 16, complete sequence, NC_000016.8 (GI:51511732); *Homo sapiens* myosin, heavy polypeptide 11, smooth muscle (MYH11), NM_002474 (transcript variant SM1), NM_022870 (transcript variant SM2); *Homo sapiens* smooth muscle myosin heavy chain 11 isoform SM1, NP_002465; *Homo sapiens* smooth muscle myosin heavy chain 11 isoform SM2, NP_074035.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. WO2006061690 - DIAGNOSIS OF ARTERIAL DISEASES BY IDENTIFICATION OF A MUTATION IN THE MYH11 GENE OR PROTEIN

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Title

[EN] DIAGNOSIS OF ARTERIAL DISEASES BY IDENTIFICATION OF A MUTATION IN THE MYH11 GENE OR PROTEIN

[FR] DIAGNOSTIC DE MALADIES ARTERIELLES PAR IDENTIFICATION D'UNE MUTATION DANS LE GENE OU LA PROTEINE MYH11

Abstract

[EN]

The invention relates to an ex vivo method of diagnosing or predicting an arterial disease, or a risk of arterial disease, in a subject, which method comprises detecting a mutation in the MYH11 gene or protein (myosin heavy polypeptide 11 of smooth muscle cells), wherein said mutation is indicative of an arterial disease or of a risk of arterial disease.

[FR]

La présente invention se rapporte à une méthode *ex vivo* permettant de diagnostiquer ou de prédire une maladie artérielle ou un risque de maladie artérielle, chez un sujet, ladite méthode consistant à détecter une mutation du gène ou de la protéine MYH11 (polypeptide 11 lourd de la myosine des cellules des muscles lisses), ladite mutation indiquant une maladie artérielle ou un risque de maladie artérielle.

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Genetics

Mapping of Familial Thoracic Aortic Aneurysm/Dissection With Patent Ductus Arteriosus to 16p12.2–p13.13

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Background—Three loci have been shown to be responsible for nonsyndromic familial thoracic aortic aneurysms (TAAs) and aortic dissections (ADs). We recently described a large family in which TAA/AD associates with patent ductus arteriosus (PDA) and provided genetic arguments for a unique pathophysiological entity.

Methods and Results—Genome-wide scan was performed in 40 subjects belonging to 3 generations in this large pedigree. Using the 7 TAA/AD cases as affected, we observed positive 2-point LOD scores on adjacent markers at chromosome 16p, with a maximum LOD score value of 2.73 at $\theta=0$, a value that increased to 3.56 when 5 PDA cases were included. Multipoint linkage analysis yielded a maximum LOD score of 4.14 in the vicinity of marker *D16S3103*. Fine mapping allowed the observation of recombinant haplotypes that delimited a critical 20-cM interval at 16p12.2–p13.13. Automatic determination of aortic compliance with cine MRI showed that all subjects bearing the disease haplotype, even asymptomatic, displayed a very low level of aortic compliance and distensibility. Aortic stiffness was strongly associated with disease haplotype with a marked effect of age, indicating subclinical and early manifestation of the disease.

Conclusions—Genetic analysis of this family identified a unique locus responsible for both TAA/AD and PDA at chromosome 16p12.2–p13.13 with aortic stiffness as an early hallmark of the disease. TAA/AD with PDA is a new monogenic entity among the genetically heterogeneous group of TAA/AD disease. (*Circulation*. 2005;112:200–206.)

Key Words: aneurysm ■ aorta ■ ductus arteriosus ■ genetics ■ mapping

Among thoracic aortic aneurysm (TAA) and/or aortic dissection (AD) cases, 15% to 20% may be familial.¹ Familial clustering of TAA/AD, however, is complex and heterogeneous. In addition to the classic mendelian connective tissue disorders such as Marfan or Ehlers-Danlos vascular-type syndromes,^{2–4} several loci have been associated with nonsyndromic familial TAA/AD, namely TAAD1, TAAD2, and FAA1, which map to 5q13–q14, 3p24–p25, and 11q23.2–q24, respectively.^{5–7} Identification of the causative genes should lead to new insights into the underlying mechanisms and to the detection of at-risk subjects who could benefit from early specific medical care.

We recently described the large 3-generation French “Bourgogne” family in which TAA/AD, patent ductus arteriosus (PDA), or both occurred and provided genetic arguments for a particular entity not linked to the previously described loci.⁸ To the best of our knowledge, only 1 American and 1 Canadian family have been reported that may also have this apparently rare disorder.^{9,10}

Here, we report on the identification of a single locus at 16p12.1–p13.2 that is responsible for this aortic disease. In addition, we demonstrate its association with a marked aortic stiffness, an abnormality that occurs even in nonsymptomatic subjects bearing the disease haplotype.

Methods

Subjects

The clinical description of the Bourgogne family (Supplemental Figure in the online-only Data Supplement) has been published.⁸ For the genome scan analysis, 40 first-degree nonconsanguineous relatives from 3 generations and 9 unrelated spouses were enrolled in this study after giving written consent form. Among these 40 subjects, 28 were investigated with cine MRI after determination of their aortic compliance and distensibility. Subsequently, 29 nonsymptomatic additional relatives were also investigated for the haplotype status (total, 69 subjects) and 20 for cine MRI measurements (total, 48 subjects). The study was approved by the local ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Bourgogne No. 2000/15, 00/03/16) and the French Ministry of Health.

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Procedures

Genome-Wide Genotyping

Genomic DNA was isolated from peripheral blood obtained from all subjects with classic methods. The whole-genome scan was performed by the Centre National de Génotypage with 382 well-defined highly polymorphic microsatellite markers distributed with an average spacing of 10 cM (ABI Prism Linkage Mapping Set 2, version 2.5, Applied Biosystems).

Linkage Analysis

MLINK from LINKAGE software package (version 5.2) was used for 2-point linkage analysis.¹¹ Classic affected-only-pedigree-members bipoint linkage analyses were performed under a dominant model of inheritance shown by segregation analysis, as described previously.⁸ Subjects were classified according to 2 phenotypes: First, TAA/AD cases only were considered affected, with others classified as unknown status; second, TAA/AD and PDA cases were considered affected, with others classified as unknown. For the first analysis, 7 TAA/AD (2 subjects with both TAA/AD and PDA) from 3 generations were considered. The second linkage analysis also included 5 PDA cases and was only performed for markers belonging to chromosome 16. Multipoint analysis was performed with the GENEHUNTER2.1 package.¹²

Parameters of Linkage Analysis

Allele frequencies for each of the 382 markers were estimated with the FBAT program¹³ from a sample of 70 white French nuclear families (as part of a genome-wide scan for loci predisposing to blood pressure salt sensitivity in hypertensive sibling pairs, Mathieu F. et al, personal data). Allele frequencies were thus compared with those estimated for unrelated subjects on the pedigree (ie, spouses); consistent results were obtained. The phenocopy rate was fixed at 4 per 100 000 for TAA/AD (average incidence in general population).^{14,15} To account for the likely age-dependent penetrance of the disease,¹⁶ asymptomatic subjects were classified into 3 liability age classes, with empirically penetrance levels set as follows: 0% for those <20 years of age; 40% for those between 20 and 50 years of age, and 80% for those >50 years of age for TAA/AD. When including PDA cases in linkage analysis, we defined a supplemental liability class with a phenocopy rate fixed at 1 per 2000¹⁷ and a penetrance rate of 90%.

Refine Mapping and Haplotype Analysis

Sixteen markers within the 16p12–p13 region were used to refine mapping. Ten belonged to the ABI PRISM Linkage Mapping Set version 2.5; the remaining 6 markers (*D16S519*, *D16S3035*, *D16S420*, *D16S3093*, *D16S3022*, *D16S3080*) were selected to be evenly spaced within the critical region (<http://research.marshfield-clinic.org/genetics/>). GeneScan version 3.7 (ABI) and Genotyper version 3.7 (ABI) were used for gel analysis and genotype assignment, respectively.

Analysis of Candidate Genes

Three positional genes that were confined at the disease locus were analyzed: *NOM1* encoding the NODAL modulator 1 protein (NM_014287) that was previously called *PM5*,^{18,19} *ABCC6* encoding ATP-binding cassette subfamily C member 6 (NM_001171.2),^{20,21} and *BFAR* (bifunctional apoptosis regulator) encoding the bifunctional apoptosis regulator (NM_016561).²² Direct sequencing with the Sanger method (BigDye Terminator Cycle Sequencing Kit, Applied Biosystems) was performed to search for mutation. For the *NOM1* gene, the entire cDNA was sequenced after reverse transcription from RNA extracted from cultured skin fibroblasts. For the *ABCC6* and *BFAR* genes, all the exons were sequenced after amplification with polymerase chain reaction from genomic DNA. Intron-based, exon-specific primers were designed according to the genomic sequence (available on request). The Sequencher 4.0.5 program (Gene Codes Corp) was used to identify differences between sequences. For each gene, we analyzed peripheral DNA from 2 affected subjects carrying the disease haplotype (1 with TAA, 1 with PDA). As controls, we used DNA from 1 unaffected

individual of the family not carrying the disease haplotype and from 1 unrelated white normal volunteer.

Automatic Determination of Aortic Compliance With Cine MRI

Aortic compliance was analyzed in 48 subjects of the French Bourgogne kindred (28 subjects among the 40 genotyped for genome-wide linkage analysis and 20 additional relatives). It was measured by MRI with automatic detection of the contour of the aorta over the whole cardiac cycle as previously described.²³ Ascending aorta was imaged in the transverse plane at the level of the pulmonary trunk with a cine MRI sequence (ECG-gated cine FISP sequence) with a repetition time of 15 milliseconds. Forty to 60 images of a single slice were acquired, thereby covering the whole cardiac cycle. Cross-sectional area of the aorta was computed with an automatic contouring method, and a surface-versus-time curve was obtained for each patient. Compliance (C in mm²/mm Hg) and aortic distensibility (D in mm Hg⁻¹) of the ascending aorta were defined as follows: $C = \Delta S / \Delta P$ and $D = \Delta S / (\Delta P \times S_{min})$ respectively, where S is aortic surface (mm²) and P is blood pressure (mm Hg) as described elsewhere.^{23,24}

Statistical Analysis of Aortic Compliance and Distensibility

Values of aortic compliance and aortic distensibility in subjects without the disease haplotype (H⁻), affected subjects with TAA/AD or PDA (H⁺, symptomatic), and asymptomatic carriers of the disease haplotype (H⁺, asymptomatic) were compared by use of a classic ANOVA. Similar significant results were obtained with the nonparametric Mann-Whitney test. Statview statistical software (release 6.0, Abacus Concept) was used for statistical analysis. Data are expressed as mean and SD. A value of $P < 0.05$ was considered significant.

Results

Genome-Wide Screen and Linkage Analysis

Results of the genome-wide linkage scan are indicated in Figure 1. In the first analysis in which only TAA/AD cases were considered affected, LOD scores ≥ 1.5 were detected for several adjacent markers on chromosome 16, with a maximum value of 2.73 at $\theta = 0$ for marker *D16S3068* (the Table). No other suggestive chromosomal region was detected. If the 5 PDA cases are considered affected, stronger values were observed at this region, with a maximum LOD score of 3.56 for marker *D16S3075* (the Table and Figure 1). Multipoint analysis showed a peak LOD score of 4.14 near marker *D16S3103*. Consequently, the linkage analysis provided evidence suggestive of linkage for the TAA/AD only status and significant evidence of linkage when for TAA/AD and PDA status according to the standard thresholds proposed by Lander and Kruglyak.²⁵

When pedigrees ascertained for a disease contain founders with unknown marker phenotypes, misspecification of the allele frequencies of the marker locus might lead to an increased rate of false-positive linkage findings.²⁶ To test for the robustness of our linkage results, linkage analyses were repeated with allele frequencies of 1/n. This resulted in a loss of statistical significance for the 2-point analyses, which no longer established linkage. For the *D16S3075* marker, the LOD score was equal to 1.19 and reached 2.54 when PDA cases were included. The multipoint analysis, however, continued to detect significant linkage, with a maximal LOD score of 3.88.

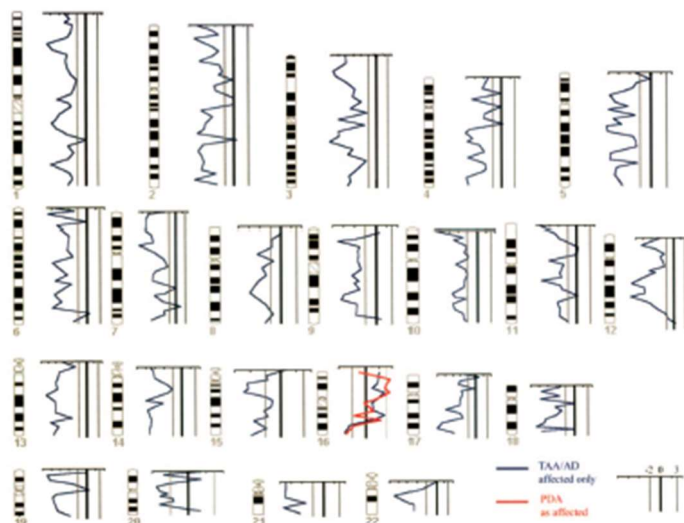


Figure 1. Results of genome-wide scan in Bourgogne family. Blue curves correspond to results of bipoint linkage analysis obtained with TAA/AD affected-only model. Red curves correspond to results obtained TAA/AD and PDA subjects are considered affected. Only in 16p region were significant LOD scores obtained.

Fine Mapping and Haplotype Analysis

Sixteen microsatellite markers allowed the observation of 2 recombinant haplotypes that delimited a critical interval to a 20-cM region (sex-averaged) bordered by the centromeric marker *D16S519* and the telomeric marker *D16S403* (<http://research.marshfieldclinic.org/genetics/>). This recombinant region was shared by all the 12 subjects with TAA/AD or PDA. Among them, 2 other crossovers (subjects III-1 and IV-5) were observed close to each boundary of the critical interval. Thus, a unique genetic locus was responsible for both TAA/AD and PDA (Figure 2 and online-only Data Supplement Figure).

Among the remaining 57 subjects of this large family, haplotype analysis led us to identify 9 carriers of the disease haplotype without TAA/AD or PDA, 33 noncarriers, and 15 subjects with a recombinant haplotype. Consequently, the overall observed penetrance of TAA/AD or PDA disease could be estimated at 57%. If TAA/AD and PDA were separated, the observed penetrance for the TAA/AD trait could be estimated to be 0% at age <20 years (0 affected, 7

asymptomatic), 43% at age between 20 and 50 years (3 subjects affected, 5 asymptomatic), and 66% after 50 years of age (4 affected, 2 asymptomatic). For PDA, the observed penetrance of 33% (7 PDAs for a total of 21 carriers) has to be taken even more cautiously because most of the children of the family have not been systematically explored and because PDA can be transient and asymptomatic.

Automatic Determination of Aortic Compliance With Cine MRI

We hypothesized that asymptomatic subjects bearing the disease haplotype could display abnormalities in aortic compliance or distensibility. Indeed, aortic MRI performed in the first subjects showed a strong decrease in the amplitude of variation of the aortic cross-sectional area during a cardiac cycle (Figure 3a). When all individuals were compared according to their phenotypic and haplotypic status, a marked reduction in aortic compliance and distensibility was observed in all haplotype-positive individuals, both symptomatic and asymptomatic (Figure 3b

Bipoint Linkage Analysis at Chromosome 16

Marker	Position From pter (Marshfield), cM	TAA/AD Only-Affected 2-Point LOD Score at θ of					TAA/AD and PDA-Affected 2-Point LOD Score at θ of				
		0.00	0.05	0.1	0.2	0.3	0.00	0.05	0.1	0.2	0.3
<i>D16S423</i>	10.36	2.10	1.91	1.70	1.24	0.73	-5.23	1.16	1.44	1.34	0.93
<i>D16S404</i>	18.07	1.76	1.61	1.44	1.07	0.64	0.22	1.88	1.92	1.61	1.08
<i>D16S3075</i>	23.28	1.88	1.72	1.56	1.20	0.78	3.56	3.29	3.00	2.35	1.59
<i>D16S3103</i>	32.07	1.70	1.53	1.35	0.95	0.53	2.57	2.36	2.13	1.61	1.00
<i>D16S3046</i>	40.65	0.98	0.90	0.82	0.63	0.40	2.11	1.96	1.79	1.41	0.95
<i>D16S3068</i>	48.53	2.73	2.49	2.23	1.67	1.03	3.49	3.20	2.89	2.21	1.43
<i>D16S3136</i>	62.11	1.63	1.46	1.29	0.91	0.51	-0.87	0.89	0.98	0.83	0.51
<i>D16S415</i>	67.62	0.98	0.91	0.83	0.64	0.40	-3.79	-0.07	0.37	0.59	0.49

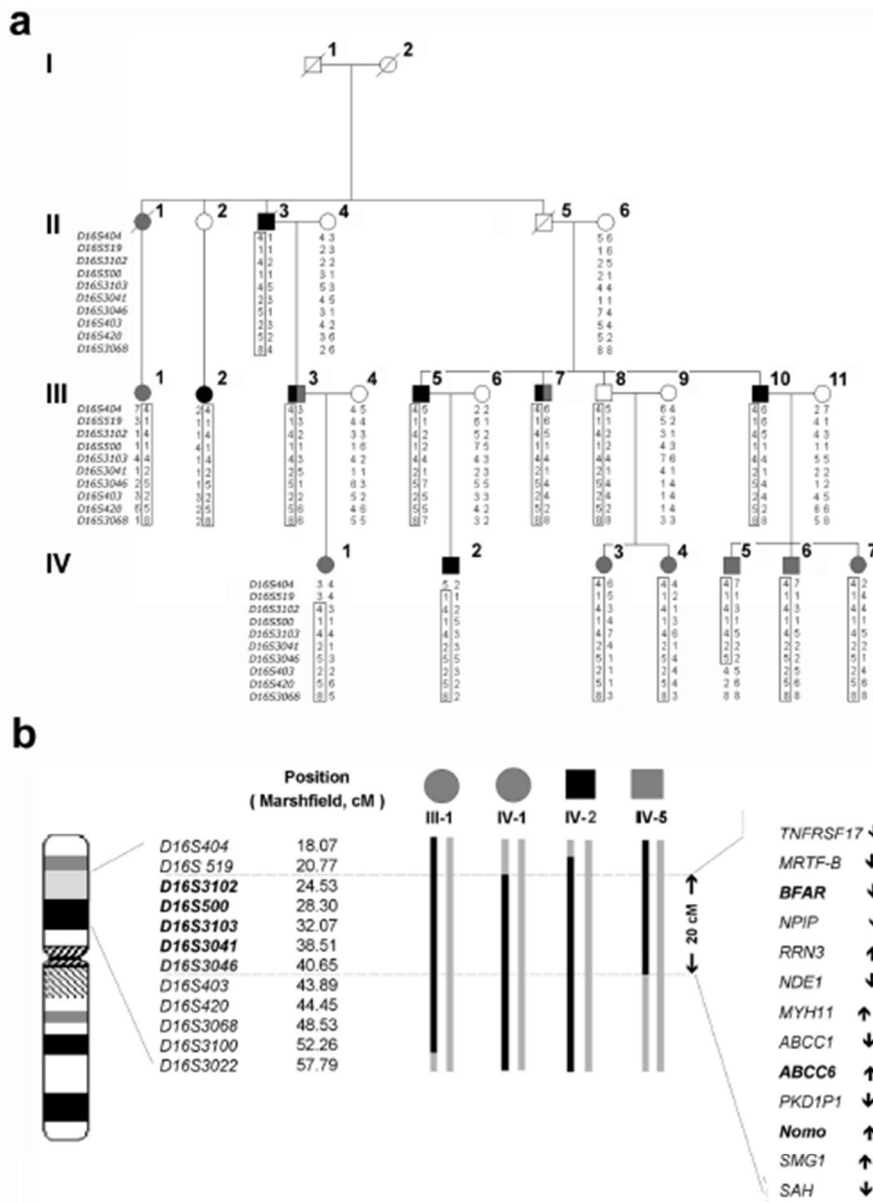


Figure 2. Haplotype analysis at disease locus. Top, Simplified pedigree of Bourgogne family. For simplicity, only data concerning symptomatic TAA/AD (black solid symbols) or PDA (gray solid symbols) subjects are represented. Genotypes for TAA/AD and PDA locus microsatellites are shown below. TAA/AD with PDA disease haplotype is boxed. Bottom, Critical TAA/AD or PDA interval. It is defined by 2 critical recombination events (subjects IV-1 and IV-5). Critical interval is closely surrounded by 2 other crossovers (subjects III-1 and IV-5).

and 3c). A marked effect of age was present in subjects with normal haplotype but not in the group bearing the disease haplotype. The effect of the genotype was particularly strong in young adult subjects (Figure 3d through

3f). In individuals <35 years of age (symptomatic or not), aortic compliance values did not overlap between carriers (n=7; range, 0.43 to 1.28 mm²/mm Hg; mean, 0.82 mm²/mm Hg) and noncarriers (n=19; range, 1.32 to 2.96 mm²/

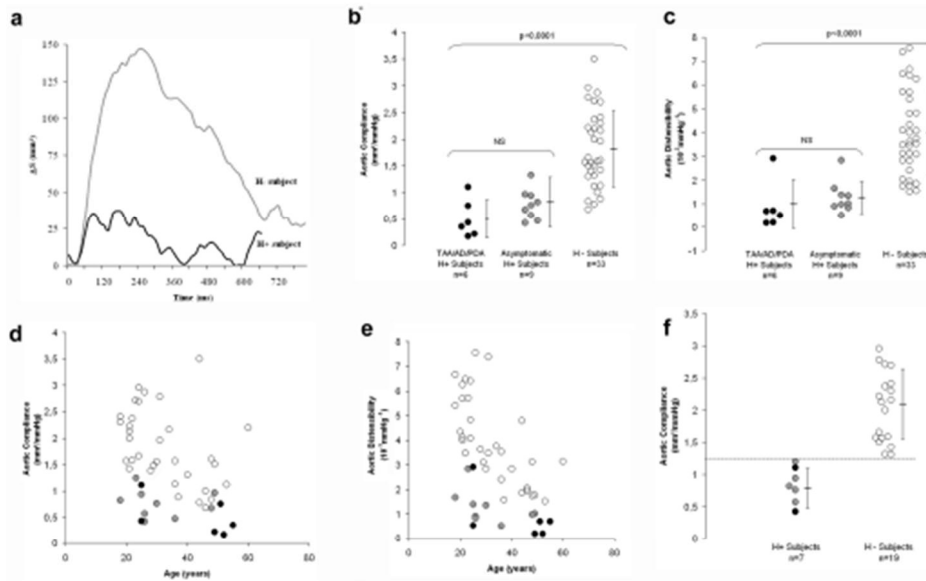


Figure 3. Genotype-phenotype relationships. a, Changes in cross-sectional area (ΔS) of ascending aorta during cardiac cycle. Gray line corresponds to typical result obtained in wild-type subject (H⁻); black line, that obtained in subject without TAA/AD or PDA but who exhibits disease haplotype (H⁺). H⁺ subject had markedly reduced systolic dilatation of aorta ($\Delta S=29$ vs 148 mm² for subject H⁻) and subsequent differences in aortic compliance (0.45 vs 2.96 mm²/mm Hg, respectively). Both subjects were similar for variables known to influence aortic compliance: age (25 versus 24 years), body surface area (1.72 versus 1.74 m²), blood pressure ($123/59$ versus $116/66$ mm Hg), absence of tobacco exposure, dyslipidemia, and diabetes mellitus. b, c, Aortic compliance (b) or distensibility (c) values in subjects without disease haplotype (H⁻; open symbols), affected subjects with TAA/AD or PDA (H⁺, symptomatic; solid symbols), and asymptomatic carriers of disease haplotype (H⁺, asymptomatic; gray symbols). For each group, means and SDs are indicated to right of individual values. Groups did not differ in sex ratio, age, and blood pressure. d-f, Marked effect of age with decreased of aortic compliance (d) or distensibility (e) was observed in subjects without disease haplotype (H⁻ subjects; open symbols). This was not the case in subjects harboring disease haplotype (H⁺, symptomatic (TAA/AD and/or PDA; solid symbols) or not (gray symbols). In this last group, subjects <35 years of age (f) had such marked decrease in aortic compliance that values did not overlap between carriers ($n=7$; range, 0.43 to 1.28 mm²/mm Hg; mean, 0.82 mm²/mm Hg) and noncarriers ($n=19$; range, 1.32 to 2.96 mm²/mm Hg; mean, 2.10 mm²/mm Hg) of disease haplotype. Limit of 1.3 (dotted line) seems to differentiate carriers and noncarriers of haplotype susceptibility.

mm Hg; mean, 2.10 mm²/mm Hg) of the disease haplotype. Thus, altered aortic compliance and distensibility that result in aortic stiffness were an early hallmark of the disease.

Candidate Gene Analysis

A large number of genes (86 known or predicted genes on Human May 2004 assembly, <http://genome.ucsc.edu/>) are contained within this region. As a first effort to identify the causative gene, we analyzed 3 attractive positional candidates. Negative results were obtained on *NOMO1*, encoding a novel protein that shares DNA homology with conserved regions of the collagenase gene family.¹⁹ Several single nucleotide polymorphisms (11 silent, 2 coding) were observed on the *NOMO1* cDNA from the 2 affected and the 2 control subjects. Presence of these variants in different parts of the cDNA at the heterozygous state excluded a microdeletion or a nonsense mutation with mRNA decay. The *ABCC6* gene was also an attractive candidate because it causes pseudoxanthoma elasticum, a disease with arterial complications.^{20,21} However, we did not observe any mutation but 3 heterozygous SNPs (c.1864T→C, c.1913C→G,

c.1919C→A) in both affected and unaffected subjects. Finally, no mutation but 1 synonymous SNP (c.1365A→G) was observed on *BFAR*, a gene thought to be at the intersection between the extrinsic (ie, TGF- β receptors) and intrinsic (ie, BCL-2 family protein) pathways for induction of apoptosis,²² a mechanism known to occur during PDA closure²⁷ and Marfan syndrome.²⁸

Discussion

We chose a 2-step approach with first a stringent phenotype (TAA/AD alone) and then a larger one (TAA/AD and/or PDA) as a positive disease status. Initially, positive LOD scores were obtained with several adjacent markers at chromosome 16 using 7 TAA/AD cases on 3 generations. No other suggestive region was observed, in agreement with our previous segregation and candidate gene/loci analysis.⁸ Using the 5 PDA cases as additional affected subjects, we obtained significant positive bipoint and multipoint LOD scores for the same 16p12.2–p13.13 region, which definitely assessed a true linkage.²⁹ Thus, the genetic analysis of this family identified a unique genetic locus responsible for both TAA/AD and PDA, illustrating the interest in deciphering particular men-

delian traits among a more complex group of heterogeneous diseases. The size of the Bourgogne family allowed this finding without genetic heterogeneity.

Until now, TAA/AD with PDA seemed to be a rare recognizable entity, described only in an American family and possibly a Canadian family.^{9,10} In our study, 6 of 11 PDA cases were first discovered by our echo Doppler screening in nonsymptomatic subjects without auscultatory signs.⁸ These results provide argument for a systematic search of PDA when screening aortic abnormalities in familial or apparently sporadic TAA/AD cases. It is interesting to note that in the case of TAA/AD, a periodic imaging of the aortic root is recommended that does not allow identification of PDA.^{30,31} Consequently, the proportion of this recognizable entity among the genetically heterogeneous familial TAA/AD may be underestimated. Personal or family history of either TAA/AD or PDA should indicate the possibility of this particular entity and consequently suggest an appropriate screening in relatives.

The identification of the locus involved in the Bourgogne family gave the possibility of an individual indirect genetic test. Whereas 33 of the 69 subjects did not carry the disease haplotype, 9 asymptomatic subjects carried the at-risk haplotype. Investigation performed after written informed consent to our research protocol showed the absence of PDA or TAA in these individuals. To obtain further insight into the possibility of subclinical vascular abnormalities, aortic compliance was measured in 48 individuals (42 asymptomatic, 6 TAA/PDA) with MRI, a very precise, noninvasive technique.²³ Our results show that aortic stiffness is an early hallmark of the disease, occurring in all subjects harboring the disease haplotype compared with their normal relatives.

The severity of the disease in some members of the family,⁸ the possibility of an indirect genetic diagnosis in relatives, and the strong decrease in aortic distensibility in positive subjects argue for the indication of a lifelong β -blockade therapy to prevent TAA/AD in that particular disease. This treatment is now recommended for prevention of aortic dissection in inherited diseases such as Marfan syndrome, Ehlers-Danlos syndrome, and annuloaortic ectasia.³¹ It is interesting to note that aortic stiffness has been shown to be an early and independent predictor of TAA/AD development in Marfan disease^{24,32} and is reduced by β -blockade therapy. MRI is well suited to detect these changes.³³ In that regard, noninvasive measurement of aortic stiffness derivatives such as aortic compliance and distensibility should help to assess and monitor the individual risk of aortic dilatation and rupture.²⁴

An accurate genetic test will need identification of the disease gene. Until now, we restricted the locus to a 20-cM interval, defined proximally by marker *D16S519* and distally by marker *D16S403*. Among the 15 subjects harboring a recombinant haplotype, only 4 were investigated with cine MRI to allow determination of their aortic compliance or distensibility. Among them, a 30-year-old woman had a normal aortic compliance. Taking a complete penetrance of the disease on this biomarker would define the interval more distally by marker *D16S3041* and thus restrict it to 18 cM. Values obtained for the 3 other individuals were either

intermediate or difficult to interpret because of age and associated diseases and treatment. It is therefore difficult to restrict the locus on the sole assumption of a complete penetrance of the disease gene on aortic compliance and on the value observed in 1 individual. MRI investigation in other individuals with recombinant haplotypes should help to refine mapping and to identify the causative gene.

In conclusion, identification of TAA/AD causative genetic defects should open avenues to an understanding of the underlying mechanisms thought to be complex and heterogeneous. Recent advances in Marfan disease have brought novel arguments for a targeted treatment of the disease.²⁹ Among the heterogeneous inherited aortic diseases, we provide here arguments for a recognizable entity associating aortic stiffness, TAA/AD, and PDA, which maps to 16p12.2–p13.13. Further analysis may identify its genetic basis and might contribute to promote new strategies for clinical management and treatment of thoracic aortic diseases.

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ARTICLE

Familial thoracic aortic aneurysm/dissection with patent ductus arteriosus: genetic arguments for a particular pathophysiological entity

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Thoracic aortic aneurysm and aortic dissection (TAA and AD) are an important cause of sudden death. Familial cases could account for 20% of all cases. A genetic heterogeneity with two identified genes (*FBN1* and *COL3A1*) and three loci (3p24–25 or *MFS2/TAAD2*, 5q13–q14 and 11q23.2–24) has been shown previously. Study of a single family composed of 179 members with an abnormally high occurrence of TAA/AD disease. A total of 40 subjects from three generations were investigated. In addition to five cases of stroke and three cases of sudden death, there were four cases of AD and four cases of TAA in adults. In all, 11 cases of patent ductus arteriosus (PDA) were observed, two of which were associated with TAA and one with AD. Segregation analysis showed that the distribution of these vascular abnormalities was more likely compatible with a single genetic defect with an autosomal dominant pattern of inheritance. There were no clinical signs of Marfan, Ehlers–Danlos vascular type or Char syndromes. Genetic linkage analysis was performed for seven genes or loci implicated in familial TAA/AD disease (*COL3A1*, *FBN1*, 3p24–25 or *MFS2/TAAD2*, 5q13–q14 and 11q23.2–q24), Char syndrome (*TFAP2B*) or autosomal recessive PDA (12q24). Using different genetic models, linkage with these seven loci was excluded. Familial TAA/AD with PDA is likely to be a particular heritable vascular disorder, with an as yet undiscovered Mendelian genetic basis.

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Keywords: genetics; linkage; aorta; aneurysm; dissection; ductus arteriosus

Introduction

Thoracic aortic aneurysm (TAA) and/or aortic dissection (AD) (MIM 132900) are an important cause of sudden

death.^{1–2} A retrospective study showed that they may account for 20% of all TAA and AD cases, and that several genes and several modes of inheritance are likely to be involved in this heterogeneous phenotypic entity.³ TAA/AD is a common manifestation in Marfan syndrome (MFS) and less usual in Ehlers–Danlos syndrome (EDS) vascular type.⁴ To date, two genes have been identified: *COL3A1* (type III procollagen)^{5,6} and *FBN1* (fibrillin-1)^{7,8} Two loci for nonsyndromic familial TAA/AD have been mapped to 5q13–q14 and 11q23.2–24 and called TAAD1⁹ and FAA1,

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respectively.¹⁰ More recently, another locus for nonsyndromic familial TAA/AD was mapped to 3p24–25 and termed TAAD2.¹¹ It overlaps a previously mapped second locus for MFS (*MFS2*).¹²

Another particular vascular syndrome that associates TAA/AD and patent ductus arteriosus (PDA) has been suggested in a single family.¹³ We report here the study of a large three-generation French family with further evidence for this peculiar inherited pathophysiological entity transmitted with an autosomal dominant mode of inheritance. Genetic linkage analysis excludes the responsibility of the previously described loci in syndromic and nonsyndromic familial TAA/AD, Char syndrome and recessive PDA.

Subjects and methods

Subjects

This family was identified from a proband who underwent genetic counseling following the sudden death of his pregnant sister caused by an acute AD and because of the abnormal repetition of serious vascular events in his family. A total of 40 first-degree relatives from three generations were enrolled in the University Hospital of Dijon (France) for a clinical investigation protocol composed of two parts:

- a medical questionnaire involving available medical records, state of health and family history; a standardized clinical and ophthalmologic examination focused on classic signs of connective tissue disorders.
- a complete cardio-vascular examination, a transthoracic echocardiography and a thoracic MRI were carried out. Standard biochemical blood measurements with determination of fasting total and LDL–HDL cholesterol, triglycerides and glucose were also monitored. DNA from peripheral blood samples was obtained for all the 40 relatives and nine unrelated spouses.

A written consent form was obtained from all the subjects enrolled in this study – both approved of by the local ethic committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Bourgogne n° 2000/15; 00/03/16) and the French Ministry of Health.

Methods

Transthoracic echocardiography, thoracic MRI and transesophageal echocardiography

We used the well-defined method described by Roman *et al.*¹⁴ for the transthoracic echocardiography measurements of the aortic root. Thoracic MRI was performed with a 1.5 T magnetic resonance whole-body imager. Gated spin-echo T1-weighted and cineMRI sequences were obtained in multiple oblique planes parallel and perpendicular to the

aortic longitudinal axis. The aortic diameters were measured in the best plane at each level of the thoracic aorta (aortic sinuses, sinotubular junction, ascending aorta, horizontal aorta, isthmus and descending aorta). We considered the limit values given by Higgins.¹⁵ Transesophageal echocardiography were carried out only when previous examinations were inconclusive.¹⁶ A positive status for TAA was retained when echocardiography and MRI measurements were concordant and exceeded the above-mentioned limit values. Discordant measurements were defined when the difference exceeded 2 mm between the two methods (unknown status).

Segregation analysis

Segregation analysis was performed using regressive models for binary traits based on logistic regression proposed by Bonney.¹⁷ Maximum likelihood was maximized under several models using the computer program REGRESS.¹⁸ When they nested, the restrictive model was tested *versus* the more general one by using the likelihood ratio test.¹⁹ In the non-nested case, we used the Akaike information criterion (AIC) defined by: $-2 \ln(\text{Likelihood}) + 2p$, where p is the number of estimated parameters.

DNA analysis and polymerase chain reaction

Genomic DNA was harvested from peripheral lymphocytes using standard procedures. Polymorphic markers were amplified using previously described conditions.²⁰ The type and position of the markers are given in Table 2.

Linkage analysis

Classical affected-only genetic linkage analysis of seven candidate loci was performed under a dominant model of inheritance. Two analyses were performed: in one case, only TAA and/or AD affected subjects were considered and in the other, PDA cases were included with the TAA/AD cases as affected subjects.

For the TAA/AD status, patients with AD (III:5, IV:7, IV:20, IV:22) and those with a TAA established with concordant echocardiography and MRI (IV:9, IV:11, IV:13, V:13) were considered to be affected. Others were considered to be of unknown status.

For the PDA status, patients with symptomatic or asymptomatic PDA discovered by screening (Table 1 and Figure 1) were considered to be affected. Patients without PDA demonstrated by echo-Doppler (IV:21, V:1, V:2, V:7, V:8, V:19) were considered to be unaffected. As there was no systematic screening for PDA in the family, the status of other individuals was considered to be unknown.

The phenocopy rate was fixed at 4/100 000 for TAA/AD (average incidence in general population).^{1,2} Empirically, penetrance levels for carrier subjects were set at: 0% < 20 years old; 40% between 20 and 50 years old and 80% > 50 years old for TAA/AD, to take into account the likely age-dependent penetrance of the disease.²¹ When

Table 1 Cardiovascular characterization of the 'Bourgogne' family

Subject	Sex	Year of birth	Height (cm)	Weight (kg)	Blood pressure (mmHg)	Body surface area (m ²)	Clinical data – medical history	Indexed ζ with BSA of aortic sinuses (cm/m ²) / absolute ζ of ascending aorta (mm) with TTE	ζ of aortic sinuses/ascending aorta/horizontal aorta/aethmus (mm) Spin echo T1 weighted MRI
II:1	F	—	—	—	—	—	SD at 20 years old	—	—
II:2	M	—	<175	—	—	—	—	—	—
III:1	F	1917	—	—	—	—	Died from stroke at 65 years old	—	—
III:3	F	1920	149	—	—	—	PDA treated in the childhood. Died from a breast cancer at 57 years old	—	—
III:5	M	1923	172	67	130/70	1.79	Type III AD treated in 1976	—	—
III:7	M	1925	<175	—	—	—	Died in 1978 (53 years old) from stroke	—	—
III:9	F	1927	<160	—	—	—	Died in 1992 following the surgery of a right intracranial carotid aneurysm	—	—
III:11	F	1929	<160	—	—	—	Died from stroke at 66 years old	—	—
IV:1	M	1939	<175	—	—	—	SD during a great effort at 49 years old, violent thoracic pain (no autopsy)	—	—
V:3	F	1946	164	73	110/60	1.79	PDA treated at 10 years old	2/30	35/28/23/20
V:5	F	1955	156	51	115/70	1.48	Borderline measurements	2.1/28	28/22/20/19
V:7	F	1948	155	—	140/70	—	Chronicle type III AD	—	—
V:9	M	1948	174	64	130/70	1.76	PDA and ascending TAA detected by this study without AI	2.2/34	40/34/25/32
V:10	F	1947	<160	—	—	—	SD at 42 years old at the time of an effort (no autopsy)	—	—
V:11	M	1949	172	68	95/60	1.8	TAA detected by this study without AI	2.4/35	50/36/28/26
V:13	M	1951	166	70	120/80	1.79	TAA without AI, PDA spontaneously closed (27 years old)	2.2/39	43/40/29/24
V:19	F	1956	150	47	130/70	1.39	Discordant aortic measurements	2.2/27	27/21/14/20
V:20	F	1959	<165	—	—	—	PDA treated at 9 years old Died from an acute type III AD at 8 month of gestation	—	—
V:21	M	1964	171	74	120/70	1.86	Borderline/discordant measurements	2.1/38	38/38/24/20
V:22	M	1969	173	65	120/70	1.78	Ascending dissecting aneurysm type II (Figure 2a and b)	3.5/60	65/60/32/30
V:26	F	1956	—	—	—	—	Died in 1977 (21 years old) from a left intracranial carotid dissection	—	—
V:27	F	1964	160	57	130/70	1.58	Borderline/discordant measurements	2.1/34	35/29/20/18
V:28	F	1966	152	47	120/70	1.43	Borderline/discordant measurements	2.3/33	32/28/18/17
V:4	M	1971	155	100	120/60	1.92	Borderline/discordant measurements	1.8/36	38/35/24/24
V:6	M	1979	174	68	130/75	1.81	Borderline/discordant measurements	2.1/32	37/30/18/18
V:9	F	1975	160	47	120/60	1.46	PDA treated at 2 years old	1.7/26	34/28/20/22
V:13	M	1975	179	73	120/60	1.91	TAA without AI	2.2/39	41/39/24/28
V:15	M	1974	176	68	110/60	1.83	Borderline/discordant measurements	2.1/35	38/36/28/25
V:21	M	1992	—	—	—	—	PDA treated at 1 year old	—	—
V:22	M	1994	110	17	95/53	0.75	PDA detect by this study and treated at 6 years old	2.66	—
V:23	F	1997	—	—	—	—	PDA detect by this study and treated at 3 years old (Figure 2c)	—	—

TAA, thoracic aortic aneurysm; AD, aortic dissection; PDA, patent ductus arteriosus; SD, sudden death; —, not known or not performed; TTE, transthoracic echocardiography; AI, aortic insufficiency.

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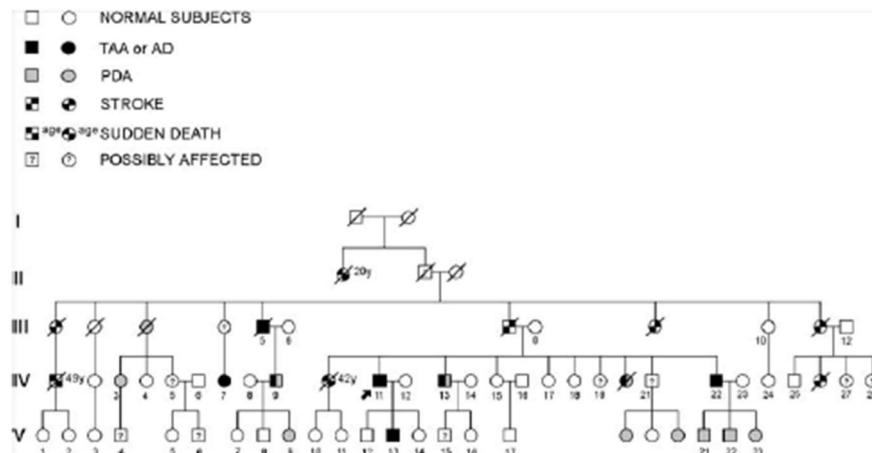


Figure 1 The 'Bourgogne' family tree affected by TAA/AD and PDA. To simplify the figure, only subjects enrolled in this study have been numbered.

including PDA cases in linkage analysis, we defined a supplemental liability class with a phenocopy rate fixed at $1/2000^{22}$ and a penetrance rate of 90%. The linkage analyses were performed using the M-LINK program implemented in the Linkage Package.²³

Results

Characterization of the pedigree (Figure 1)

There were eight cases of TAA ($n=4$) and AD ($n=4$) in three generations, compatible with an autosomal dominant pattern of inheritance. The main characteristics and medical history of the affected subjects are indicated in Table 1 and Figure 2. During the screening, four asymptomatic TAA were diagnosed (IV:9, IV:11, IV:13 and V:13). All corresponded to a dilatation of the aortic root with measurements in excess of 2.1cm/m^2 (aortic sinuses) at echocardiography and 40 mm (aortic sinuses) at MRI. In all these individuals, a normal tricuspid aortic valve was observed. In addition to the TAA reported above, a large number of vascular events occurred in this family (Figure 1): five cases of stroke, two of which occurred due to a documented intracranial carotid aneurysm (III:9 and IV:26), and three cases of unexplained sudden death (II:1, IV:1, IV:10), two of which occurred during an unusually violent effort.

Among the subjects investigated, we found 11 cases of PDA (Table 1). None had a history of neonatal problems. Subject IV:20 who died from AD was surgically treated for PDA. All the PDA cases seem to cosegregate in an autosomal dominant pattern of inheritance with cases of TAA/AD (Figure 1).

It is important to note that for all 40 subjects examined, except aortic abnormalities, there were neither signs of MFS, EDS vascular type and Char syndrome,²⁴ nor valve abnormalities. There was no family history of arterial hypertension or dyslipidemia. Histological examinations of the aorta were performed postoperatively for subjects III:5, IV:11 and IV:22. Lesions consisted of medial degeneration with disruption of the medial elastic fibers, smooth muscle disorganization with deposits of mucopolysaccharide-like material. These abnormalities favor the hypothesis of an intrinsic vascular abnormality.

Segregation analysis

Segregation analysis was performed using the computer program REGRESS¹⁸ that allows to estimate the genetic parameters of inheritance (q : the disease allele frequency and α_{AA} , α_{Aa} , α_{aa} : the genotype-specific baseline parameters). The sporadic model was rejected against the codominant one ($\chi^2(3\text{ df})=9.47$; $P=0.02$). A model of the dominant pattern of inheritance was significantly better than a codominant one ($\chi^2(1\text{ df}=3,7$; $P=0.05$). The recessive model was not rejected against the codominant one ($\chi^2(1\text{ df}=0.05$; $P=0.99$).

The best genetic model was a dominant biallelic locus with an allele disease frequency of 0.03. Thus, the possibility of two distinct genetic defects remains possible but unlikely.

Genetic linkage analysis

Contributions of all the candidate loci were tested using at least two informative microsatellite markers at each locus (Table 2). Using first the affected-only TAA/AD phenotype



Figure 2 TAA/AD in one affected subject and PDA in his daughter. Preoperative MRI of subject IV:22 showing an aortic dissecting aneurysm type II. (a) Gated spin-echo T1 weighted; (b) cineMRI, the arrows show the aortic dissection and aortic valve incompetence. Operative picture of the trans-catheter PDA closure (Amplatz's duct) of the daughter, subject V:23 (c).

(PDA as unknown), two-point linkage analysis excluded linkage ($Z < -2$) for all loci (Table 3). Changing values of penetrance or disease frequency had no effects on the exclusion (data not shown). When PDA-affected status was included, the exclusion values were even stronger. Multi-point linkage analysis confirmed the exclusion of linkage for all the loci tested (data not shown). Consequently, another gene is implicated in this family.

Discussion

The careful analysis of 40 members of one large French family in which cardiovascular events, TAA, AD and PDA occurred at a high frequency provides strong arguments for a particular pathological entity that associates TAA/AD and PDA. Such an entity was suggested by Glancy *et al*¹³ who described a single family where TAA/AD and PDA occur in three generations with an apparent autosomal dominant inheritance. In both Glancy's report and ours, no other

features of typical connective disorders were observed, strongly suggesting a similar genetic defect. Teien *et al*²⁵ also described the presence of TAA/AD and PDA cases within a small family. The analysis of our pedigree further demonstrates the likelihood of a unique gene causing PDA and TAA/AD, and allows the exclusion of the genes causing MFS and EDS vascular-type syndromes and of three loci previously linked to nonsyndromic TAA/AD.⁹⁻¹¹ Histological examinations of aortic samples from three affected members of our family revealed medial degeneration that is known to occur but not specifically, in connective tissue diseases such as MFS and vascular EDS, in association with TAA/AD. Linkage to these loci, as well as linkage to the loci on chromosomes 3,¹¹ 5⁹ and 11 was ruled out.¹⁰ Moreover, DNA sequencing of *COL3A1* cDNA in three affected subjects detected no mutation within the entire coding sequence (data not shown). The exclusion of linkage was even stronger when subjects with PDA were considered to be affected rather than as unknown.

The identification of the genes involved in the pathogenesis of TAA/AD disease can be seriously hampered by: (1) the late onset of the disease; (2) the high mortality with a high incidence of sudden death; (3) the incomplete and age-dependent penetrance; (4) the difficulties inherent to the echo-Doppler and MRI screening; (5) the likely genetic heterogeneity; and (6) the occurrence of various cardiovascular events within the same family. Conversely, genetic analysis of particular large families such as those studied herein can more easily lead to substantial results. The large number of subjects with PDA ($n=11$) compared to the average incidence of 1/2000 in general population,²² its presence in three subjects with TAA or AD, and its cosegregation with TAA/AD (Figures 1 and 2) and the results of the segregation analysis strongly suggest the presence of a unique autosomal dominant genetic defect. None of the cases observed in this family occurred in the presence of particular neonatal circumstances such as those usually observed in sporadic PDA.^{26,27} Besides, none of the examined subjects exhibited dysmorphism or hand anomalies that may indicate Char syndrome²⁴ or other polymalformative conditions. Gelb *et al*²⁸ described in a unique family a new heart-hand syndrome resembling Char syndrome with mild dysmorphism, hand anomalies, PDA, bicuspid aortic valves and aortic root abnormalities. These characteristics were not observed in our family, especially bicuspid aortic valves, known to be an important cause of TAA/AD type I/II.^{29,30} Thus, familial TAA/AD with PDA is likely to be a particular pathophysiological entity. From the analysis of our family, it is difficult to distinguish if TAA, AD and PDA or even stroke with carotid artery aneurysms/dissections aneurysm correspond to some phenotypic heterogeneity or to incomplete penetrance of the same genetic defect. One of the difficulties is due to the possibility of asymptomatic or paucisymptomatic PDA and to a possible underestimation of its presence unless a

**Table 2** Microsatellite markers at the candidate loci and genes

Locus/marker	Nature	Heteroz	Position	Position from p-ter (cM)
<i>COL3A1</i>				
HUMCOL3A1B ³⁴	VNTR	0.73	COL3A1 (Intron 25)	190.0
D2S118 ³⁵	(AC)n	0.79	<0.01 cM/COL3A1	190.0
<i>FBN1</i>				
HUMFBN1-I (mts-1) ³⁶	(AC)n	0.58	Intragenic at 1.1 cM from <i>FBN1</i> locus	38.1
D1S5117 ³⁵	(AC)n	0.78		37.0
<i>MFS2-TAAD2</i>				
D3S2338 ^{1,12}	(AC)n	0.93	Both distant from 10.5 cM, surrounding the <i>MFS2</i> locus	42.1
D3S1266 ^{1,12}	(AC)n	0.79		52.6
<i>TFAP2B</i>				
D6S269 ³⁵	(AC)n	0.71	Both distant from 1.15cM, surrounding <i>TFAP2B</i>	73.1
D6S465 ³⁵	(AC)n	0.61		74.2
<i>5q13-q14-TAAD1</i>				
D5S2029 ⁹⁻³⁵	(AC)n	0.89	All linked to the 5q13-q14 locus	92.4
D5S641 ⁹⁻³⁵	(AC)n	0.85		92.4
MAP1B ³⁷	(AC)n	0.71		92.4
<i>11q23.2-q24-FAA1</i>				
D11S4195 ¹⁰⁻³⁵	(AC)n	0.75	Spaced of 3 cM in the 11q23.2-q24 locus	113.4
D11S924 ^{10,35}	(AC)n	0.79		115.5
D11S4132 ^{10,35}	(AC)n	0.82		116.1
<i>12q24 (with PTPN11):</i>				
D12S78 ^{33,35}	(AC)n	0.86	<i>PTPN11</i>	118.5
D12S86 ^{33,35}	(AC)n	0.93	Spaced of 13.5 cM	140.3
D12S324 ^{33,35}	(AC)n	0.64	Surrounding the 12q24 locus	153.8

Table 3 Results of Two-point linkage analysis at candidate loci and genes

Loci tested	Marker	PDA as unknown two-point LOD score at θ					PDA as affected two-point LOD score at θ				
		0.00	0.10	0.20	0.30	0.40	0.00	0.10	0.20	0.30	0.40
<i>COL3A1</i>	HUMCOL31B	-2.90	-0.42	-0.09	-0.03	-0.04	-2.66	-0.33	0.05	0.12	0.07
	D2S1118	-6.20	-0.78	-0.35	-0.17	-0.08	-6.12	-0.87	-0.44	-0.23	-0.10
<i>FBN1</i>	FBN1-I	-7.38	-1.66	-0.75	-0.30	-0.08	-7.85	-3.25	-1.68	-0.80	-0.28
	D15D117	-0.73	-0.09	-0.15	-0.08	-0.01	-2.41	-1.58	-0.74	-0.38	-0.19
<i>MFS2/TAAD2</i>	D3S2338	-10.71	-1.71	-0.77	-0.34	-0.11	-10.71	-1.26	-0.35	-0.04	-0.03
	D3S1266	-3.90	-0.75	-0.37	-0.16	-0.05	-4.24	-0.38	-0.12	-0.01	-0.03
<i>TAAD1</i> <i>5q13-q14</i>	D5S2029	-9.55	-1.13	-0.46	-0.15	-0.02	-13.47	-2.30	-0.93	-0.32	-0.05
	D5S641	-5.21	-0.65	-0.27	-0.09	-0.04	-9.76	-1.44	-0.63	-0.24	-0.05
	MAPFAM	-4.62	-0.63	-0.27	-0.11	-0.03	-3.84	-0.19	-0.01	-0.01	-0.00
<i>FAA1</i> <i>11q23.2-q24</i>	D11S4195	-4.39	-0.12	0.05	0.08	0.06	-6.25	-0.95	-0.28	-0.05	0.00
	D11S924	0.05	0.06	0.05	0.01	-0.01	-2.63	-0.45	-0.21	-0.13	-0.08
	D11S4132	-4.06	-0.65	-0.30	-0.13	-0.03	-6.11	-2.21	-1.11	-0.55	-0.22
<i>TFAP2B</i>	D6D26	-8.57	-3.40	-1.90	-1.10	-0.41	-10.77	-3.23	-1.76	-0.95	-0.40
	D6S465	-7.66	-1.32	-0.63	-0.30	-0.11	-7.99	-1.33	-0.55	-0.23	-0.09
<i>PTPN11</i> <i>12q24</i>	D12S78	-11.35	-1.66	-0.68	-0.24	-0.04	-9.96	-0.48	0.74	0.57	0.27
	D12S86	-4.98	-0.29	-0.45	-0.35	-0.18	-6.62	-1.54	-0.46	-0.02	0.10
	D12S324	-2.23	-0.37	-0.43	-0.33	-0.77	-0.32	-0.32	0.33	0.49	0.35

systematic investigation is conducted. Indeed, six out of 11 of our PDA cases were discovered by echo-Doppler thanks to our screening. The systematic follow-up of the family members might allow us to evaluate the risk of TAA/AD for offspring with apparently isolated PDA. Identification of the causal gene will help to study its role in the overall PDA and TAA/AD cases. Currently, the only identified causal gene is *TFAP2B*, a transcription factor that is expressed in neural crest cells and responsible for CHAR syndrome.²⁴ The absence of facial abnormalities and other malformations in our family as well as in the ones described by Teien *et al*²⁵ and Glancy *et al*¹³ strongly suggests that another causal gene and genetic linkage analysis ruled out the implication of that locus in our family (Table 3), but not the influence of possible coactivators.³¹ Recently, Mani *et al*³² reported a recessive locus at 12q24 that commonly contributes to PDA. Our genetic linkage analysis assuming a dominant model and a variable penetrance excluded this locus. Incidentally, this genetic analysis also excluded the responsibility of the *PTPN11* gene, whose defects have been implied in the Noonan syndrome in which TAA and PDA might exist.³³

PDA might also be one of the primary signs of neural crest malfunction, even though in that case it is usually accompanied by aortic arch anomalies, as in the DiGeorge syndrome. We thus analyzed the karyotype of subject IV:13 affected by both PDA and AAT using standard fluorescent hybridization *in situ*. No 22q11.2 microdeletion was observed. An alternative mechanism relating PDA and TAA/AD might be a gene expressed in the vascular wall that would affect the physiological changes of this aortic arch artery with advancing gestation, prevent its regression and apoptosis and at the same time favor a long-term fragility of the aortic vascular wall.

In conclusion, familial TAA/AD and PDA association may be a new recognizable entity where subjects with PDA could have a particular risk of TAA/AD. Further genetic analysis should help to elucidate the molecular mechanisms responsible for the TAA/AD/PDA syndrome.

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Missense Mutations of Conserved Glycine Residues in Fibrillin-1 Highlight a Potential Subtype of cb-EGF-like Domains



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ABSTRACT: In six index cases/families referred for Marfan syndrome (MFS) molecular diagnosis, we identified six novel mutations in the *FBN1* gene: c.1753G>C (p.Gly585Arg), c.2456G>A (p.Gly819Glu), c.4981G>A (p.Gly1661Arg), c.5339G>A (p.Gly1780Glu), c.6418G>A (p.Gly2140Arg) and c.6419G>A (p.Gly2140Glu). These variants, predicted to result in Glycine substitutions are located at the third position of a 4 amino acids loop-region of calcium-binding Epidermal Growth Factor-like (cb-EGF) fibrillin-1 domains #5, #9, #24, #25 and #32. Familial segregation studies showing cosegregation with MFS manifestations or *de novo* inheritance in addition to *in silico* analyses (conservation, 3D modeling) suggest evidence for a crucial role of the respective Glycine positions. Extending these analyses to all Glycine residue at position 3 of this 4 residues loop in fibrillin-1 cb-EGF with the UMD predictor tool and alignment of 2038 available related sequences strongly support a steric strain that only allows Glycine or even Alanine residues for domain structure maintenance and for the fibrillin functions. Our data compared with those of the literature strongly suggest the existence of a cb-EGF domain subtype with implications for related diseases. © 2009 Wiley-Liss, Inc.

KEY WORDS: cb-EGF-like domain, Marfan Syndrome, Fibrillin 1 (*FBN1*) gene, fibrillinopathies

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INTRODUCTION

Human fibrillin-1, the protein involved in Marfan syndrome (MFS; MIM#154700) is characterized by a specific modular organization with structural and non-structural functions in the extracellular matrix. The components of this organization consist in repeated cysteine rich structural modules that include: multiple copies of an epidermal growth factor-like (EGF) module of which 43/47 match with the consensus sequence of class I calcium-binding EGF (cb-EGF) (Downing, et al., 1996), TB (or 8-cysteine) or hybrid modules. About 60% of the reported mutations of the *FBNI* gene are missense. More than 75% of them are located in the cb-EGF modules of fibrillin-1 and involve mostly their obligatory residues (in particular, cysteine residues implicated in disulfide bond formation or other residues implicated in calcium-binding and/or intra- intermolecular interactions) (Collod-Beroud, et al., 2003; Downing, et al., 1996). Interpretation of the remaining missense mutations can be very challenging and requires additional arguments (familial segregation, epidemiological studies, functional data...) to assess their causality and thus, complicates the outcome of genetic testing with uninformative results. Reciprocally, the observation of deleterious mutations can help to identify additional key positions and therefore to assess clinical significance of missense variants.

To date, among 228 non-related index cases/families referred and analyzed in our laboratory for MFS molecular diagnosis, we have identified six missense mutations in the *FBNI* gene in six index cases, which were predicted to result in Glycine substitutions sharing an identical position in their corresponding cb-EGF domain (1.3% of tested chromosomes). This position corresponds to the p.Gly1127Ser mutation that has been intensively studied elsewhere (Francke, et al., 1995; Smallridge, et al., 2003; Whiteman, et al., 1998; Whiteman, et al., 2001). We provide here arguments to generalize previous findings concerning the p.Gly1127Ser mutation and demonstrate a crucial structural role for Glycine (or Alanine) at this position in class I cb-EGF domains.

PATIENTS AND METHODS

Patients

Pedigrees and main clinical data are presented in Fig. 1A and 1B. All the patients were of French Caucasian origin.

Case 1 /pedigree MFS 00021

A 39-year-old female, who was treated since 26 years of age with beta-blockers for a paroxysmal supraventricular tachycardia was referred for a MFS diagnosis (-1 Ghent criteria) because of a suggestive familial history of MFS and a bilateral ectopia lentis operated on at the age of 29 years. She also presented significant striae, positive wrist and thumb signs. A computed tomography (CT) of the hips and the lumbosacral region revealed that she had a protrusio acetabulae. The rest of the investigation was normal. Her father and her brother (Fig. 1A) died from a type A aortic dissection, respectively at the age of 59 and 34 years. Both were reported to be of tall stature (194 and 202cm, respectively) with suggestive evidence of skeletal (arachnodactyly and dolichostenomelia) and ocular (strong myopia, which complicate in blindness in the father) involvements. There was no other familial antecedent.

Case 2 /pedigree MFS 00055

A 15-year-old female from healthy parents was referred for a MFS diagnosis (-1 Ghent criteria) after a comprehensive Ghent criteria assessment that identified a skeletal system involvement (scoliosis >20°, wrist and thumb signs, flexum at the elbows, characteristic face, high arched palate with crowding of teeth) and a major dural criterion on MRI. She had no familial history of MFS.

Case 3 /pedigree MFS 00126

A 60-year-old male was referred to confirm the diagnosis as well as for familial screening (Ghent criteria fulfilled). He had a familial history of MFS, a bilateral ectopia lentis operated on at the age of 16 years and 3 spontaneous pneumothorax events. Ghent criteria evaluation revealed that he also presented a scoliosis of more

than 20°, significant pes planus and a high arched palate with crowding of teeth. CT of the lumbosacral region also showed a significant meningocele on S3. Ectopia lentis and other suggestive MFS manifestations were also reported in his son, sisters and mother (Fig. 1A and 1B).

Case 4 / pedigree MFS 00231

A 59-year-old father and his 14-year-old son were both referred to confirm the diagnosis (Ghent criteria fulfilled). They showed a similar score for Ghent criteria: a major criterion for the skeletal system (reduced upper to lower segment ratio, wrist and thumb signs, reduced extension at the elbows, protrusio acetabulae, moderate pectus excavatum, joint hypermobility and a high arched palate with crowding of teeth), a major criterion for the cardiovascular system (type A aortic dissection at 40 years of age for the father and aortic root dilatation for the son) and recurrent herniae. There were no other evident MFS manifestations in the family (notably in the parents, who died from a breast cancer and Alzheimer disease at the age of 52 and 80 years, respectively for the mother and the father).

Case 5 / pedigree MFS 00249

A 41-year-old female with no familial history of MFS was referred for a MFS diagnosis (-1 Ghent criteria). Ghent criteria assessment identified a major criterion for the skeletal system (pectus carinatum, scoliosis >20°, wrist and thumb signs, protrusio acetabulae, high arched palate with crowding of teeth), a strong bilateral myopia (>6.5 diopters), a mitral insufficiency with a mild valve prolapse and significant striae.

Case 6 / pedigree MFS 00405

A 12-year-old female with a familial history of MFS manifestations including ectopia lentis (in the father and in an aunt) was referred for a MFS diagnosis (-1 Ghent criteria): major ocular criterion (ectopia lentis) and skeletal system involvement (wrist and thumb signs, high arched palate with crowding of teeth, joint hypermobility). Her paternal grandmother died suddenly at the age of 58 years. She was operated on heart and ascending aorta at the age of 34 and 48 years.

***FBNI* mutation screening in probands and family study**

Genomic DNA or blood samples from patients were sent to the “Laboratoire de Génétique Moléculaire, CHU Montpellier” from other French centers. DNA was harvested from peripheral blood using various methods. Mutation screening allowing the scanning of the 65 *FBNI* exons and flanking regions including the splice sites up to the branching regions was performed in probands with a robot-assisted and single condition PCR/direct sequencing strategy as described elsewhere (Tjeldhom, et al., 2006; Stheneur, et al., 2009). For several amplicons, at least one of the two primers was newly designed. Mutations were validated with two independent methods (performed from an independent dilution of the DNA and with other primers (Comeglio, et al., 2001; Sakai, et al., 2006; Howarth, et al., 2007)): 1- bidirectional sequencing and 2- dHPLC (performed on a WAVE DNA fragment analysis system (Transgenomic, Elancourt, France) for the c.1753G>C, c.2456G>A, c.4981G>A, c.6418G>A and the c.6419G>A mutations) or High-Resolution Melting analysis (HRM) (performed on a LightCycler 480 (Roche Diagnostics Corporation) for the c.5339G>A mutation). Both methods were then applied in proband relatives to establish familial segregation of each variant and in 95 French Caucasian controls. In all familial cases, haplotype analysis were performed in view to check for the good concordance of the samples origin and to complete Ghent criteria assessment using at least 8 microsatellites markers at the *FBNI* locus (Judge, et al., 2001). Detailed protocols are available upon request.

Mutation numbering refers to the *FBNI* cDNA GenBank reference sequence: NM_000138.3, with the A of the ATG translation initiation codon as nucleotide +1 (www.hgvs.org/mutnomen).

Conservation patterns and *in silico* predictions

Orthologs of the *FBNI* gene were identified with the NCBI and Ensembl websites (<http://www.ncbi.nlm.nih.gov/> and <http://www.ensembl.org/>) and sequences were aligned with the human fibrillin 1 precursor reference sequence accession NP_000129.2 using ClustalW (Chenna, et al., 2003). Jalview Alignment Editor (Clamp, et al., 2004), and homemade Perl (<http://www.perl.org/>) scripts were used to visualize and analyze

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the respective alignments. The UMD-predictor tool (Frederic, et al., 2009) was used to assess the impact at both RNA and protein level for all possible single nucleotide substitutions of the 31 fibrillin-1 Glycine codons that correspond to the third amino acid position of all the fibrillin-1 cb-EGF modules identified with a 4 amino acid loops between obligatory Cysteine residues 2 and 3. This includes the six missense mutations reported here and the 6 previously described (see further).

3D Modeling of the fibrillin-1 Glycine substitution effects

Supramolecular structure and NMR data available in the Protein Data Bank (<http://www.rcsb.org/>, PDB ID:1EMN) (Downing, et al., 1996) was used for the cb-EGF#32 domain (p.Gly2140Arg and p.Gly2140Glu). For p.Gly585Arg, and p.Gly1661Arg, located in the cb-EGF#5 and #24 domains of the fibrillin-1, respectively, a homology model was created based on PDB template 1LMJ (40 and 45% sequence identity, respectively)(Smallridge, et al., 2003). PDB template 1UZP (40% and 54% sequence identity with cb-EGF-like domain #9 and #25, respectively)(Lee, et al., 2004) was used to build the model to analyze p.Gly819Glu and p.Gly1780Glu. Template search was performed with Domain Fishing 1.0 (Contreras-Moreira and Bates, 2002) and Modeller program (version 8.2, <http://salilab.org/modeller/>, (Eswar, et al., 2007)) was used for homology modeling. The quality of the resulting structures was assessed using the evaluation tools available on the CBS web site (<http://bioserv.cbs.cnrs.fr/>). Mutagenesis and visualization were performed with PyMOL (<http://pymol.sourceforge.net>).

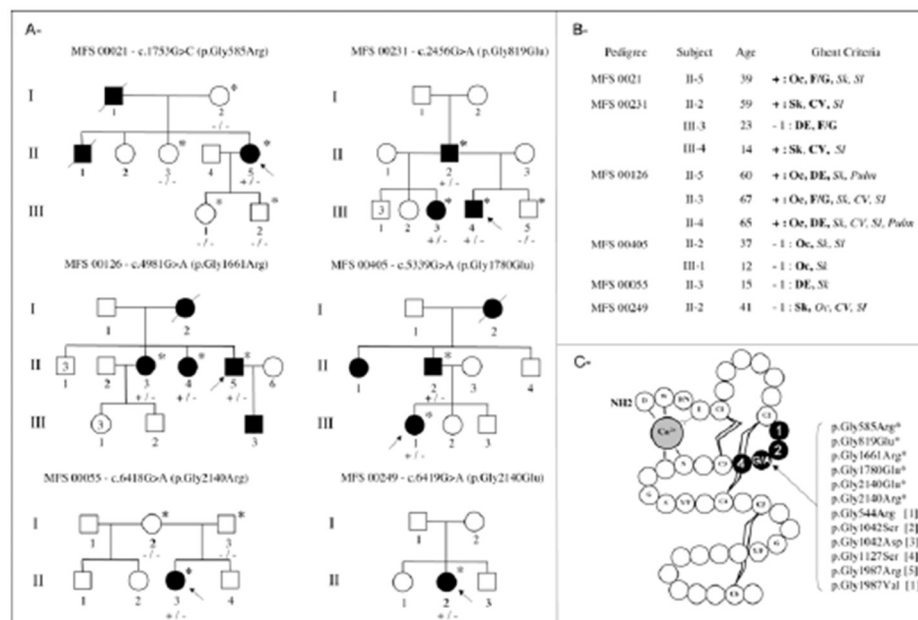


Figure 1. Clinical data and familial investigation of the six Glycine missense mutations suggesting a class I cb-EGF subtype. (A)- Affected pedigrees with familial segregation of each Glycine mutation. Pedigree/Generation/subject number and status concerning Marfan (MFS) manifestations in each family member are indicated. Squares, male subjects; circles, female. Affected and unaffected subjects are represented by solid and open symbols, respectively. Slashes denote deceased. Subjects with samples available for this study are indicated with an asterisk. Presence and absence of the Glycine missense mutation is represented by +/- or -/-, respectively. (B)- Main clinical data and Ghent criteria evaluation for MFS in affected subjects. +: criteria are fulfilled (i.e. the diagnosis of MFS is established); -1: a criterion is missing before testing to fulfill the criteria

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(incomplete MFS). Skeletal, Ocular, Cardio-Vascular, Dural, Skin and Integuments, Pulmonary and Familial/Genetic systems are indicated in the following order with bold characters if considered as a major criterion or in italics if the system is involved (minor criterion): Sk, Oc, CV, DE, SI, Pulm and F/G. (C)- Class I cb-EGF subtype suggested by conservation pattern and UMD-predictor analysis (adapted from (Handford, et al., 1991)). The Glycine missense mutations reported here (asterisk) or elsewhere: [1], (Stehneur, et al., 2009); [2], (Attanasio, et al., 2008); [3], (Sakai, et al., 2006); [4], (Francke, et al., 1995) [5], (Comeglio, et al., 2001); involve a Glycine residue located at position 3 of a loop region composed of four residues (solid symbols) between obligatory Cysteine residues 2 and 3.

RESULTS

Mutation identification and segregation studies

Six missense mutations were identified in the 6 unrelated French index cases, in whom no other mutation suspected to be deleterious was identified (Fig. 1). The c.1753G>C (p.Gly585Arg), c.2456G>A (p.Gly819Glu), c.4981G>A (p.Gly1661Arg), c.5339G>A (p.Gly1780Glu), c.6418G>A (p.Gly2140Arg) and c.6419G>A (p.Gly2140Glu) mutations are, to date, not reported in the literature, in the currently updating UMD-*FBN1* online database (Colod-Beroud, et al., 2003) and were absent in 190 ethnically-matched control chromosomes. Data concerning MFS diagnosis criteria assessments (Ghent criteria) in positive tested relatives, familial investigations and mutation analyses are given in Fig. 1. With the exception of pedigree MFS 00249 for which no familial sample was available, each mutation cosegregated with manifestations of the disease in pedigrees MFS 00021, MFS 00126, MFS 00231 and MFS 00405. In pedigree MFS 00055, the c.6418G>A (p.Gly2140Arg) mutation was shown to arise *de novo*, although a possible germinal mosaicism in the parents could not be ruled out. Results of the haplotype study were concordant with the origin of the samples, data of the pedigrees and disease status.

Conservation patterns and *in silico* predictions

These missense mutations involved a conserved Glycine residue, located in a loop-region between obligatory Cysteine residues 2 and 3, that is present in 31/43 cb-EGF-like modules in fibrillin-1. Alignment of these 43 modules revealed two subtypes: one with a loop involving 4 residues (32 sequences including a Glycine (n=31) or an Alanine (n=1) at position 3 in cbEGF#42) and a second (the 11 remaining sequences) involving either a shorter or longer loop (*i.e.* cbEGF#15, #18-19, #26, #34-35, #38-41, #43). Orthologous alignments of fibrillin-1 in 19 different species (including *Homo sapiens*) revealed a similar pattern on a set of 833 sequences matching class I cb-EGF domains. A Glycine is at position 3 in 592/611 (97%) loops involving 4 residues. The only alternative residue is Alanine (100%, *i.e.* 19/611-592), the second smallest amino-acid after Glycine. A comparison of the respective distribution of Glycine and Alanine (excluding Glycine) from the position 1 (Glycine 115/611=19%, Fischer's exact test: $P<0.0001$ and Alanine 42/611-115=8%, $P<0.0001$), from the position 2 (Glycine 120/611=20%, $P<0.0001$ and Alanine 5/611-120=1%, $P<0.0001$) and position 4 (Glycine 0/611=0%, $P<0.0001$ and Alanine 9/611-0=1.5%, $P<0.0001$) showed that both residues are specific of the third position. Amino-acid sequence alignments of fibrillin-2 and fibrillin-3 confirmed these findings. Finally, we assessed an NCBI alignment of 10421 cb-EGF sequences identified with the PFAM website (<http://pfam.sanger.ac.uk>, for PF07645). Only 8050/10421 contained the obligatory 6 Cysteines of the module and were taken into account. Of these, 2006 showed a loop composed of 4 residues between Cysteines 2 and 3. Glycine (1713/2006) and Alanine (143/2006) are found in position 3 of these loops in 85 and 7%, respectively (each alignment text file is available on request). The high conservation pattern of this Glycine position was also confirmed by the UMD-predictor analysis. Output of this analysis (Supp. Table S1) showed that all potential Glycine substitutions are predicted to be "pathogenous" except for the Alanine residues for which depending on the cb-EGF module predictions and scores were ranging from "probably polymorphism" to "probably pathogenous" or "pathogenous" and from 59 to 82/100, respectively.

All these data suggest an important role for the domain maintenance of this position and a subtype of the fibrillin class I cb-EGF described by Downing et al (Downing, et al., 1996), Fig. 1.

3D Modeling of the fibrillin-1 Glycine substitution effects

The possible effect of these Glycine substitutions on the structure of their corresponding modules was assessed by *in silico* 3D Modeling analysis (Fig. 2). The Ramachandran plot obtained for the Glycine residue1661 showed

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that the Phi-Psi angles are impossible to adopt for an Arginine residue. Therefore, the conformation of the mutant domain is likely to be strongly altered by the presence of this mutation. For the Glycine residues 585, 819, 1780 and 2140, the predicted change to Arginine or Glutamic Acid involves the same mechanism. Indeed, in each case, the mutant residue is predicted to induce steric clashes with its surroundings. The p.Arg585 breaks a loop involving p.Lys612, p.Lys599 and p.Pro600. Thus, the structure of the cb-EGF-like#5 domain is likely to be strongly affected to retain the volume of the Arginine residue (*i.e.*: van der Waals volume of Glycine is 48\AA^3 compared to 148\AA^3 for Arginine). The p.Arg2140 and p.Glu2140 mutants are predicted to interfere with a crucial structural component of the cb-EGF-like domains, a disulfide bridge involving p.Cys2153-p.Cys2164, and p.Cys2137-p.Cys2151, respectively (Fig. 2). A similar effect is predicted with p.Gly819Glu and p.Gly1780Glu, which are likely to disrupt respectively p.Cys832-p.Cys845 and p.Cys1806-p.Cys1793 as well as p.Cys1777-p.Cys1791 covalent bonds (data available on request).

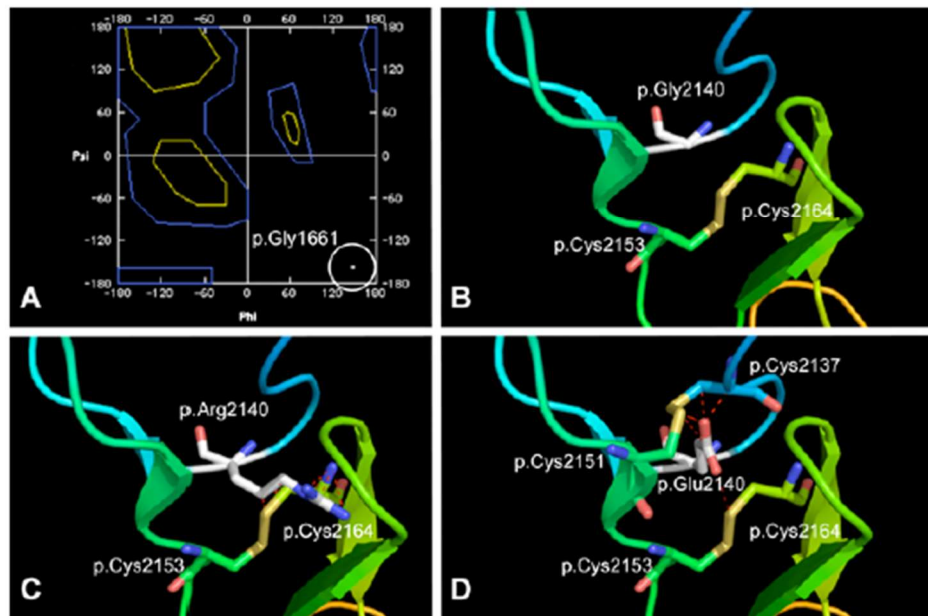


Figure 2. Prediction of the impact of mutations on cb-EGF-like domains. (A)- Ramachandran plot of predicted p.Gly1661: Phi and Psi angles are not accessible to other amino-acids. Plot drawn with Swiss-Pdb Viewer (<http://spdbv.vital-it.ch/>). (B)- prediction of p.Gly2140 position in the domain cb-EGF-like#32. (C)- According to PyMOL (<http://pymol.sourceforge.net/>), the most probable rotamer for p.Arg2140 would interfere with a disulfide bond involving p.Cys2153 and p.Cys2164. (D)- A similar mechanism is predicted with the mutant p.Glu2140. (C ; D)- Red dots indicate too short interatomic distances between residues. Figures showing predictions of p.Gly585Arg, p.Gly819Glu and p.Gly1780Glu effects in cb-EGF#5, #9, #25 are available upon request.

DISCUSSION

To date, we have sequenced the 65 *FBNI* exons and flanking regions in 228 non-related patients referred for molecular diagnosis of Marfan syndrome. We have identified in 6 of them (1.3 % of tested chromosomes) a single nucleotide substitution predicting a missense mutation of Glycine residues as the only unclassified and potentially deleterious variant. These mutations were absent in 190 ethnically-matched control chromosomes. They cosegregated with manifestations of the disease within the pedigrees or occurred *de novo*. Study of conservation patterns showed that the corresponding Glycine position is highly conserved in orthologous, paralogous and also in

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cb-EGF like modules sequences that include a 4 amino-acids loop between Cysteine residues 2 and 3. These results were also sustained by the UMD-predictor (Frederic, et al., 2009) analysis that in addition showed no argument for an impact on splicing. Interestingly, the alternative residue in the alignments and for which the predictions made by UMD-predictor were variable at this position was Alanine (Supp. Table S1). Alanine is the second smallest amino-acid after Glycine. This suggests that the third position of the loop could have particular local strains with steric exclusion for larger residues. Thus, we studied the possible effect of these Glycine substitutions on the structure of their corresponding modules by *in silico* 3D Modeling analysis and we bring arguments for a key position in terms of steric hindrance. All this results suggest a potential cb-EGF type I subtype (Fig. 1) with potential implication for related diseases.

To our knowledge, six missense mutations in the *FBNI* gene implicating a Glycine residue in such a position (p.Gly544Arg, p.Gly1042Ser, p.Gly1042Asp, p.Gly1127Ser, p.Gly1987Arg, p.Gly1987Val in cb-EGF#4, #11, #13, #30, respectively), in MFS or incomplete MFS subjects have been described and support our arguments (Attanasio, et al., 2008; Collod-Beroud, et al., 2003; Comeglio, et al., 2001; Francke, et al., 1995; Sakai, et al., 2006; Stheneur, et al., 2009). Only the p.Gly1127Ser mutation has been exhaustively studied: it was shown to result in a local cb-EGF like#13 domain folding disruption, a normal synthesis but a reduced fibrillin deposition into the extracellular matrix and proposed to cause disease via an extracellular dominant negative effect (Francke, et al., 1995; Whiteman, et al., 1998; Whiteman and Handford, 2003; Whiteman, et al., 2001). In their studies, Whiteman et al. (Whiteman, et al., 1998; Whiteman, et al., 2001) have previously noted that the p.Gly1127 position in cb-EGF like#13 was highly conserved and located in a turn of a two stranded antiparallel beta-sheet. However, neither Glycine nor Alanine were reported as mandatory at this position in the cb-EGF type I model (Downing, et al., 1996), thereby allowing to class variants and to facilitate the molecular diagnosis workflow of related diseases. This could be explained because of a variable composition in terms of type and number of amino acids in the loop between Cysteine residues 2 and 3. Here, the subtype of cb-EGF type I model, suggested by alignments of 8050 sequences (Fig. 1) and highlighted by 6 related missense mutations with some genetics/independent arguments for pathogenicity, shows how the findings previously made for p.Gly1127Ser could be extended. The p.Gly1127Ser mutation, associated with a mild effect on protein folding, was reported in 10 related-patients with a moderate phenotype (Francke, et al., 1995). None of the patients reported here as well as those described elsewhere when sufficient data are available to assess severity of the phenotype (Attanasio, et al., 2008; Comeglio, et al., 2001; Sakai, et al., 2006) have a severe and a typical MFS phenotype, suggesting that these *FBNI* gene mutation class could be encountered in incomplete or atypical MFS. It is difficult to conclude definitely on the precise genotype/phenotype correlations associated with this mutation class. First, because of the marked intra- and inter-familial variability in MFS manifestations and age of onset of the symptoms, that is notably illustrated in pedigree MFS 00231 (c.2456G>A (p.Gly819Glu), Fig. 1). The father (subject II-2), who suffered from a type A aortic dissection at 40 years of age, fulfilled the Ghent criteria clinically. His 14-years-old affected son (subject III-3) displayed a similar phenotype and had an aortic root dilatation unlike her 23-year-old daughter (subject III-2), which also inherited the mutation. Following a comprehensive Ghent criteria assessment, she only presented a high arched palate with crowding of teeth, a suggestive MFS facial appearance, a wrist sign without thumb sign, a strong myopia and a dural ectasia on MRI (not fulfilling Ghent criteria). Considering that she is adult (but also that she may develop an aortic complication), the penetrance of the c.2456G>A mutation towards MFS is incomplete suggesting the importance of modifying factors. Second, we could not extend the clinical and molecular investigations because of a lack of participation of the relatives to fully assess the phenotypes associated with this class of mutation. However, from our study and as reported before we claim that this singular missense mutation class affecting Glycine residues located at the third position of this cb-EGF-like subtype (if resulting in a substitution other than Alanine) should be at least considered as a strong risk factor for a wide range of MFS manifestations, including aortic complications. Aortic dissection and sudden death are highly prevalent within families reported with such missense mutations. To our opinion, the medical management and follow-up should not differ from classic MFS.

We have shown a key residue-position in a potential subtype of class I cb-EGF like module. Both, Glycine and Alanine residues appear mandatory for the structure maintenance and therefore for the fibrillin-1 functions. These findings have implications for genetic testing in MFS, other fibrillinopathies and in other cb-EGF-like related-disorders.

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Parental mosaicism in Marfan and Ehlers–Danlos syndromes and related disorders

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Abstract

Marfan syndrome (MFS) is a heritable connective tissue disorder (HCTD) caused by pathogenic variants in *FBNI* that frequently occur de novo. Although individuals with somatogonadal mosaïcisms have been reported with respect to MFS and other HCTD, the overall frequency of parental mosaïcism in this pathology is unknown. In an attempt to estimate this frequency, we reviewed all the 333 patients with a disease-causing variant in *FBNI*. We then used direct sequencing, combined with High Resolution Melting Analysis, to detect mosaïcism in their parents, complemented by NGS when a mosaïcism was objectivized. We found that (1) the number of apparently de novo events is much higher than the classically admitted number (around 50% of patients and not 25% as expected for *FBNI*) and (2) around 5% of the *FBNI* disease-causing variants were not actually de novo as anticipated, but inherited in a context of somatogonadal mosaïcisms revealed in parents from three families. High Resolution Melting Analysis and NGS were more efficient at detecting and evaluating the level of mosaïcism compared to direct Sanger sequencing. We also investigated individuals with a causal variant in another gene identified through our “aortic diseases genes” NGS panel and report, for the first time, on an individual with a somatogonadal mosaïcism in *COL5A1*. Our study shows that parental mosaïcism is not that rare in Marfan syndrome and should be investigated with appropriate methods given its implications in patient’s management.

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SPRINGER NATURE

Introduction

Marfan syndrome (MFS, [OMIM #154700]) is caused by heterozygous variants in *FBNI*, which alter the function of the encoded fibrillin 1 protein, a component of the extracellular microfibrils [1]. This heritable connective tissue disorder (HCTD) is dominantly inherited and characterized by high penetrance and variable expressivity with some patients displaying only discrete signs and some with early-onset MFS [2]. The broad phenotypic expression includes manifestations mainly in the cardiovascular (i.e., aortic root aneurysm), ophthalmologic (i.e., *ectopia lentis*), and skeletal systems (i.e., arachnodactyly, scoliosis, *pectus* deformity). Diagnosis is established following the Revised Ghent Nosology [1].

The frequency of de novo MFS-affected individuals is classically reported to be around 25% [3]. True de novo individuals (disease-causing variant in only one gamete) are hard to distinguish from MFS sporadic individuals occurring from mosaïcism. In this latter situation, there are two levels of possible mosaïcism [4]. On one hand, in the

proband when a disease-causing variant appears during the early stages of the embryologic development (also named post-zygotic event) leading to somatic mosaicism. On the other hand, in one of the two parents, the disease-causing variant can appear during gametogenesis (germline or gonadal mosaicism) or, in the same way, during the early stages of the embryologic development affecting both soma and germ cells (somatogonadal mosaicism). This last mechanism, which has been previously reported in MFS [5–9] induces a risk of recurrence in siblings of an apparently sporadic proband [6]. Parental molecular analysis revealed the majority of reported individuals with post-zygotic mosaicism in *FBNI*. Despite the fact that most patients with proven somatogonadal mosaic were asymptomatic or displayed discrete MFS signs [5, 8], some of them displayed aortic dilatation [6, 7].

Diagnosis of *FBNI* mosaicism is a challenge for the clinician dealing with patients harboring subtle signs of MFS. It is also challenging for the diagnostic laboratory due to the limits of detection sensitivity for a mosaic variant detection. Multiple technologies have been tested and developed in order to be more sensitive in mosaic variant detection [10–13].

Although somatogonadal mosaicism in MFS appears to be rare, its frequency is not known and has only been published in case reports. In particular, no systematic molecular parental studies have been performed in the way that was done in vascular Ehlers–Danlos Syndrome (vEDS) or in developmental disorders [14, 15] except as a communication at the 2020 ESGH meeting [16]. To try to estimate the frequency of de novo and parental mosaic variants in MFS, we reviewed all the individuals with a MFS confirmed by a disease-causing variant in *FBNI* in our center. We report here three novel parents with a somatogonadal mosaicism for the disease-causing in *FBNI* variant identified in their child. This allows us to estimate, to an accuracy of around 5%, the frequency of parental mosaicism in MFS.

Mosaicism has been reported in other genes of HCTD, in particular in Loey's–Dietz syndrome with mosaic variants in *TGFBR1* and *TGFBR2* [17, 18]. We therefore, reviewed all affected individuals for other HCTD genes but also familial thoracic aortic aneurysm/dissection (FTAAD) genes studied in our center. We found a parental mosaicism in *COL5A1*, an occurrence which has never before been reported to our knowledge.

Materials and methods

Patients

This study was designed in compliance with the tenets of the Helsinki Declaration. Informed consent was obtained from all

individuals included in this study, which was approved by our local ethic committee (Nîmes University Hospital).

We included all the 333 probands with a (likely) pathogenic variant in *FBNI* referred for molecular diagnosis in our center during the past 15 years (2004–2019) in order to determine the frequency of mosaicism in this disorder. The diagnosis was performed initially by direct sequencing (of *FBNI*, *TGFBR1*, and *TGFBR2* from 2004 to 2013) and by NGS (detailed below and in Supplementary Material 1).

We also collected data of patients with HCTD harboring a disease-causing variant in a gene other than *FBNI* (genes list in Supplementary Material 1). However, because of the low number of patients for each of these genes, they were not included in our statistical analyses.

Clinical data and family history were obtained through a questionnaire completed by the medical referent. Probands with neither data on their family history nor molecular parental studies were excluded. The condition was considered as “familial” in presence of another affected relative and as “sporadic” when family history was not suggestive. It should be noted, as the objective was to determine whether the variant was likely to be inherited or not, that the proband's offspring was not taken into consideration. The variant was considered inherited if one of the parents or another relative (if parental transmission was suspected) was heterozygous for the variant.

We also reviewed all the siblings who benefited from molecular analysis after parental studies showing de novo occurrence, in order to look for a recurrence.

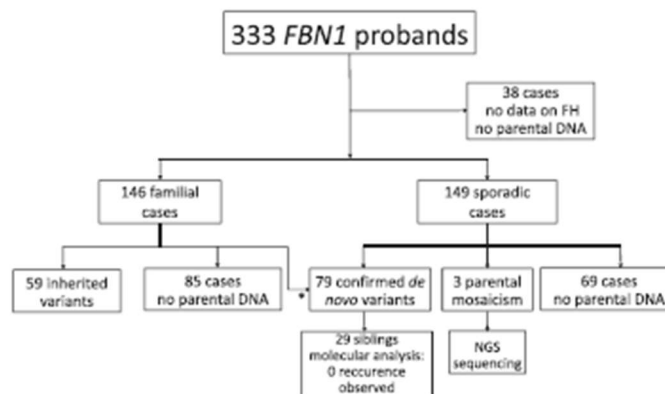
Molecular testing of relatives

We routinely use two methods for molecular testing of relatives in our center: direct sequencing and High Resolution Melting Analysis (HRMA). It should be noted that the analysis of six microsatellites markers in the *FBNI* locus is systematically performed to confirm the paternity and maternity as well as DNA contamination.

Direct sequencing was performed for both parents of 163 probands (141 with disease-causing variant in *FBNI*, 22 in another FTAAD gene). We performed targeted PCR amplification using specific primers on the proband's and his/her relatives' genomic DNA extracted from blood and cheek cells (when available). PCR products were then sequenced using an ABI3130XL or an ABI3500XL (Applied Biosystems, Foster City, California, U.S.A.). DNA sequence variants were identified using SeqPilot software (JSI medical systems, Eitenheim, Germany).

HRMA, which was developed secondarily in our lab (2012), was performed when applicable in the parents of 81 out of 163 probands (62 with disease-causing variant in *FBNI*, 19 in another FTAAD gene). This second method was developed as a complement to direct sequencing

Fig. 1 Flow chart of the study. FH family history, NGS next-generation sequencing. * Two cases reclassified as *de novo* after parental molecular study.



because of its better sensitivity, in particular for mosaic variants detection although it has some limitations (i.e., single nucleotide polymorphism near the variant) [11]. We performed targeted real-time PCR with specific primers (different couples than those used for direct sequencing) using Light Cycler 480 (Thermo Fisher scientific, Waltham, Massachusetts, USA). HRMA was also used for semi-quantitative analysis in mosaic patient as described elsewhere [11]; to estimate the mosaicism using HRMA, we performed a dilution range of the familial variant in a non-mutated DNA.

When we detected a somatogonadal mosaic variant by either HRMA, direct sequencing or both in samples of parents, we then performed a NGS analysis in order to have a quantitative estimation of the mosaicism level and a comparison between these different methods. The NGS FTAAD panel used includes 35 genes (list in Supplementary Material 1). This also allowed checking for DNA contamination. Four parents were identified: three with a mosaic variant in *FBN1* (M0078, M0277, M0336) and one with a mosaic variant in *COL5A1* (M1144). These novel *FBN1* and *COL5A1* variants reported here have been deposited for public access in the Global Variome shared LOVD at www.lovd.nl/FBN1 and www.lovd.nl/COL5A1 with individual IDs #00306166 to #00306169.

Library preparation was performed using 3 µg of DNA. Capture of all the coding exons and their flanking intronic sequences were performed using Sureselect technology (Agilent, Santa Clara, California, USA). All the samples were run on the Ion chef & S5™ system (Thermo Fisher Scientific). Run parameters and data output from each run were obtained and compared against specifications outlined by the manufacturer. Bioinformatics data analysis steps were performed using NextGENe software (Softgenetics, State College, Pennsylvania, USA) and Alamut Visual (Integrative Biosoftware, Rouen, France).

Results

Estimation of the *de novo* variant frequency and parental mosaicism in MFS

We identified 333 pedigrees with a disease-causing variant in *FBN1*. For 38 out 333, we had no information on family history with no available parental DNA and thus, they were excluded from our study (Fig. 1). Among the 295 patients included, half had a family history evocative of MFS (146/295) and half were sporadic (149/295). In 141 probands, molecular analysis (direct sequencing combined with HRMA for 62 probands) was performed in both parents. The variant was inherited from a heterozygous parent in 59 of them (41.8%) and appeared *de novo* in 79 probands (56.0%). In three pedigrees (MF0014, MF0050, MF0140), analyses revealed a mosaicism for the variant in a parent (2.1%). When taking into account only the analyses made by both HRMA and direct sequencing, somatogonadal mosaicism in one parent was observed at a frequency of 4.8% (3/62; 1.1–13.6% for a 95% CI). In addition, when the variant was inherited, we tried to extend the study of the familial variant (when DNA was available) to earlier generations. We did not detect any other affected individuals with mosaicism (data not shown). It should be noted that the absence of DNA contamination was checked by NGS analysis and by the use of six microsatellites at the *FBN1* locus.

In two families (MF020, MF272), despite a familial history suggestive of MFS, the *FBN1* disease-causing variant occurred *de novo* (NM_000138.5: c.2948C>T and c.5060G>A, respectively). In the MF020 family, the half-sister was reported with a mild mitral insufficiency, arachnodactyly (thumb without wrist sign), and joint hypermobility. In the MF272 family, the prescriber suspected a MFS in the sister, who displayed a slight

Table 1 Clinical data of parents with somatogonadal/germline and somatic mosaicism in *FBNI* and their affected children.

Family	Patient	Age ^a (years)	Cardiovascular	Ocular	Systemic features	Systemic score
MF0014	M0018	28	TAAD (surgery)	–	Scoliosis, PD, AraD, PP, CFD ^b	8
	M0078	55	–	–	Dolichostenomelia	1
MF0050	M0083	Birth	TAAD (+6 SD), severe MI, tricuspid valve prolapse	Myopia (high)	Scoliosis, PD, PP, AraD, joint laxity, distal arthrogryposis, CFD ^b	9
	M0279	In utero	ND	ND	AraD, arthrogryposis	ND
	M0277	38	TAAD (+2, 5 SD), BAV	ND	AraD	ND
MF0140	M0235	37	ND	ND	ND	ND
	M0336	65	–	Myopia (mild), LE: amblyopia, peripapillary atrophy	Scoliosis, AraD, skin striae	5

Normal examination, *AraD* arachnoidactyly, *BAV* bicuspid aortic valve, *CFD* craniofacial dysmorphism, *LE* left eye, *MI* mitral insufficiency, *ND* not determined, *PP* pes planus, *PD* pectus deformity, *TAAD* thoracic aortic aneurysm or dissection.

^aAge at diagnosis.

^bSystemic score and craniofacial dysmorphism as evaluated in the revised Ghent nosology for the Marfan syndrome (Loeys et al. [1]).

marfanoid habitus (normal ophthalmologic examination and echocardiography). No other findings suggestive of MFS were reported in other relatives of the two families. Notably, parental echocardiography was normal. Both direct sequencing and HRMA confirmed that the variant was de novo and not found in the suspected affected sibling. Sample origins were checked by microsatellite analyses. This allowed the elimination of any clinical doubt, and no further molecular analysis was performed.

In three families (MF0014, MF0050, MF0140), parental segregation analysis revealed a somatogonadal mosaicism of the, respectively, *FBNI* (NM_000138.5): c.1095C>A; c.3158G>A and NG_008805.2(NM_000138.5):c.2678-1G>C variants in one parent (M0078, M0277, M0336). Clinical data of these pedigrees are summarized in Table 1 and Fig. 2.

In MF0014, a sample of cheek cells was available for the parent M0078. HRMA revealed a lower level of mosaicism in the cheek cells (variant allele frequency (VAF): 10%) than in blood cells (VAF: 20%) for the disease-causing variant c.1095C>A in *FBNI* (Fig. 3B).

In MF0050, we confirmed a recurrence of an early-onset MFS. The proband in question (M0083) was initially suspected to have Beals syndrome [MIM#121050] at birth because of congenital arthrogryposis, suggestive craniofacial, and marfanoid features. MFS was later suspected because of the development of an aortic aneurysm during the first year (Valsalva sinus: 24 mm +5 Z-score, body surface area 0.42 m² at 1 year of age) and a high myopia. At the last evaluation at 11 years of age, the proband's thoracic aortic aneurysm was stable with 37.5 mm at the Valsalva sinus (+6 Z-score, body surface area 0.9 m²) and he developed a severe mitral insufficiency as well as a progressive scoliosis (Cobb angle 48°). His brother (M0279)

died in utero at 38 weeks of amenorrhea without a cause identified during the postmortem examination (hypotrophic placenta was noted). This examination also revealed craniofacial features (prefrontal edema, hypertelorism, small chin, low set hair in the forehead), club hands with *adductus* thumbs, arthrogryposis of elbows, hips, and knees. A molecular study confirmed the presence of the heterozygous c.3158G>A *FBNI* variant in his genomic DNA. The echocardiography of their father (M0277), who harbors the variant in the mosaic state (VAF: 7% in blood, Fig. 3), shows a mild aortic aneurysm at 41 mm (+2.5 Z-score, body surface area 2.0 m²).

Mosaicism in other HCTD

We identified 51 families with a FTAAD caused by genes other than *FBNI*. The diagnosis was confirmed with the identification of a disease-causing variant in one of the following genes: *TGFBR2* (*N* = 21), *TGFBR1* (*n* = 8), *SMAD3* (*n* = 7), *TGFB3* (*n* = 4), *ACTA2* (*n* = 2), *FBN2* (*n* = 2), *LOX* (*n* = 2), *MYH11* (*n* = 1), *MYLK* (*n* = 1), *SKI* (*n* = 1), and *COL5A1* (*n* = 1). Among them, 22 molecular analyses of both parents were performed and we discovered a paternal mosaicism in *COL5A1* identified in one family (MF0519). We did not detect any other variant mosaicisms.

In family MF0519 (pedigree in Fig. 2), the proband was an 8-year-old boy (M1089) who displayed a classical EDS (cEDS) with skin hyperextensibility, atrophic scarring, *molluscoid* pseudotumors, and generalized joint hypermobility (Supplementary Material 2). Molecular analysis confirmed the diagnosis with a disease-causing *COL5A1* (NM_000093.5) variant c.3179G>A. It was found at mosaic state in the 39 year-old father's DNA (VAF:11% in blood, Fig. 3). Careful clinical examination did not find any

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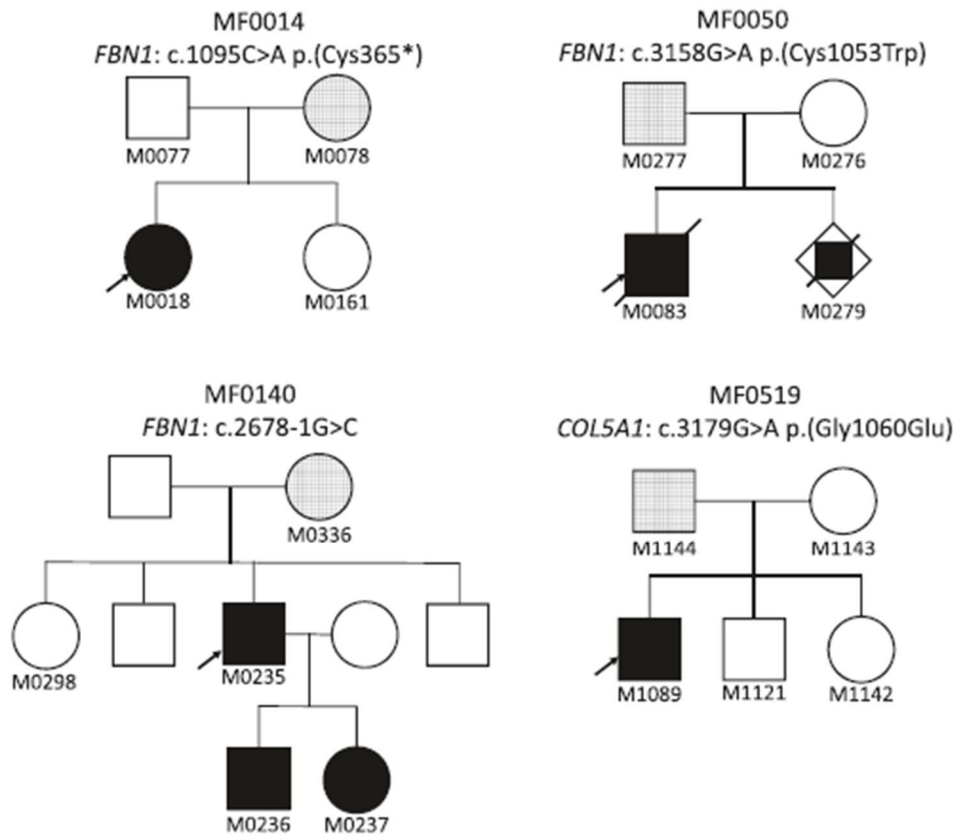


Fig. 2 Pedigrees of the four families with parental mosaicism. Tested individuals are mentioned by their anonymous identification.

Affected heterozygous patients are indicated with filled symbols. Patients with mosaicism are indicated by hatched symbols.

skeletal, skin or other diagnostic criteria in the father. Both of his other children did not inherit the variant.

Comparison of direct sequencing, HRMA, and NGS to detect a mosaic variant

HRMA and NGS were both able to detect the four mosaic variants. Direct sequencing failed to detect the *FBN1* c.3158G>A variant with the lowest level of mosaic (M277) (Fig. 3). Estimation of the VAF was concordant between HRMA and NGS (0–6% difference).

Discussion

This study shows that the frequency of parental mosaicism in MFS is not that rare. We can estimate the parental

mosaicism as around 4.8% (1.1–13.6%; 95% CI) with the retrospective analysis of data collected in our clinical laboratory. Interestingly, this estimation of MFS mosaicism is concordant with the conclusions of Yan et al. who reported, at the 2020 ESHG Meeting, a parental mosaicism of 3.6% in a population of 563 patients with suspected MFS or thoracic aortic aneurysm/dissection [16]. Until then, observations of mosaicism had only been reported through individual studies and were therefore considered as rare phenomena [5–9, 19–22]. However, this has an important impact for MFS for both the assessment of the recurrence risk in siblings and medical follow-up of mosaicism-affected individuals. The recurrence risk is indeed correlated to the timing of variant’s appearance: from high (50%) if the variant is constitutive in the parent, to null if the variant results from a post-zygotic event in the proband [4, 14]. Somatogonadal mosaicism in a parent confers an

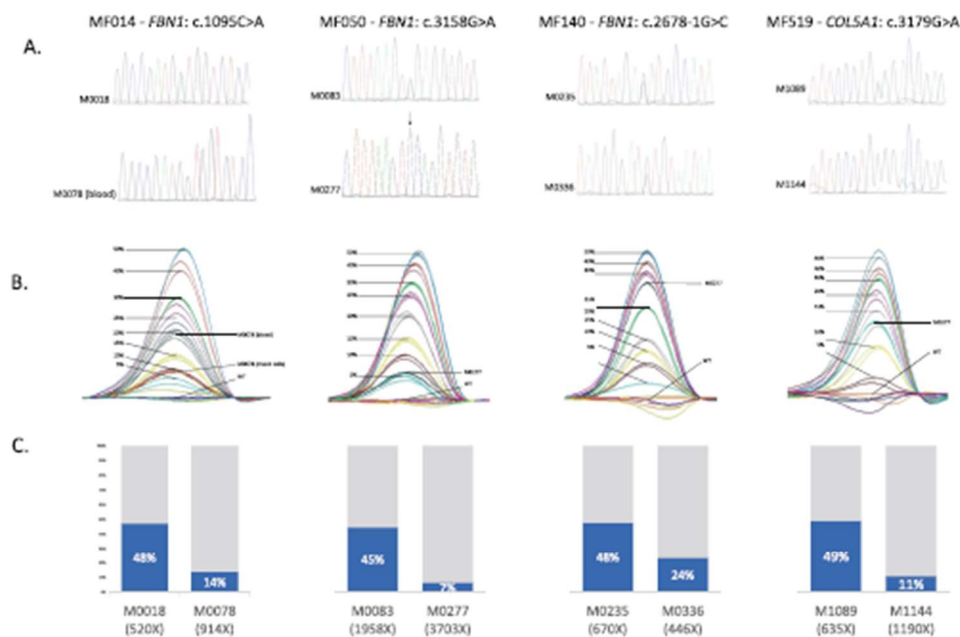


Fig. 3 Molecular analyses of the probands and in their parents with a mosaic variant. **A** Electropherograms of direct sequencing showing the causal variant present at heterozygous state in the proband and in mosaic in the parent's blood. Note the absence of visualization of the variant in family MF050 (as indicated by arrow). **B** Melting curves of High Resolution Melting Analysis showing the dilution

series for variants in *FBNI* or *COL5A1* for each family, with the superposition of the profile of mosaic patients. WT wild type. **C** Variant allele fraction (VAF) in blood obtained by next-generation sequencing showing the level of mosaicism (depth at the position is specified below patient number).

intermediate and undefined risk between 0 and 50%. Campbell et al. [23], modeled the multiple factors involved in estimating this recurrence risk (parent of origin and age for male, sexual dimorphisms during gametogenesis, mosaicism, sibling structure, etc.). They show that the detection of a somatic parental mosaicism in blood is the most important factor. It considerably increases the risk of recurrence, which can be estimated according to their calculations to 5.21% when a mosaic is detected in one parent (regardless of gender and age) in the case of a single affected child (the risk is modulated according to the number of children and their status). The recurrence risk is much lower when no mosaics are detected in the blood of the parents (depending on sex and age for fathers, 0.048–2.6% for a single affected child). This estimate of the recurrence risk although to be considered with caution undoubtedly explains why despite a significant frequency of mosaicism detected in our study, recurrence cases are rarely observed in clinical practice. Therefore, clinical evaluation of siblings as well as molecular testing should be systematic because there is always a risk that an apparently de novo situation hides a parental germline mosaicism. It is also an

important risk assessment to make for prenatal and pre-implantation genetic diagnosis considerations.

In a large majority of parents, only blood was analyzed and we cannot exclude the possibility of a gonadal mosaicism absent from blood. This phenomenon is frequent in other diseases and can be clinically relevant. For example, in Cornelia de Lange syndrome, disease-causing mosaic variants in *NIPBL* are detected in cheek cells but not in blood in 15–25% of patients [24]. In 1999, Rantamäki et al. reported an individual with germline mosaicism associated with a recurrence of MFS in two siblings [9]. The disease-causing *FBNI* variant was not detected in either of the parents despite molecular analysis of different tissues for each of them. Due to a lack of other samples, this comparison between different tissues was performed only for parent M0078 (cheek cells and blood). The *FBNI* variant was detected in both tissues with a lower level of mosaicism in cheek cells than in blood (Fig. 3B).

This study was retrospective and therefore the technologies used for the molecular analysis of parents varied, with only direct sequencing being used in some patients and direct sequencing combined with HRMA in others. Our

results confirm the limits of direct sequencing to detect mosaic variants [11–13] as it failed to detect the variant in MF050 with the lowest estimated mosaic level (VAF: 5–10%, Fig. 3). Variants with a similar mosaic level could have been missed when analyzed only by direct sequencing. This underlines the importance of using the most-sensitive technique to detect such variants. HRMA was associated with direct sequencing in order to increase sensitivity in familial variant detection [11–13]. High deep NGS can detect variants with an allelic balance below 1% [4, 10] and thus offers a better sensitivity to detect mosaic variants than direct sequencing. Digital droplet PCR is also a more efficient method. The latter two methods are however not routinely applied in clinical practice to screen for mosaic variants during parental molecular analysis (due to cost and time constraints). We thus use HRMA, which has the advantage of being a simple low-cost, single-step, closed-tube method whenever possible as the second independent technique for the screening of relatives. Furthermore, NGS may help to solve rare highly suggestive MFS patients with negative molecular test results, which may be explained by an uncharacterized mosaic variant. Our routine bioinformatics pipelines are adjusted not to retain variants with an allelic ratio lower than 20, as variants beneath this threshold are difficult to differentiate from the background noise and their analyses are not compatible with routine molecular analyses. This artificial limit is removed when analyzing mosaic variants. Furthermore, we observed that low-grade mosaicism (VAF < 20%) is not expected to cause typical MFS.

Although there are no apparent correlation between the level of mosaicism and the clinical severity in the few patients described in the literature and herein with a variant mosaicism in *FBNI* [5–9, 19–22], clinical signs tend to be milder in patients with a mosaic variant than in heterozygous patients. Only one of our patients (with a VAF of 7%) displayed cardiovascular signs with a mild aortic root dilatation (patient M0277, Table 1). Another affected individual with mild aortic root dilatation was previously described (low level of mosaicism) [6] and one patient who made a type B aortic dissection [7]. Other cardiovascular anomalies were reported, in particular mild mitral and tricuspid insufficiency [6, 22]. Two patients had an eye examination (M0078, M0336) that did not reveal any anomaly beyond a mild myopia in one of them (M0336). Interestingly, Šípek et al. described one patient with an unilateral *ectopia lentis* related to a somatic mosaicism (VAF: 10–25% in blood) [22]. The three MFS mosaic patients that we report displayed various systemic signs (i.e., arachnodactyly, scoliosis, *striae*) but the systemic score according to the revised Ghent's nosology [1] remained negative (<7). Therefore, despite the milder clinical presentation of patients harboring a mosaic variant in

FBNI, an appropriate follow-up is necessary to prevent complications, in particular aortic dissection. The question of whether a life-long betablockade therapy is indicated or not, independently of aortic dilatation, is unsolved. Furthermore, we recommend an echocardiography at least once during a parental study because of the residual risk of missing a mosaicism during the molecular study (notably by direct sequencing only) and in view of observations of aortic aneurysms/dissection with low levels of mosaicism (M0277) [6, 7]. On the contrary, in the absence of affected offspring, we can assume that this milder phenotype would result in undiagnosed patients.

One of our patients (M00277) displayed mosaicism for the *FBNI*:c.3158G>A [p.(Cys1053Tyr)] variant, located in the exons 24–32 region (formerly “neonatal region”) associated with early-onset MFS [25] accordingly to the severe phenotype noticed in his children. Such a situation was previously reported in two families [20, 22]. Our study is, however, the first to our knowledge to report a molecularly proven recurrence of early-onset MFS. In order to refine the estimation of the recurrence risk, other studies have analyzed sperm samples of mosaic fathers [26, 27]. Although it can help some couples to have this estimation, in most instances, it does not add valuable information. A lower allele fraction in a sperm sample than in a blood sample does not remove the recurrence risk.

Variants of various types have been found to be associated with parental mosaic events, most of them being single nucleotide variations or insertion/deletions [5–9, 19, 20, 22]. However, a complete *FBNI* deletion [21] has been also reported. We found parental transmission in both sexes: a total of six maternal mosaicism and four paternal mosaicism have been reported, including our new patients [5–8, 20–22]. Thus, there does not seem to be a unique mitotic mechanism resulting in parental mosaicism.

The frequency of de novo variants that we observed (47.0–50.0%) is higher than the classically admitted 25% of de novo variants [3] but similar percentages have been observed in a large international cohort of 1013 probands with MFS [2]. Faivre et al. discussed the important number of de novo mutations found in the probands of their study and raised the possibility that this higher proportion “does not reveal a higher-than-reported mutation rate but reflects practice and screening results from the contributing centers worldwide” [2]. It is likely that large characteristic Marfan pedigrees have been first screened.

Suggestive family histories with de novo variants were observed twice in our patients. Reevaluation of the two suggestive affected relatives with the negative molecular analysis showed no major signs of MFS according to the revised Ghent nosology. In addition, in multiple families, molecular analyses revealed that the variant was inherited from one parent referred to as asymptomatic. Careful retro-

phenotyping showed MFS clinical involvement in most of the latter studies. Thus, systematic molecular testing combined with careful clinical evaluation (including echocardiography) of all first-degree relatives is essential.

Previous studies report somatogonadal mosaicism in other HCTD, for example in Loeys–Dietz syndromes [17, 18] and vEDS [14]. We report here, to our knowledge, the first patient with a somatogonadal mosaicism in *COL5A1* responsible for the cEDS diagnosed in his son. Although the mosaicism was estimated with a VAF of 10–15% in blood, clinical examination did not reveal any signs of cEDS in the father.

In summary, our results show that half of MFS-affected individuals are sporadic, which is more frequent than usually expected. A significant proportion of these could be explained by parental post-zygotic mosaicism, which represents around 5% of probands with MFS. This first published estimation of parental mosaicism in MFS highlights the major impact for clinical practice of such mosaic variants and the importance of using analyses with enough sensitivity to detect them in our routine practice.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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ARTICLE

Homozygosity for a null allele of *COL3A1* results in recessive Ehlers–Danlos syndrome

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So far, mutations in the human *COL3A1* gene have been associated with the predominantly inherited Ehlers–Danlos syndrome (EDS), vascular type. Genotype–phenotype correlation perspectives collapsed, as haploinsufficiency, which was long suggested to confer a milder or unrecognized phenotype, was reported in four patients with a phenotype similar to that of vascular EDS. Here, we study a case of recessive EDS in a young consanguineous girl of healthy parents. She fulfilled the vascular EDS criteria for laboratory testing. Total sequencing of *COL3A1* cDNA identified a homozygous nucleotide duplication (c.479dupT) resulting in a premature termination codon (p.Lys161GlnfsX45). Studies in genomic DNA showed that this mutation was inherited from each parent. The expression analysis (RT-PCR, quantitative-PCR, immunohistochemistry, WB) showed strong mRNA decay and an absence of type III collagen in the proband. The expected *COL3A1* haploinsufficiency in her healthy ascendants did not lead to the manifestations of vascular EDS. This case provides evidence of a stochastic effect of *COL3A1* haploinsufficiency in humans, which could be explained by the relation between nonsense-mediated mRNA decay efficiency and the resulting dominant-negative effect depending on the position of the mutation and/or modifying factors. It opens up new perspectives for the understanding of *COL3A1* genotype–phenotype correlations, which is required while considering targeted therapy.

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Keywords: Ehlers–Danlos syndrome; *COL3A1*; recessive

Introduction

The Ehlers–Danlos syndrome (EDS) is defined by the triad: articular hypermobility, skin extensibility and tissue fragility. The vascular type (also known as type IV, MIM#130050, <http://www.ncbi.nlm.nih.gov/Omim/>)¹ is the most severe type, with a median survival below 50 years of age in the largest cohort published to date.² It is a

rare predominantly inherited disorder, caused by mutations in the *COL3A1* gene, which result in the synthesis of defective pro α 1(III) chains of type III procollagen.

The *COL3A1* gene belongs to the highly homologous family of fibrillar collagens, which have several aspects in common:³ a triple-helical domain characterized by repeating Gly-X-Y triplets encoded by 43 exons (in *COL3A1*, exon 4 and 5 are fused in a single exon 4) that invariably begin with a glycine codon and have a similar pattern of size. Thus, the deletion of a single exon or splice-site mutation mostly results in an in-frame-shortened protein. A complex posttranslational processing with the removal of precursor-specific telopeptides and trimer units that further aggregate with other collagens results in

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ordered fibril structures and finally in periodic bundles of collagen fibers.⁴ Thus, because type III collagen is a homotrimer, the synthesis of an equal amount of chains from a normal and a mutated allele (if stable) predicts the assembling of a 7:1 ratio of abnormal/normal molecules through a dominant-negative effect, leading to a strong disorganization of collagen fibers. Almost all reported mutations in the *COL3A1* gene (see the 'database of human type I and III collagen mutations' <http://www.le.ac.uk/genetics/collagen/>)⁵ match with this model. Although biases related to the screening methods for mutation detection are possible, this mutation spectrum, the analogy with the *COL1A1* and *COL1A2* mutations spectrum observed in osteogenesis imperfecta (MIM#120150 and 120160, <http://www.ncbi.nlm.nih.gov/Omim/>), and the findings from a mouse knockout model for *COL3A1*⁶ have long suggested that *COL3A1* null alleles could confer attenuated phenotypes. However, in 2001, the study by Schwarze *et al*⁷ questioned these genotype-phenotype correlations. In this study, four patients with a mutation described to result in haploinsufficiency had a clinical course similar to that in patients with classical missense or splice-site mutations. Consequently, the hope of an efficient gene-targeted therapy that could be based on the same premise as that of osteogenesis imperfecta⁸ collapsed. Here, we study a recessive case of vascular EDS observed in a consanguineous daughter of unaffected parents.

Participants and methods

Participants

The parents of the proband were uncle (II.3) and niece (III.1). The pedigree is depicted in Figure 1. The proband (IV.3) was an 11-year-old Caucasian female. She was delivered by cesarean section at 36 weeks of gestation because of the premature rupture of membrane. Neonatal examination noticed normal parameters and bilateral clubfeet (talipes equinovarus). The ages at which she started to sit and walk were 15 and 30 months, respectively, and language acquisition was normal. She had Absence epilepsy, and her brain MRI revealed a diffuse cortical dysplasia with a predominantly frontal location and ventricular dilatation. A cardiac ultrasound showed a pulmonary valve stenosis. On account of easy bruising, a search for thrombopathy and coagulopathy was undertaken, which showed normal results. The proband came to our medical attention at the age of 10 years because of a clinical assessment suggesting EDS (Figure 1). She had thin, translucent skin with marked dystrophic scars, early-onset varicose veins and articular hypermobility, notably of small joints. Her growth parameters were 30 kg 700 (0SD); 133 cm (-0.5SD) and 54 cm (head circumference; +1SD). Her face was long with up-slanting palpebral fissures, slightly hypoplastic alae nasi and thin lips with a flat philtrum. She also exhibited multiple gingival recessions.

A few weeks later, she presented acute abdominal pain related to small-bowel occlusion. Extreme intestinal, arterial and tissue fragilities were put to light during surgery that included the resection of approximately 1 m of necrotic and perforated jejunum. Owing to hemorrhagic shock, an ileostomy by default was carried out. An attempt at restoration of bowel continuity was carried out after the first ileostomy failed. Three small-bowel perforations occurred during manual manipulation. She died 6 days after the intervention from laparotomy suture break down and total evisceration. The parents declined a post-mortem examination.

The familial investigation failed to record any suggestive signs or medical event that could suggest an EDS manifestation. The careful physical exam of both 43-year-old parents and the 13-year-old brother (IV.2) was totally negative for the Villefranche criteria.¹ Premature death was traffic-related in participant II.1 and because of cancer in participants II.4 (throat) and III.2 (breast). The first born of the proband's parents (IV.1) died at 3 months of age from severe hypoxemia secondary to a diaphragmatic hernia (pathological data unavailable). No data were recorded for the common ancestral couple (participants I.1 and I.2).

Mutation analysis

Genetic and laboratory tests were carried out in the proband and her parents under conditions established by the French law, and appropriate written informed consents were collected. Blood samples were obtained from the proband and her parents. Skin fibroblasts and a frozen surgical sample of bowel (jejunum) were also collected from the proband. The parents refused skin biopsy. Human dermal fibroblasts (HDF) and a frozen jejunum sample were obtained as controls from the European Collection of Cell Culture (number: 06090715, 19 years old, Caucasian female) and from the Biological Resource Center of the Montpellier University Hospital (CHU Montpellier, France), respectively.

Total RNA was extracted using the RNeasy mini kit (Qiagen, Courtaboeuf, France) after fibroblasts were incubated for 24 h in the presence or absence of cycloheximide (CHX (100 µg/ml), Fluka, Buchs, Switzerland). Total RNA was converted into complementary DNA by means of an RT-PCR carried out by priming with random hexamers and SuperScript II Reverse transcriptase, according to the manufacturer's instructions (Invitrogen, Cergy-Pontoise, France). A control PCR was carried out to check for residual genomic DNA. Flexigene DNA and QIAamp DNA mini kits (Qiagen) were used to extract genomic DNA from blood and fibroblasts, respectively. Total cellular proteins were harvested from the cultured fibroblasts as described elsewhere.⁹

For mutation screening in the proband, all the coding sequences of the *COL3A1* gene (cDNA) were sequenced bidirectionally in 10 overlapping fragments. For mutation confirmation in the proband and her parents, sequencing

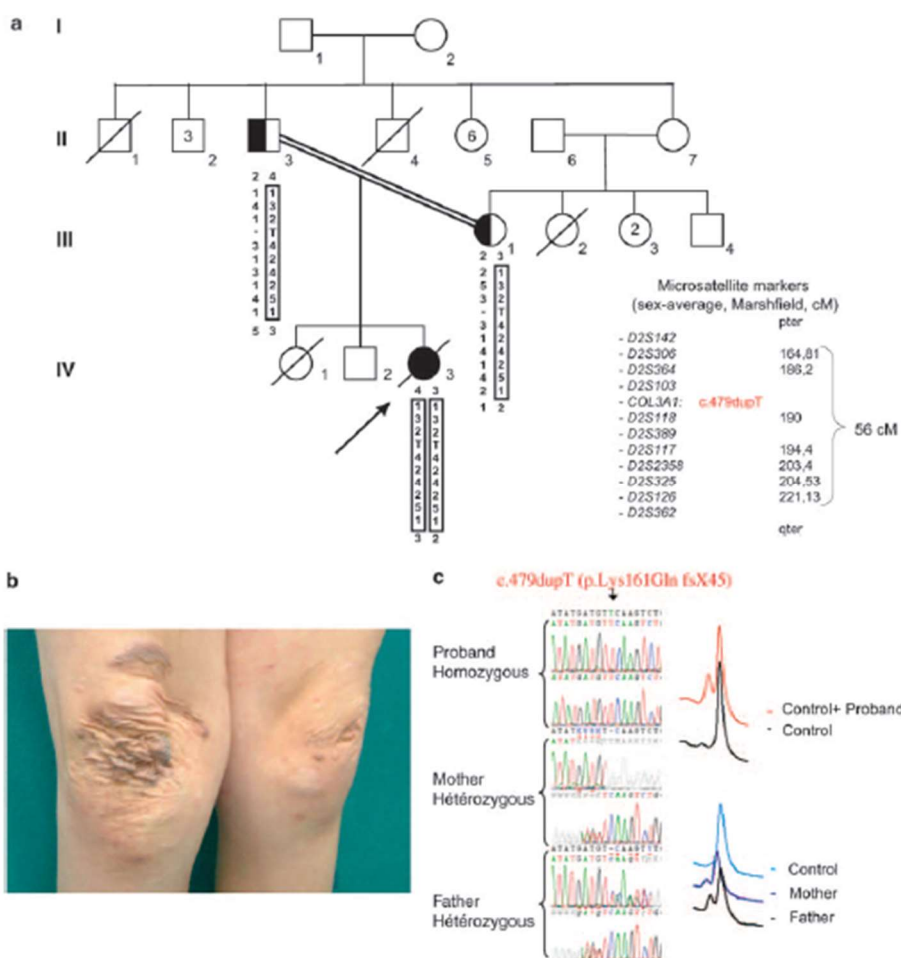


Figure 1 Pedigree, haplotype, clinical assessment and mutation status. (a) Haplotype study showing a large 56 cM chromosome 2 region (black boxed), inherited by descent in the proband (IV.3). Markers were purchased from Applied Biosystems, Courtabouef, France. Genotyping and analysis were carried out according to the manufacturer's instructions. Presence of the mutation, c.479dupT, is represented as a T in haplotype. (b) Characteristic Ehlers-Danlos syndrome (EDS) skin lesions of the Proband. (c) Electropherograms of COL3A1 exon 5 genomic sequences around the c.479dupT, and its detection with denaturing high-performance liquid chromatography (dHPLC, Transgenomic, Courtabouef, France) in genomic DNA (additional details in online-only material).

and denaturing high-performance liquid chromatography were carried out in genomic DNA. Chromosome 2 haplotype analysis was carried out to confirm biparental transmission. Finally, we carried out an expression analysis using RT-PCR, real-time quantitative PCR (LC-480, Roche, Mannheim, Germany), immunohistochemistry and immunoblotting. Methods, conditions, primer sequences

and antibody references are given in legends to figures and/or in online-only material. Exon numbering was carried out on the basis of the Reference Sequence NM_000090.3 (51 exons), as recommended by the Human Genome Variation Society guidelines.¹⁰ It is to be noted that, as stated above, our exon 4 may also be described as exon 4/5 in a historic numbering system (with 52 exons).



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COL3A1 mutation in recessive EDS
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Results

COL3A1 transcript sequences of the proband were homozygous and showed a duplication in exon 5: c. 479dupT. This duplication led to a frameshift and a premature stop codon (PTC) in exon 7: p.Lys16fsGlnfsX45. It was found in the heterozygous state in the DNA of her healthy parents, validating the homozygosity found in the proband. Moreover, the proband was found homozygous by descent for a large 56cM portion of chromosome 2, between micro-satellite markers, *D2S306* and *D2S126* (Figure 1). The predicted frameshift resulted in a sequence that did not contain a Gly-X-Y motif with a PTC at the very beginning of the triple-helical domain. To confirm that no collagen III could be synthesized in the proband, we performed several expression analysis experiments. First, the deposition of the RT-PCR products on 3% agarose gel showed that products were only detected in the proband after treatment with CHX (nonsense-mediated mRNA decay (NMD) inhibitor) (Figure 2). Second, real-time PCR and relative

quantification analysis showed a normalized ratio of 1.133 (± 0.211) in the HDF control in the absence of CHX, whereas this ratio was 0.0203 (± 0.00155) in the proband (that is, *COL3A1* transcripts were $<2.5\%$ of the control). In agreement with the first experiment, CHX exposure reestablished the expression level, with a normalized ratio of 1.728 (± 1.034) and 0.733 (± 0.18) in the control and proband, respectively (Figure 2). Third, western blotting showed no signal in the proband with an antibody against the N-terminal domain of the type III procollagen. Finally, the absence of collagen III synthesis in the proband was also sustained *in situ* by means of immunohistochemistry on jejunum samples (Figure 2).

Discussion

Mutations that confer an unusual pattern of inheritance in a gene related to a well-known genetic disease can

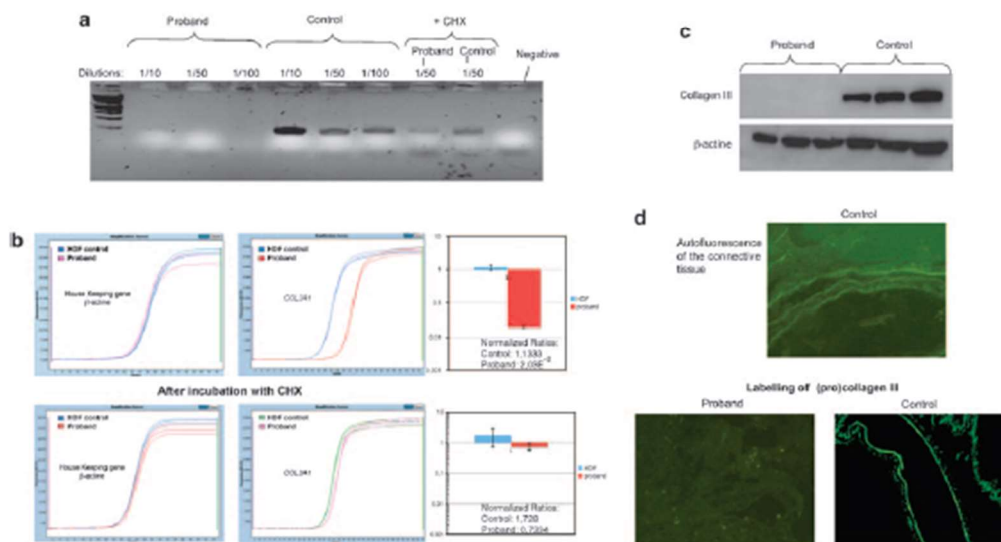


Figure 2 Expression analysis in proband. (a) RT-PCR products separated on an agarose gel. Amplification of 25 cycles of *COL3A1* exons 1–2 in the cDNA of a control individual (human dermal fibroblasts (HDF)) and the proband at different dilutions (1/10; 1/50 and 1/100) and with or without cycloheximide (CHX) treatment (only 1/50 dilution is shown). Although a specific transcript is detected in the control, none is detected in the proband without CHX treatment. (b) Real-time RT-PCR. PCR products of *COL3A1* exons 5–8 amplicon (primers in online only material) were quantified using the LightCycler 480 system with SYBR Green I Master Mix according to the manufacturer's instructions (Roche, Mannheim, Germany). The expression level of *COL3A1* was normalized to that of housekeeping genes, β -actin and *GAPDH* (Eurogentec, Angers, France). A calibrator sample (HDF) was used and experiments were repeated thrice. In each experiment, the samples were run in triplicates. The *COL3A1* expression ratio was calculated using the E-Method (Roche, Eurogentec, Angers, France). (c) Western blot analysis: Immunodetection of Type III procollagen by an antibody against the N-telopeptide (S17, Santa Cruz, Santa Cruz, CA, USA) and by an antibody against the reference gene, β -actin (AC-15, Santa Cruz). We loaded 25, 40 and 60 μ g of total proteins that were extracted from both the medium and the cell layer of the skin-cultured fibroblast from the proband and from the control. No (pro)collagen III was detected in the proband. (d) Immunohistochemistry: *in situ* detection of (pro)collagen III in frozen jejunum samples of the proband and control (48 years old, Caucasian female). In the proband, only autofluorescence of the connective tissue is detected after labeling with the S17 antibody (Santa Cruz), whereas the control showed intense labeling (concordant data were obtained with antibodies against the full-length collagen III triple-helical domain (FH-7A, Santa Cruz; data not shown)).

sometimes highlight a particular mechanism that is useful for correlating genotype to phenotype. For example, the efficiency of the NMD pathway can alter the pattern of inheritance in several genetic disorders.¹¹ We report here the characterization of a recessive inheritance of EDS linked to a *COL3A1* null allele, inherited by descent in a consanguineous child. Recessive inheritance of EDS type IV has been suggested in the past.¹² However, there were not enough data to discriminate real recessive cases from *de novo* occurrences, mosaicisms or even from marked phenotypic variability. It is interesting that the proband did not present a typical vascular-type EDS phenotype. First, she had diffuse cortical dysplasia and absence of epilepsy. This could suggest a phenotypic overlap with the EDS variant caused by mutations in the gene encoding Filamin A (MIM#300537). Moreover, this variant, as well as the vascular type, includes cardiovascular manifestations. However, the proband's MRI of the brain showed no periventricular nodular heteropia (a hallmark of this EDS variant). Considering the fact that numerous etiologies remain possible (including recessive disease), this overlap has to be interpreted with caution. Second, she had pronounced atrophic scars such as those found in classical EDS type. However, there are striking parallels between our observation and that in the mouse knockout model described by Liu *et al.*⁶ Homozygous mutant mice had a low-survival rate, with most deaths occurring within 2 days after birth. This could be consistent with the premature death of the proband's sister. The surviving homozygous mutant mice lived one-fifth of the normal life span. In addition to aneurysms, they displayed marked skin lesions, frequent intestinal enlargement and occasionally fatal intestinal rupture. This correlates with our observation in humans (although we cannot exclude another gene defect), suggesting a distinct phenotype for recessive inheritance. Finally, similar to heterozygous mice, the heterozygous parents were phenotypically normal. We could not extend the familial investigation to other relatives, however no evident vascular EDS manifestations were recorded. Therefore, it is likely that the common ancestor and potentially other relatives carrying the mutation also match the mouse model. Thus, heterozygous as well as homozygous individuals for *COL3A1* null alleles could present a distinct and unrecognized phenotype. Taken together, these points also support the classic dominant-negative mechanism as being the main basis for genotype–phenotype correlations in vascular EDS.

How could we conciliate these findings with the study of Schwarze *et al.*?⁷ It is unlikely, even if possible, that the authors failed to detect a mutation in *trans* in their reported patients (non-detected recessive cases). Therefore, two hypotheses remain to explain the discrepancy.

The first hypothesis refers to the relation between NMD efficiency and the resulting dominant-negative effect. Our findings, as well as those of Schwarze *et al.*,⁷ present

evidence of a strong mRNA decay by NMD. If admitted that NMD is rarely, if ever, 100% efficient,¹³ the pathogenicity of the mutation would be linked to its position. In their study, c.1832_1833delAA and c.555delT mutations occurred in the Gly-X-Y triple-helical domain. If translated, the little remnant peptide could be sufficient to alter the complex post-translational processes of the type III collagen through a dominant-negative effect. It is also consistent with their findings regarding the c.4294C>T mutation, which result in a truncated peptide (PTC in the last *COL3A1* exon). Finally, another explanation is necessary to modelize the effect of the remaining c.413delC mutation. Indeed, this mutation, similar to the c.479dupT mutation reported here, also predicts an absence of the Gly-X-Y major triple-helical domain. However, c.413delC also disrupts the Gly-X-Y repeats of the minor triple-helical domain of the N-telopeptide, which is known to be required for N-terminal proteinase recognition and cleaving.¹⁴ This is not the case with c.479dupT, which occurs in the microunfolded region (last part of the N-terminal domain). c.413delC could thus lead to a stronger interference with N-propeptide removal and this may explain the phenotype discordances.

The second hypothesis would be that (a) modifier gene(s)/factor(s) confer(s) a protective effect in the family described here. Such a mechanism has been documented to explain the marked variability of symptoms in other diseases. For example, for a same mutation in the *COL7A1* gene, different alleles of a frequent functional variant in the gene encoding the matrix-metalloproteinase 1 (also known as collagenase) have been described to produce different forms of recessive dystrophic epidermolysis bullosa.¹⁵ Such modifier genes could also support the marked variability observed in some vascular EDS pedigrees and in the discrepancy between haploinsufficiency consequences in the Schwarze study and in ours. *Cis*-acting elements could be more easily identified if there is a correlation between phenotype and variations in the expression of the wild-type allele. However, up to now, we have had no fibroblast cultures of the clinically unaffected heterozygous parents. It is interesting that these two hypotheses are not exclusive. Recently, NMD efficiency has been shown to vary between cell types, tissues and individuals with the growing idea that the NMD pathway could act as a modulator of genetic disease severity.¹⁶

The mechanisms by which mutations in the *COL3A1* gene produce diseases are still poorly understood. So far, all the mutations reported in *COL3A1* have been linked to an autosomal dominant pathway of inheritance. For the first time, we have described herein a recessive EDS case caused by an autozygous nonsense mutation in the *COL3A1* gene. This observation opens up new perspectives for genotype–phenotype correlations. Understanding why heterozygosity for a *COL3A1* null allele can result in an apparently normal phenotype in a mouse and, here, in humans could



offer seductive therapy perspectives for this life-threatening disease.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on *European Journal of Human Genetics* website (<http://www.nature.com/ejhg>)

*Letter to the Editor***Vitamin K Deficiency Embryopathy****To the Editor:**

Menger et al. [1997] presented the cases of three children with vitamin K deficiency embryopathy due to a disorder of embryonic vitamin K metabolism. We have had the opportunity to study a prenatal case, detected by ultrasound, with the same entity.

CLINICAL REPORT

Our case is a fetus of a young nonconsanguineous Portuguese couple. The mother, 25 years old and pregnant for the first time, was referred for a second opinion following the discovery of a thickness of the neck during an ultrasound study at 16 weeks of pregnancy. Ultrasound study at 17 weeks showed moderate lymphedema, mild dilatation of the cerebral ventricles, and hyperechogenicity of fetal bowel. The pregnancy ended spontaneously at 18 weeks and 3 days. The mother's tests carried out before the uterine evacuation showed a 36% prothrombin level (PTL) with factor II at 25%, factors VII and IX at 18%, and factor V at 91%. Considering that the patient had not taken any oral anticoagulants, these data indicated a vitamin K deficiency. After an injection of 10 mg of vitamin K, the evacuation took place.

The female fetus had pronounced frontonasal hypoplasia, a cystic hygroma, and metaphyseal spicules visible on the radiographs, and abdominal calcification (Fig. 1). Autopsy showed that frontonasal ossification was extremely deficient (Fig. 2), with long bones being abnormally short for gestational age. On the X-ray films, the metaphyses of the long bones (Fig. 3) are spiculated and when observed with a microscope the osseous trabeculae are abnormally wide.

The mother had undergone several abdominal operations for an idiopathic chronic intestinal obstruction which resulted in an ileocaecal resection, a duodenojejunostomy, and a cholecystectomy. After that the patient was followed by a nutritionist. The last tests 6 months before the beginning of the pregnancy were normal apart from a positive breath test, which led to the patient being treated with erythromycin and small

quantities of copper, zinc, and selenium. The PTL was 78%. At the beginning of the pregnancy the patient was taking erythromycin, arginine, α -tocopherol, folic acid, and ferrous sulphate sesquihydrate.

The biological tests following the evacuation showed that there was a rapid improvement in the vitamin K-dependent factors 24 hr after the injection of vitamin K1 (PTL = 68%, factors VII + X at 52% and factor V at 100%). There were also deficiencies in vitamin E (3.65 mg/l; minimum, 4 mg/l), retinol (0.07 mg/l; minimum, 0.4 mg/l), vitamin C (0.4 mg/l; minimum, 0.8 mg/l), serum carotene levels (α -carotene, 12 μ g/l; minimum, 130 μ g/l and β -carotene, 20 μ g/l; minimum, 205 μ g/l), and copper and zinc. There was no infection, in particular no viral infection.

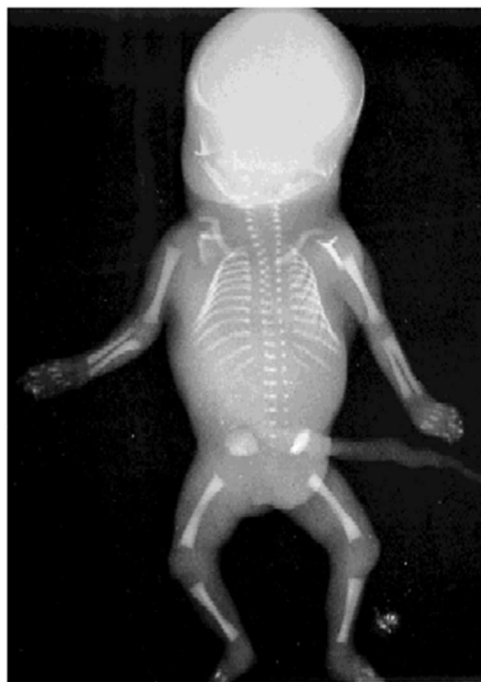


Fig. 1. Radiograph of the fetus.

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Fig. 2. Photograph of the fetus.

DISCUSSION

Our patient's deficiency in vitamin K has several causes: Her syndrome was linked to surgery and long-term antibacterial malabsorption treatment. During pregnancy daily needs of vitamin K increase by 20%, and a weak placental transfer may also occur [Suzuki et al., 1989; Israels and Israels, 1995].

This observation is the first to be made of a prenatal detection of this ailment. In our case the condition was particularly serious and the fetus did not survive. This case complements those described by Menger et al. [1997], supporting the hypotheses of Howe et al. [1995] drawn from their study of rats, among others [Howe and Webster, 1992].

The teratogenic period of the warfarin embryopathy is between the sixth and ninth weeks of amenorrhea [Howe et al., 1992]. Therefore we can postulate that besides the mechanism described by Franco et al. [1995] concerning arylsulfatase E, there is also an increased need of vitamin K, which is essential for frontonasal and metaphyseal ossification. Thus, we confirm that it is necessary to watch closely and supplement every women of child-bearing age who might have a vitamin K deficiency in order to prevent such an embryopathy, a vitamin K deficiency being certainly teratogenic at an early stage of pregnancy.



Fig. 3. Radiograph showing the long bones.

The long-term intake of erythromycin can be considered an aggravating factor in malabsorption of vitamin K caused by surgery, probably because it alters the bacterial intestinal flora that produces menaquinones (vitamin K2). Lipsy [1994] describes this aggravating phenomenon in relation with other antibiotics (containing methyl tetrazole-thiol) that inhibit vitamin K metabolism, notably the gamma carboxylation of glutamic acid. Phenytoin [Howe et al., 1995] and alcohol [Howe et al., 1992] have also been postulated to disturb vitamin K metabolism and have also some similar effects in their phenotype translation in children who have been exposed to them during pregnancy.

CONCLUSION

Vitamin K deficiency is teratogenic at an early stage of human pregnancy. We think it is important to more accurately describe these situations in order to prevent the embryopathies they can cause in women whose pregnancies have not been diagnosed yet.

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Muscle and Bone Impairment in Children With Marfan Syndrome: Correlation With Age and *FBN1* Genotype

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ABSTRACT

Marfan syndrome (MFS) is a rare connective tissue disorder caused by mutation in the gene encoding the extracellular matrix protein fibrillin-1 (*FBN1*), leading to transforming growth factor-beta (TGF- β) signaling dysregulation. Although decreased axial and peripheral bone mineral density (BMD) has been reported in adults with MFS, data about the evolution of bone mass during childhood and adolescence are limited. The aim of the present study was to evaluate bone and muscle characteristics in children, adolescents, and young adults with MFS. The study population included 48 children and young adults (22 girls) with MFS with a median age of 11.9 years (range 5.3 to 25.2 years). The axial skeleton was analyzed at the lumbar spine using dual-energy X-ray absorptiometry (DXA), whereas the appendicular skeleton (hand) was evaluated using the BoneExpert system (with the calculation of the Bone Health Index). Muscle mass was measured by DXA. Compared with healthy age-matched controls, bone mass at the axial and appendicular levels and muscle mass were decreased in children with MFS and worsened from childhood to adulthood. Vitamin D deficiency (<50 nmol/L) was found in about a quarter of patients. Serum vitamin D levels were negatively correlated with age and positively correlated with lumbar spine areal and volumetric BMD. Lean body mass (LBM) Z-scores were positively associated with total body bone mineral content (TB-BMC) Z-scores, and LBM was an independent predictor of TB-BMC values, suggesting that muscle hypoplasia could explain at least in part the bone loss in MFS. Patients with a *FBN1* premature termination codon mutation had a more severe musculoskeletal phenotype than patients with an inframe mutation, suggesting the involvement of TGF- β signaling dysregulation in the pathophysiologic mechanisms. In light of these results, we recommend that measurement of bone mineral status should be part of the longitudinal clinical investigation of MFS children. © 2015 American Society for Bone and Mineral Research.

KEY WORDS: MARFAN SYNDROME; FIBRILLIN-1; TGF- β ; BONE MINERAL DENSITY; DXA; PUBERTY

Introduction

Marfan syndrome (MFS) (MIM 154700) is a rare autosomal dominant connective tissue disorder involving the cardiovascular (aortic root dilatation and mitral valve prolapse), ocular (ectopia lentis and myopia), and skeletal (disproportionate tall stature, arachnodactyly, pectus deformity, and scoliosis) systems.⁽¹⁾ It is caused by mutation in the gene encoding the extracellular matrix protein fibrillin-1 (*FBN1*). Although it was first believed that quantitative and/or qualitative abnormalities in *FBN1* underlie the different manifestations, recent studies have demonstrated the implication of transforming growth factor-beta (TGF- β) signaling

dysregulation in the pathophysiology of MFS.⁽²⁾ Thus, *FBN1* regulates the bioavailability of TGF- β through its interaction with latent TGF- β binding proteins (LTBPs), and loss of *FBN1* leads to a release of TGF- β , which activates the TGF- β signaling pathway. Interestingly, downregulation of TGF- β signaling, via TGF- β neutralizing antibodies or losartan, an angiotensin II receptor antagonist, rescues the different manifestations of MFS in *Fbn1*-deficient mice, including aortic root dilatation.⁽³⁾ Several trials are currently being conducted worldwide to test the potential benefit of losartan in patients with MFS.⁽⁴⁾

Skeletal findings are frequent in MFS and are thought to be caused by disproportionate overgrowth of the long bones.⁽⁵⁾

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Several studies of adults with MFS have also reported decreased axial and peripheral bone mineral density (BMD),^(6–13) suggesting an increased risk of fractures. However, the precise incidence of fractures in patients with MFS is still unknown.

Three studies using dual-energy X-ray absorptiometry (DXA) suggested that low bone mass is already present in MFS children,^(7,9,14) but they were limited by the small number of patients and the absence of information about calcium intake, vitamin D status, and bone turnover markers. Moreover, in two of these studies, patients with MFS (who have tall stature) were compared with healthy age-matched individuals of normal height. Many parameters for bone mass are height-dependent and, therefore, problems with the interpretation of results can arise when comparing groups that differ in height.⁽¹⁵⁾ Last, the effect of MFS on muscle-bone relationships has never been specifically addressed.

The goal of the present study was to evaluate the bone and muscle characteristics in children, adolescents, and young adults with MFS. The axial skeleton was analyzed at the lumbar spine using DXA, whereas the appendicular skeleton (hand) was evaluated using the BoneXpert system. Muscle mass was measured by DXA.

Materials and Methods

Patient population

This cross-sectional study comprised patients followed by the multidisciplinary team for MFS at Purpan University Hospital in Toulouse, France. Inclusion criteria were a diagnosis of MFS according to the new Ghent nosology⁽¹⁶⁾ and age from 5 to 25 years. Patients receiving corticosteroids or any other medications affecting bone metabolism were excluded.

A total of 48 patients (22 girls, 26 boys) with a median age of 11.9 years (range 5.3 to 25.2 years) were included. Forty patients (83%) had a mutation in the *FBN1* gene.

For the purpose of the present analysis, three patient groups were distinguished according to growth status. The "children group" consisted of 23 children who had not started puberty, the "adolescent group" consisted of 15 children who had entered puberty (defined as Tanner stage ≥ 2), and the "young adult group" consisted of 10 patients who had achieved their final height (defined as growth velocity < 2 cm per year or bone age > 16 years in girls and 18 years in boys).

The study protocol was approved by the Research Ethics Committees of the Purpan University Hospital in Toulouse, France. Written informed consent was obtained from parents and from children older than 12 years. Assent was obtained from children younger than 12 years.

Personal history and clinical evaluation

Cardiac, ocular, and orthopedic histories were recorded for all subjects. Echocardiographic measurements (outflow tract diameter, sinuses of Valsalva, sinotubular junction, and tubular ascending aorta) were also collected and converted to Z-scores.⁽¹⁷⁾

Height was measured using a Harpenden stadiometer (Holtain, Crymch, UK). Weight was determined using digital electronic scales for infants and mechanical scales for older children and adults (Healthometer, Bridgeview, IL, USA). Body mass index (BMI) was calculated as the ratio of weight in kg divided by the square of height in meters. Height, weight, and BMI measurements were converted to age- and sex-specific Z-scores on the basis of published reference data. Pubertal

development was assessed by physical examination and scored by the Tanner criteria. The onset of puberty was defined clinically by the appearance of breast development in girls and a testicular volume of 4 mL or more in boys.

Biochemical measurements

Blood samples were drawn in the fasting state.

Total, HDL, and LDL cholesterol, triglyceride concentrations, as well as plasma total calcium, phosphate, and alkaline phosphatase were measured using standard colorimetric or colorimetric enzymatic methods on the Cobas 8000 modular analyzer series, using the C701 module, from Roche Diagnostics (Mannheim, Germany); the results were converted to age- and sex-specific Z-scores.⁽¹⁸⁾

Serum concentration of total 25OH vitamin D was measured by chemiluminescent immunoassay method on the Cobas 8000 modular analyzer series, using the E602 module, from Roche Diagnostics. Bone alkaline phosphatase was measured by enzyme immunoassay supplied by Quidel (San Diego, CA, USA), and serum CTX was measured with IDS-iSYS immunoassay system using chemiluminescent detection; the results were converted to age- and sex-specific Z-scores on the basis of published reference data.⁽¹⁹⁾

Sequence analysis of the *FBN1* gene

Mutation screening, allowing the scanning of the 65 *FBN1* exons and flanking regions including the splice sites up to the branching regions, was performed in patients with a robot-assisted and single-condition PCR/direct sequencing strategy, as described elsewhere.⁽²⁰⁾

Mutation numbering refers to the *FBN1* cDNA GenBank reference sequence: NM_000138.4, with the A of the ATG translation initiation codon as nucleotide +1 (www.hgvs.org/mutnomen).

Two major mutation categories were distinguished: premature termination codons (PTCs) and inframe mutations. PTC mutations were classified as those that would be likely to produce no or an instable/truncated *FBN1* remnant peptide (deletion of the whole gene, frameshifts, stop codons, and out-of-frame splice mutations), whereas inframe mutations were classified as missense mutations, inframe deletions/duplications, or inframe splice mutations.

Bone health index

The radiographs of the left hand and wrist were collected as Dicom-files. Images were analyzed using the BoneXpert system (BoneXpert, version 2.1.0.12, Visiana, Holte, Denmark) that automatically generates the following outcome variables: bone age based on Greulich and Pyle's method, Z-scores of bone age (compared with a healthy reference population), the bone health index (BHI), and Z-scores of BHI.⁽²¹⁾ The Z-score is computed relative to normal children of the same bone age and sex, except when the bone age is above 15 for girls and 17 for boys; then the Z-score is computed relative to normal children of the same age and sex.

BHI is calculated using the cortical thickness (T) of the three middle metacarpals and the metacarpal width (W) and length (L) to compensate for the variation in stature of growing children, using the following formula: $BHI = \pi \times T \times (1 - T/W) / (L \times W)^{0.33}$.

As a result, BHI can be considered as an estimate of cortical volume at the metacarpal level, corrected for the length and width of the bones.

Dual-energy X-ray absorptiometry

Bone mineral content (BMC), fat body mass (FBM), and lean body mass (LBM) of the total body, as well as lumbar spine (L₂ to L₄) areal bone mineral density (LS-aBMD, unit: mg/cm²) were measured by DXA using a Lunar Prodigy device (GE Healthcare, Piscataway, NJ, USA). Fat body mass was expressed as a percentage of total body mass. In two patients who had hardware inserted for scoliosis surgery, lumbar spine densitometry could not be performed. An estimate of three-dimensional bone density, commonly called volumetric BMD (vBMD, unit: mg/cm³), was calculated as described by Kroger and colleagues⁽²²⁾ using the formula: vBMD = aBMD × [4 / (π × width)], width being the mean width of vertebral bodies L₂ to L₄.

The LS-aBMD results were transformed to age-specific Z-scores using data provided by the densitometer manufacturer. The lumbar spine vBMD (LS-vBMD), percentage of fat body mass, and absolute lean body mass results were transformed to age-specific Z-scores using data published by van Der Sluis and colleagues.⁽²³⁾

Statistical analyses

The raw results were transformed to age- and sex-specific Z-scores from the average result in the reference population using the published reference data cited in the description of the measurement techniques. The expected mean result of these transformed values in a healthy population is 0. The significance of the difference from 0 was calculated by the one-sample t test.

Differences between the groups were tested for significance using Mann-Whitney's U test for pairwise group comparisons and the Kruskal-Wallis test for comparisons between more than two groups. After comparison between the prepubertal, pubertal, and adult groups using the Kruskal-Wallis test, the three groups were compared two by two using Mann-Whitney's U test with Bonferroni's adjustment to adjust for multiple testing; in this case, a p value <0.0167 (0.05 divided by three) was considered significant.

Group differences in dichotomous variables were tested for significance using the chi-square test.

Associations are given as Pearson correlations or Spearman rank correlations, as appropriate.

Multiple regression analysis was used to assess potential predictors of 25OH D levels. Age, sex (coding: male = 1; female = 2), Marfan severity (coding: absence of ectopia lentis and aortic root dilatation = 1; presence of ectopia lentis or aortic root dilatation = 2; presence of ectopia lentis and aortic root dilatation = 3), and season (coding: 1 = autumn, 2 = winter, 3 = spring, 4 = summer) were introduced as independent variables.

Multiple regression analysis was also used to assess potential predictors of total body BMC (TB-BMC). Age, sex (coding: male = 1; female = 2), Marfan severity (coding: absence of ectopia lentis and aortic root dilatation = 1; presence of ectopia lentis or aortic root dilatation = 2; presence of ectopia lentis and aortic root dilatation = 3), type of *FBN1* mutation (coding: PTC mutation = 1; inframe mutation = 2), 25OH D levels (nmol/L), and LBM (gr) were introduced as independent variables.

The effect of potential predictor variables was assessed in the stepwise mode.

All tests were two-tailed, and throughout the study p < 0.05 was considered significant. These calculations were performed using the SPSS software, version 11.5 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Clinical, biochemical, and radiological characteristics were similar in girls and boys (Supplemental Table S1), and, therefore, the results of the patients were analyzed as a single group.

Medical and surgical patient histories

The cardiac, ocular, and orthopedic histories are summarized in Table 1.

No patient reported a history of nontraumatic fractures; however, 5 patients had experienced fractures of wrists (2 patients), clavicle, radius, or tibia with trauma.

Clinical and biochemical data (Table 2)

As expected, compared with age- and sex-specific reference ranges, MFS patients on average were taller (median Z-score: 3; p < 0.0001) and had lower BMI (median Z-score: -1.4; p < 0.0001). Thirty-six patients (75%) had tall stature defined as a height Z-score above 2, and 17 patients (35%) had leanness defined as a BMI Z-score below -2. Tall stature and BMI Z-scores were unchanged from childhood to adulthood.

All patients had appropriate pubertal development for age. Fasting lipid blood work was available in 22 (46%) patients. Compared with the age- and sex-specific reference ranges, MFS patients on average had normal triglycerides levels (median Z-score: -0.2, range: -2.8 to 2.7; p = 0.7411), lower cholesterol levels (median Z-score: -0.6, range: -2.4 to 0.5; p = 0.0002), lower LDL levels (median Z-score: -1.4, range: -3.6 to -0.4; p < 0.0001), and normal HDL levels (median Z-score: 0.4, range: -1.2 to 2.1; p = 0.0852).

Mineral homeostasis parameters (ie, serum total calcium, phosphate, and alkaline phosphatase) were in the normal range for all patients.

The median serum vitamin D concentration was 72 nmol/L (range: 15 to 217 nmol/L) with vitamin D deficiency (defined as concentrations <50 nmol/L) in 12 patients (27%) and vitamin D insufficiency (defined as concentrations from 50 to 75 nmol/L) in 11 patients (25%).

Serum vitamin D levels were negatively correlated with age (r = -0.332; p = 0.0273) (Fig. 1). Frequency of vitamin D deficiency increased from childhood to adulthood (18% in the

Table 1. Cardiac, Ocular, and Orthopedic Histories of Patients With Marfan Syndrome

	n	Total
Ectopia lentis (%)	47	23 (49%)
Lens surgery (%)	46	8 (17%)
Aortic root dilatation (%)	45	18 (40%)
Outflow tract diameter (Z-score)	45	1.1 (-1.3; 5.6)
Sinuses of Valalva (Z-score)	45	1.7 (-2.4; 4.4)
Sinotubular junction (Z-score)	45	0.5 (-2.5; 6.3)
Tubular ascending aorta (Z-score)	45	0.5 (-3; 5.1)
Beta blocker therapy (%)	43	27 (63%)
Aortic dissection (%)	48	0
Cardiac surgery (%)	48	1 (2%)
Scoliosis (%)	47	20 (43%)
Pectus (%)	48	32 (67%)
Pneumothorax (%)	48	5 (10%)

Values are medians (range).

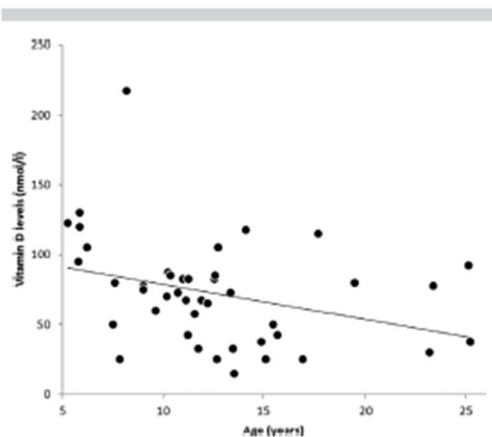


Fig. 1. Relationship between serum 25-hydroxy vitamin D levels and age in patients with Marfan syndrome ($r = -0.332$; $p = 0.0273$).

prepubertal group, 29% in the pubertal group, and 50% in the young adult group), but this difference was not statistically significant.

Serum vitamin D levels were also positively correlated with LS-aBMD Z-scores ($r = 0.477$; $p = 0.0014$) and LS-vBMD Z-scores ($r = 0.351$; $p = 0.0239$) but not with BHI.

Multiple regression analysis was performed to evaluate which putative determinants (age, sex, MFS severity, and season) were independently associated with 25OH D levels. This revealed that age ($p = 0.0455$) and MFS severity ($p = 0.0386$), but not season or sex, were significant independent predictive factors of 25OH D levels. In this model, the regression equation was: serum 25OH D levels (nmol/L) = $-1.3 \times \text{age (years)} + 7 \times \text{MFS severity} + 38.6$.

Compared with age- and sex-specific reference ranges, MFS patients had higher levels of bone alkaline phosphatase (median Z-score: 0.5; $p = 0.011$) and CTX (median Z-score: 2.2; $p < 0.0001$) (Table 2). Bone turnover markers were correlated with all bone parameters (ie, TB-BMC, LS-aBMD, LS-vBMD, and BHI).

Radiological measurements (Table 2)

Compared with age- and sex-specific reference data, FBM was normal ($p = 0.8025$) and LBM was significantly decreased ($p < 0.0001$). LBM Z-scores were negatively correlated with age ($r = -0.541$; $p = 0.0001$), underlining that the decrease in muscle mass was exacerbated from childhood to adulthood (Fig. 2).

TB-BMC, LS-aBMD, LS-vBMD, and BHI Z-scores were significantly decreased ($p = 0.0086$, 0.0057, 0.0360, and 0.0062, respectively) (Table 2). This decrease was more pronounced at the end of growth.

Muscle and bone parameters showed no difference between patients with and without beta blocker therapy and with and without aortic root dilatation (Supplemental Tables S2 and S3). In contrast, compared with patients with ectopia lentis, patients with normal ocular examination had significantly lower LBM, LS-aBMD, and LS-vBMD Z-scores ($p = 0.0085$, 0.0046, and 0.0117, respectively). Although TB-BMC Z-score values tended to be lower in patients without ectopia lentis, this did not reach significance ($p = 0.060$) (Supplemental Table S4).

Genotype-phenotype relationships

As shown in Table 3, patients with PTC mutations had significantly lower TB-BMC, LS-aBMD, and LS-vBMD Z-scores compared with patients with inframembranous mutations. The LBM Z-score was lower in the patients with PTC mutations, but this did not reach significance.

The type of mutation and the main ocular, cardiac, and orthopedic manifestations of the patients are summarized in Supplemental Table S5.

Table 2. Clinical, Biochemical, and Radiological Results of Patients With Marfan Syndrome

Variable	n	Total	p^1	n	Prepubertal	n	Pubertal	n	Young adult	p^2
Sex (female/male)	48	22/26		23	5/18	15	10/5	10	8/2	
Age (years)	48	11.9 (5.3; 25.2)		23	9.0 (5.3; 13.5) ^{a,b}	15	12.7 (10.8; 16.6) ^c	10	19.4 (15.7; 25.2)	<0.0001
Height (Z-score)	48	3.0 (-2.0; 4.9)	<0.0001	23	3.1 (-0.3; 4.9)	15	3.1 (0.2; 4.9)	10	2.4 (-2.0; 4.3)	0.1752
Body mass index (Z-score)	48	-1.4 (-3.7; 2.2)	<0.0001	23	-1.6 (-3.6; 2.2)	15	-0.7 (-3.7; 1.5)	10	-1.6 (-3.6; 1.2)	0.5842
Serum 25OH vitamin D (nmol/L)	44	72 (15; 217)		23	77 (25; 217)	15	67 (15; 117)	8	60 (25; 115)	0.2005
BAP (Z-score)	40	0.5 (-2.6; 3.7)	0.011	20	0.5 (-1.1; 2.6)	11	0.1 (-2.6; 3.7)	9	0.6 (-1.2; 3.4)	0.8196
CTX (Z-score)	39	2.2 (-1.0; 4.8)	<0.0001	20	2.3 (-1.0; 4.8)	13	2.2 (-1.0; 3.8)	6	1.1 (-0.5; 2.7)	0.4035
Fat body mass % (Z-score)	44	0.2 (-4.5; 2.7)	0.8025	23	0.4 (-4.5; 2.2)	15	0.0 (-2.4; 2.0)	9	0.3 (-2.1; 2.7)	0.5183
Lean body mass (Z-score)	43	-1.0 (-4.1; 3.5)	<0.0001	23	-0.8 (-2.0; 1.5) ^b	15	-1.3 (-3.2; 3.5)	8	-2.1 (-4.1; 0.0)	0.0736
Total body BMC (Z-score)	44	-0.6 (-2.5; 2.7)	0.0086	20	-0.5 (-1.6; 2.0)	15	-0.2 (-2.5; 2.7)	9	-0.9 (-2.4; 1.1)	0.4569
LS-aBMD (Z-score)	45	-0.9 (-2.9; 2.4)	0.057	23	-0.9 (-2.0; 2.4)	15	-0.9 (-2.8; 0.7)	9	-1.1 (-2.9; 0.8)	0.4896
LS-vBMD (Z-score)	44	-0.9 (-3.2; 2.0)	0.0360	23	-0.6 (-1.6; 2.0) ^b	15	-0.6 (-3.2; 1.1)	9	-1.7 (-2.9; 0.2)	0.0440
Bone health index (Z-score)	36	-0.8 (-2.9; 1.5)	0.0062	19	-0.7 (-2.5; 1.5)	12	-0.8 (-2.9; -0.1)	5	-1.0 (-1.9; 0.4)	0.7759

BAP = bone alkaline phosphatase; BMC = bone mineral content; LS-aBMD = areal bone mineral densitometry of the lumbar spine; LS-vBMD = volumetric bone mineral densitometry of the lumbar spine.

Values are medians (range). The p^1 values represent the comparison with normal reference data (one-sample *t* test). The p^2 values represent the significance of the difference between the three groups (Kruskal-Wallis test).

Results of post hoc analyses: ^asignificantly different between prepubertal and pubertal groups; ^bsignificantly different between prepubertal and adult groups; ^csignificantly different between pubertal and adult groups.

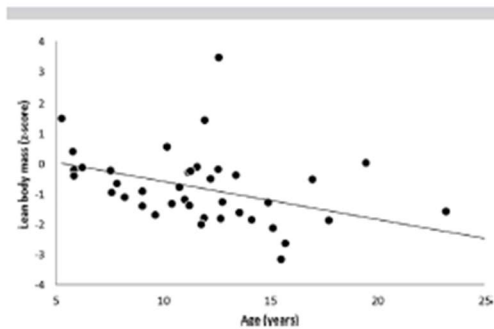


Fig. 2. Relationship between lean body mass and age ($r = -0.541$; $p = 0.0001$).

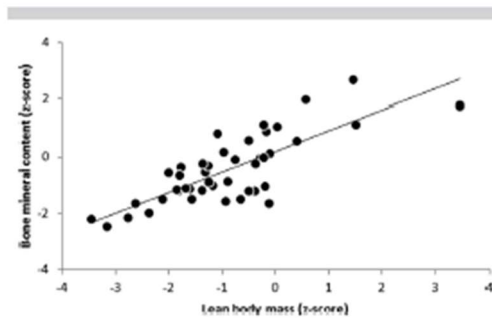


Fig. 3. Relationship between lean body mass and total body bone mineral content ($r = 0.797$; $p < 0.0001$).

Relationships between muscle mass and bone mineral content

LBM Z-scores were positively associated with TB-BMC Z-scores ($r = 0.797$; $p < 0.0001$) (Fig. 3). Multiple regression analysis was performed to evaluate whether LBM values were independent predictors of BMC values after accounting for age, sex, MFS severity, type of mutation, and vitamin D levels. This revealed that age, sex, and LBM were independently associated with whole-body BMC ($p < 0.0001$ for LBM, $p = 0.0123$ for age, and $p = 0.0191$ for sex), but not with MFS severity, mutation category, or vitamin D levels.

The regression equation was: $BMC = 0.054 \times LBM$ (gr) $+ 26.746 \times \text{age}$ (years) $+ 220.269 \times \text{gender} - 658$.

Discussion

In this study, we found that about a quarter of the children and young adults with Marfan syndrome had vitamin D deficiency (< 50 nmol/L). Serum levels of 25OH D decreased with age, and lower serum 25OH D levels were associated with lower LS-BMD Z-scores.

The proportion of MFS patients with vitamin D deficiency was similar to the findings in children and adolescents with bone

fragility⁽²⁴⁾ and osteogenesis imperfecta,⁽²⁵⁾ but was lower than that reported in healthy children living at the same latitude.^(26,27) This difference with healthy children might be because of a generally higher awareness of the importance of vitamin D in patients with MFS. The finding that the serum 25OH D levels were lower in the teenagers of our population than in the younger children has already been described in North American and European youths.⁽²⁷⁻²⁹⁾ Similar to the findings in healthy school children,^(30,31) we observed that serum 25OH D levels were independently associated with LS-BMD Z-scores in MFS patients, underlining the importance of supplementing these patients to prevent bone loss.

Compared with healthy age-matched controls, the children and young adults with MFS had lower bone mass at the axial and appendicular levels, which is in accordance with previous studies of children with MFS.^(7,9,14) Interestingly, this finding was unchanged for bone size-independent parameters (ie, LS-vBMD and BHI), which take into account the tall stature of MFS patients. This decrease in bone mineral content was already present in younger children and worsened from childhood to adulthood, leading to decreased peak bone mass.

Childhood is a critical period for the acquisition of bone mass.⁽³²⁾ This bone mass increases until the age of 25 years,

Table 3. Clinical, Biochemical, and Radiological Results of Patients According to Genotype

Variable	n	PTC mutations	n	Inframe mutations	p
Sex (female/male)	16	9/7	22	9/13	
Age (years)	16	14.5 (5.8; 25.2)	22	11.7 (5.3; 25.1)	0.0978
Height (Z-score)	16	2.7 (-0.3; 4.9)	22	3.0 (-2.0; 4.9)	0.6898
Body mass index (Z-score)	16	-1.9 (-3.7; 0.7)	22	-0.6 (-3.6; 1.9)	0.0625
Serum 25OH vitamin D (nmol/L)	15	65 (15; 130)	21	72 (25; 217)	0.7121
BAP (Z-score)	11	0.6 (-1.1; 2.8)	20	0.6 (-2.7; 3.7)	0.5915
CTX (Z-score)	11	1.9 (-1.0; 3.8)	19	2.3 (-1.0; 4.8)	0.1752
Fat body mass % (Z-score)	14	-0.1 (-2.4; 2.1)	21	0.3 (-4.5; 2.0)	0.1430
Lean body mass (Z-score)	14	-1.6 (-3.2; 0.0)	21	-0.7 (-3.5; 3.5)	0.0923
Total body BMC (Z-score)	14	-1.2 (-2.5; 1.1)	21	-0.1 (-2.2; 1.8)	0.0027
LS-aBMD (Z-score)	14	-1.5 (-2.8; 0.7)	21	-0.7 (-1.9; 2.4)	0.0018
LS-vBMD (Z-score)	14	-1.5 (-3.2; 1.1)	21	-0.4 (-1.6; 2.0)	0.0016
Bone health index (Z-score)	10	-0.4 (-2.9; 1.2)	19	-1.0 (-2.6; 1.5)	0.5059

Values are medians (range). The p values represent the significance of the difference between the two groups (Mann-Whitney test).

when it reaches its maximum value, the peak bone mass. Peak bone mass is correlated with the risk of developing osteoporosis in later life: The lower the peak bone mass is, the greater the risk of osteoporosis. Although none of our patients had sustained pathological fractures, they may be at risk in later life. The incidence of fractures in MFS adults is unknown and needs further evaluation.

A striking result of our study was the correlation between bone involvement and genotype. Two major mutation categories have been reported in the *FBN1* gene: PTC mutations, predicting haploinsufficiency (quantitative defect), and inframe mutations, predicting a dominant negative effect (qualitative defect). Several studies have reported that PTC mutations are associated with a more severe skeletal (ie, pectus deformity, scoliosis, joint laxity, dolichostenomelia, and arachnodactyly) and skin phenotype, whereas inframe mutations are associated with ectopia lentis.^(33,34) In line with these findings, we observed that the decrease in bone mass was more severe in the patients with PTC mutations. Interestingly, low bone mass and skeletal fragility have been described in Loey-Dietz syndrome, an overlapping syndrome with MFS caused by loss-of-function mutations in the TGF- β receptor, paradoxically leading to TGF- β activation, whereas ectopia lentis is absent in this syndrome.^(35,36) This suggests that abnormal TGF- β signaling activation may underlie bone involvement, whereas structural defects in *FBN1* fibers may underlie ocular involvement. The relationship between bone involvement and TGF- β signaling dysregulation has also been suggested by mouse models. Indeed, *Fbn1*-deficient mice develop osteopenia owing to TGF- β -driven osteodystogenesis,⁽³⁷⁾ which is in accordance with the higher bone resorption marker levels⁽³⁸⁾ observed in our study. In this mouse model, losartan treatment had no impact on bone loss. Similarly, low bone mass was also observed in mice with increased TGF- β production,⁽³⁹⁾ whereas mice with reduced TGF- β signaling had increased bone mass.⁽⁴⁰⁾

We demonstrated that children and young adults with MFS had lower muscle mass compared with healthy age-matched controls. Because muscle size correlates with height,⁽⁴¹⁾ it is likely that the decrease in muscle mass would be more severe if our patients were compared with height-matched healthy subjects.

Poor muscle development was noted by Marfan in his original description. Since then, several studies have reported a decrease in skeletal muscle mass and strength in adults with MFS.^(42–45) Histological analyses of skeletal muscle from patients with MFS and *Fbn1*-deficient mice have shown a decrease in the number and size of myofibers associated with an increase in fragmented fibers.^(42,46) More recently, it was shown that the TGF- β signaling pathway is involved in muscle growth regulation and that chronic activation of TGF- β is linked to muscle atrophy and impaired muscle regeneration.⁽⁴⁷⁾ Interestingly, when the TGF- β signaling pathway was downregulated via TGF- β neutralizing antibodies or losartan, *Fbn1*-deficient mice exhibited normal muscle architecture and regeneration.⁽⁴⁶⁾ Depending on the risk of aortic dissection, most patients with MFS report that they modify their physical activities as a result of their diagnosis,⁽⁴⁸⁾ and this may worsen the primary muscle defect.

In our study, muscle mass was an independent predictive factor of bone mass, suggesting that muscle hypoplasia could explain at least in part the bone loss in MFS. Indeed, the mechanostat theory postulates that bone adapts to the mechanical forces to which it is subjected in order to keep the strain on the bone at a constant set point (functional

muscle-bone unit).⁽⁴⁹⁾ Thus, a decrease in muscle mass corresponds to a decrease in bone strength. In this case, individuals with MFS may benefit from physical training. It can, therefore, be proposed that in the absence of severe cardiovascular or musculoskeletal disease, patients with MFS should participate in sports and exercise, with the exception of contact sports. Exercise programs starting in childhood need to be investigated for individuals with MFS, keeping in mind the relationship of such exercise to quality of life, peak bone mass, and cardiovascular complications.

Several recent clinical trials conducted in patients with MFS demonstrated that losartan was as efficient as atenolol in slowing down aortic root dilatation.⁽⁵⁰⁾ A secondary effect of this treatment may be improved muscle and bone mass.

Although our study is original, it has several limitations. The sample size was limited, despite it being greater than that of previous studies of children with MFS. Moreover, physical activity and muscle strength were not investigated.

Compared with healthy age-matched controls, bone mass at the axial and appendicular levels and muscle mass were decreased in children with MFS and worsened from childhood to adulthood.

Muscle mass was an independent predictive factor of bone mass, suggesting that muscle hypoplasia could explain at least in part the bone loss in MFS. Patients with a *FBN1* premature termination codon mutation had a more severe musculoskeletal phenotype than patients with an inframe mutation, suggesting the involvement of TGF- β signaling dysregulation in pathophysiologic mechanisms.

Measurement of bone mineral status should be part of the longitudinal clinical investigation of MFS patients. In light of our findings, we recommend optimizing bone mineral acquisition in childhood by providing adequate calcium and vitamin D intake and safe weight-bearing activity.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: Study design and study conduct: TE. Data collection: TE and EH. Mutation screening: PKVK and AP. Data analysis: TE and EH. Data interpretation: TE, PKVK, and EH. Drafting manuscript: TE, PKVK, and EH. Revising manuscript content: TE, EH, PKVK, JPS, MT, IG, AP, SJ, and YD. Approving final version of manuscript: TE, EH, PKVK, JPS, MT, IG, AP, SJ, and YD. TE takes responsibility for the integrity of the data analysis.

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CLINICAL REPORT

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Clinical and genetic data of 22 new patients with *SMAD3* pathogenic variants and review of the literature

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Abstract

Background: Pathogenic *SMAD3* variants are responsible for a cardiovascular phenotype, mainly thoracic aortic aneurysms and dissections. Precocious identification of the vascular risk such as aortic dilatation in mutated patients has a major impact in terms of management, particularly to avoid dissection and sudden death. These vascular damages are classically associated with premature osteoarthritis and skeletal abnormalities. However, variable expressivity and incomplete penetrance are common with *SMAD3* variants.

Methods: To investigate the clinical variability observed within *SMAD3* patients, we reviewed the phenotypic and genetic data of 22 new patients from our Centre and of 133 patients reported in the literature. From this cohort of 155 mutated individuals, we first aimed to delineate an estimated frequency of the main clinical signs associated with *SMAD3* pathogenic variants and, then, to look for genotype-phenotype correlations, mainly to see if the aortic phenotype (AP) could be predicted by the *SMAD3* variant type.

Results: We showed, herein, the absence of correlation between the *SMAD3* variant type and the occurrence of an AP in patients.

Conclusion: Therefore, this report brings additional data for the genotype-phenotype correlations of *SMAD3* variants and the need to explore in more detail the effects of genetic modifiers that could influence the phenotype.

KEYWORDS

Aneurysms-Osteoarthritis syndrome, Loeys-Dietz syndrome, *SMAD3*, TGF 

1 | INTRODUCTION

Pathogenic *SMAD3* variants are responsible for a wide spectrum of clinical manifestations including Aneurysms-Osteoarthritis

Syndrome (Aubart et al., 2014). The *SMAD3* gene represents 5–10 percents of the transforming growth factor   (TGF ) vasculopathies (TGF vs), caused by a defect in the TGF  pathway genes such as *TGFBR1*, *TGFBR2*, *TGFBI2*, *TGFBI3* and

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SMAD2. There is a clinical overlap between these TGF β s and Marfan syndrome (MFS) in which the aortic aneurysm/dissection events dominate the cardiovascular phenotype. Unlike MFS, dilatation/dissection events are frequent in other arteries and arterial tortuosity is common, particularly in head and neck vessels (Aubart et al., 2014; Laar et al., 2012; Regalado et al., 2011). In addition, the main features that distinguish *SMAD3*-related disorders (SRD) from MFS and other TGF β s are precocious osteoarthritis (<50 years old) and Charcot–Marie–Tooth-like neuropathy (Aubart et al., 2014).

The *SMAD3* gene contains two functional domains, Mad Homology 1 (MH1) and 2 (MH2), separated by a linker region (Schepers et al., 2018). The N-terminal domain MH1 is mainly involved in DNA binding. The C-terminal domain MH2 mediates oligomerization with *SMAD4* and *SMAD*-dependent downstream transcription (Schepers et al., 2018). Heterozygous variants of different nature (missense, truncating, splicing variants) have been described in SRD (Schepers et al., 2018). Interestingly, gain-of-function in overall TGF β pathway has been observed in the aortic wall of patients with aortic aneurysms, including patients with loss-of-function mutations in the TGF β pathway genes (Gomez et al., 2009). Despite the absence of hot spot identification in *SMAD3*, the majority (63) of the missense variants is reported in the MH2 domain.

Thus, the role of *SMAD3* in a wide phenotypic spectrum with different types of variants prompted us to review all the individuals with a *SMAD3* (likely) pathogenic variant from our Centre and to compare them with published cases, first to determine an estimated frequency of the main clinical signs in SRD and the presence of any genotype-phenotype correlation associated with variants of this gene.

2 | MATERIALS AND METHODS

2.1 | Patients

All the 22 patients were referred to our Reference Centre for “Marfan syndrome and related disorders” for personal and/or family history suggestive of a connective tissue disorder (mainly aortic event or dilatation and skeletal findings).

This study was designed in compliance with the tenets of the Helsinki Declaration. Informed consent was obtained from all individuals included in this study.

We also assembled all the clinical data available on individuals with a pathogenic or likely pathogenic variant in the *SMAD3* gene from an exhaustive review of the literature using the keyword “SMAD3” in the PubMed database. Patients whose vascular phenotype was unknown (in particular, the presence or the absence of an aortic dilatation) were not included in the correlation study.

2.2 | Molecular testing

Index cases benefited from multigene panels that include *SMAD3* (NM_005902.3) as well as a number of other genes associated with disorders that include heritable thoracic aortic aneurysms/dissections (Data S1). For the patient 9, for whom the *SMAD3* pathogenic variant was already known (family 4), a targeted Sanger sequencing was directly performed. Segregation analysis of any identified (likely) pathogenic variant was performed in each family (parents and other affected family members when available). All variants classified according to the ACMG recommendations (Richards et al., 2015) as pathogenic, likely pathogenic or of unknown significance (VUS) were reported here.

2.3 | Phenotype classification

Phenotypes were classified into two groups: (a) an aortic phenotype (AP) that includes the aortic events (dissection, rupture, or elective repair of the aorta) or dilatation (more than two standard deviations measured by echocardiogram at the Valsalva sinus) and (b) a mild phenotype grouping asymptomatic patients and patients without aortic damage. Patients without AP but with an aneurysm in another artery were considered to have a mild phenotype.

2.4 | Statistical analysis

From more than 300 patients reported in literature with SRD, only 133 of them were described with precise clinical data to be included in our genotype-phenotype correlations study (Arno et al., 2012; Arroyave, Carretero, & Gruosso, 2018; Backer & Braverman, 2018; Campens et al., 2015; Kaadan et al., 2018; Laar et al., 2012; Nevidomskyte et al., 2017; Overwater et al., 2018; Proost et al., 2015; Regalado et al., 2011; Schepers et al., 2018; Wischmeijer et al., 2013; Ye et al., 2013). Six out of these 133 patients bearing splice variants in *SMAD3* (Campens et al., 2015; Nevidomskyte et al., 2017; Overwater et al., 2018) were not included in our analysis because the underlying pathogenic mechanism of these variants could not be predicted with certainty.

We studied the link between the variant type and the presence of an AP with logistic regression models. As some patients belonged to the same family, we also performed an analysis on the index cases only. $P < .05$ were considered as significant. Statistical analyses were performed with STATA release 14.

We also compared patients with a missense variant in either the MH1 or the MH2 domain to patients with a truncating variant. Four patients were not included in this analysis as

they had a missense variant within the N-terminal extremity before the MH1 domain.

3 | RESULTS

3.1 | Clinical and genetic data

We identified 22 patients from eight families carrying a heterozygous (likely) pathogenic variant in *SMAD3* (Table 1; Figure 1; Data S2). Four were novel variants: c.269_271dup (p.Arg90dup) classified as pathogenic (PS2, PM1, PM2, PM4, PP1), c.736del (p.Glu246Arg*10) classified as likely pathogenic (PVS1, PM2), c.874del (p.Arg292Glufs*49) classified as pathogenic (PVS1, PM2, PP1) and c.991G>T (p.Val331Phe) classified as likely pathogenic (PM1, PM2, PM6, PP3).

Interestingly, in family 3, besides the c.1153A>G (p.Arg385Gly) variant identified in *SMAD3*, a second variant was identified in *TGFβ2*: the c.470T>C (NM_001135599.2) (p.Ile157Thr) variant, which was classified as a VUS. Indeed, it has been reported at 0.0002481% in gnomAD and is predicted damaging by in silico analyses (Polyphen2, Mutation Taster) and tolerated by SIFT software. The index case in family 3 (patient 6) who was carrying both the heterozygous variants in *SMAD3* and *TGFβ2*, displayed the most severe phenotype of the family as shown in the pedigree (Figure 2). He underwent surgery for an ascending aorta dilatation at 56 year old and suffered from aortic insufficiency, lumbar spondylolisthesis and scoliosis. He died at 60 years old from a pulmonary embolism. His elder son (patient 7) also displays a severe phenotype compared to his brothers, each carrying only the *SMAD3* (patient 8) or the *TGFβ2* variant (patient A; Figure 2, Data S2). He has an aortic dilatation (41 mm, +3.7SD at the sinus of Valsalva), recurrent inguinal hernias and a severe maxillary hypoplasia. The patient 8 carrying only the *SMAD3* variant made a dissection of the left primitive iliac artery at 30 years old; he also had a dilatation in the right iliac artery. His echocardiography was normal. The echocardiography of the son carrying the *TGFβ2* variant (patient A) did not reveal any aortic dilatation or valvular heart disease but hypertrophic cardiomyopathy with asymmetric thickening of the interventricular septum.

3.2 | Genotype-phenotype correlations

We compared 149 patients (127 + 22 patients) from 56 families (with 1–28 members) according to their aortic status and the nature of the *SMAD3* variant (truncating vs. missense variant; Data S3). After performing a statistical analysis, we did not detect any significant difference in the AP between patients carrying a truncating variant and patients with a missense variant in *SMAD3*. Both groups had indeed about 68%

of patients with an aortic disease ($P = .935$). When comparing missense variants in the MH2 domain versus truncating variants, we also did not detect any statistical difference in the AP ($P = .848$). We did not observe a statistical difference either when comparing the phenotype of patients with a missense variant in the MH1 domain to those with a truncating variant ($P = .656$). We did not detect any significant difference either when analyzing the index cases only.

4 | DISCUSSION

Pathogenic heterozygous variants in the *SMAD3* gene are a rare cause of connective tissue disorder. To our knowledge, 132 families have been reported in the literature with 102 different variants (Arno et al., 2012; Arroyave et al., 2018; Aubart et al., 2014; Backer & Braverman, 2018; Campens et al., 2015; Collins, Flor, Tang, Bange, & Zarate, 2018; Hostetler et al., 2019; Kaadan et al., 2018; Kfoury, Chen, & Lin, 2017; Laar et al., 2012; Nevidomskye et al., 2017; Overwater et al., 2018; Proost et al., 2015; Regalado et al., 2011; Schepers et al., 2018; Wischmeijer et al., 2013; Ye et al., 2013) and four total or partial deletions of *SMAD3* (Hostetler et al., 2019; Schepers et al., 2018). In this work, we report 22 additional patients from 8 families, showing high variability in terms of expression and penetrance of *SMAD3* pathogenic variants even at pediatric age (Hostetler et al., 2019; Laar et al., 2012; Wischmeijer et al., 2013).

As shown previously (Aubart et al., 2014; Hostetler et al., 2019; Laar et al., 2012), aortic aneurysm and dissection are the main vascular findings, affecting, respectively 67% (104/155) and 29% (39/133) of all patients described with a *SMAD3* variant (Table 1). In our cohort, six out of the eight index cases have aortic aneurysms and 5 out of their 14 affected relatives display arterial aneurysms. Aortic dilatation can be found at a very young age (Arroyave et al., 2018; Aubart et al., 2014; Hostetler et al., 2019; Laar et al., 2012) as it is the case for patients 5 and 14 reported here (aged 8 and 7 years old, respectively) and patient 22 who underwent a surgery for an aortic dilatation at 10 years old. Only 9% of our patients had an aortic dissection, the young age of some of our patients (5/22 are under 18) could be an explanation given that the aortic dissection has still not been reported in children to date. Nevertheless, one patient from a family with *SRD* died from an aortic dissection at the age of 18 (Wischmeijer et al., 2013). In absence of enough patients to correlate the risk of dissection with the phenotype and the gender, as it was done for *TGFβR1* and *TGFβR2* (Jondeau et al., 2016), the follow-up and treatment of aortic manifestations in *SRD* is the same as in *MFS*. A recent study, reporting a cohort of 251 patients with *SMAD3* pathogenic variants found that the AP was less severe in *SMAD3* than in *TGFβR1* and *TGFβR2*, with later onset of aortic events (Hostetler

TABLE 1 Genetic and clinical data in 22 patients with SRD and review of the literature

	Family 1	Family 2	Family 3	Family 4	Family 5	Family 6	Family 7	Family 8												
<i>SMAD3</i> variant	c.786del	c.260_271dup	c.115A>G	c.33M_33AinsCT	c.874A>T	c.788C>T	c.668delC	c.991G>T												
Protein domain (exon)	p.Gln246A>G*10	p.Ser57dup	p.Arg386Gly	p.Ala112Ile	p.Arg292Gln*49	p.Pro226Gln*18	p.Pro226Gln*18	p.Val318Phe												
	MB12 (ex 6)	MB11 (ex 2)	MB12 (ex 8)	MB11 (ex 2)	MB12 (ex 7)	MB12 (ex 6)	MB12 (ex 6)	MB12 (ex 7)	Total (185)											
Patient	1	2	3	4	5	6	7	8	22											
Age (years)	40	6	29	10	8	60	38	32	48	19	17	18	19	20	21	22	Our patients	208 (mean)		
Gender	F	M	F	M	M	M	M	M	F	M	M	M	F	M	M	M	N (56)	N (56)		
Cardiovascular findings																				
Aortic aneurysm	-	-	+	-	+	-	-	-	+	-	+	-	-	-	-	-	+	11/22 (50)	30/61 (50%)	
Aortic dissection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/22 (9)	39/131 (30)	
Aortic regurgitation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Aortic stenosis	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	21/11 (18)	16/83 (17)	
Aortic valve sclerosis	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	21/11 (18)	20/95 (21)	
Aortic valve prolapse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/22 (9)	52/122 (43)	
Aortic valve regurgitation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4/22 (18)	53/95 (56)	
Skeletal findings																				
Dolichocrania	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8/19 (42)	15/72 (21)
Brachydactyly	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7/19 (37)	25/79 (32)	
Scoliosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9/22 (41)	43/96 (45)	
Pectus deformity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6/20 (30)	24/95 (25)	
Pectus excavatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4/17 (24)	62/83 (75)	
Joint laxity	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4/17 (24)	21/81 (26)	
Osteoarthritis	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4/17 (24)	46/100 (46)	
Osteopenia	ND	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3/9 (33)	7/16 (44)	
Osteoporosis	ND	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/19 (0)	0/24 (0)	
Z-score < -1.0	(-1.3)	(1.1)	(0.2)	(-0.9)	(-0.9)	(-0.6)	(-2.2)	(-2.0)	(-2.2)	(-2.2)	(-1.2)	(-1.2)	(-1.2)	(-1.2)	(-1.2)	(-1.2)	(-1.2)	6/19 (32)	7/24 (29)	
Z-score < 2.5																			3/15 (20)	23/83 (28)
Cardiac																				
Frank tetralogy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/19 (0)	0/24 (0)	
Hypertrophic cardiomyopathy	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3/15 (20)	23/83 (28)	
Abnormal mitral valve	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/19 (11)	26/88 (29)	
High aortic/zygomatic angle	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12/40 (30)	23/83 (28)	

(Continues)

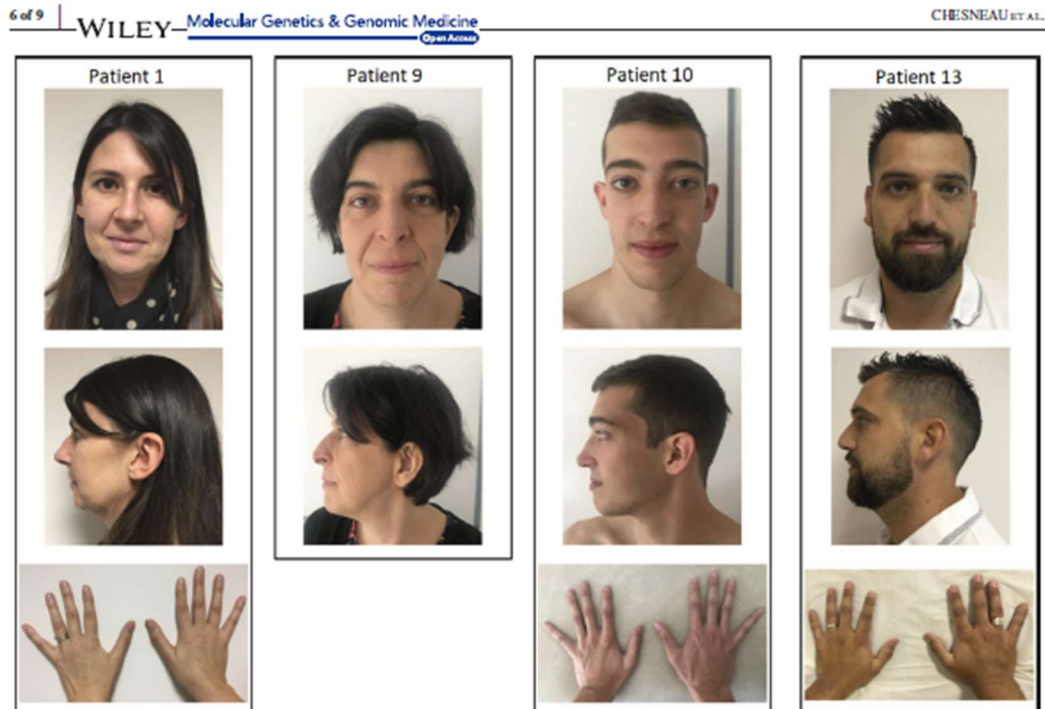


FIGURE 1 Photographs of patients 1, 9, 10 and 13, showing the high variability in craniofacial and extremities features

et al., 2019). Aneurysms and dissections can also be seen in other arteries particularly intracranial aneurysms found in almost 20% of patients (16/83, Table 1). It is important to note that arterial events can happen even in the absence of aortic involvement: 14 patients of our study displayed an extra-aortic aneurysm or dissection history without aortic anomalies (i.e. patient 8 who made an iliac artery dissection with a normal aortic diameter). Therefore, ultrasonography of supra-aortic vessels is also recommended as well as brain and abdominopelvic magnetic resonance angiography.

One of our patients (patient 22, Data S2) presents with a congenital heart malformation. Other patients have been reported with various congenital heart malformations including ventricular septal defect and hypoplastic left heart syndrome but these malformations remain rare in *SRD* (Fitzgerald, Bhat, Conard, Hyland, & Pizarro, 2014; Hostetter et al., 2019; Laar et al., 2012; Overwater et al., 2018). Four of our patients display aortic insufficiency. To our knowledge, aortic insufficiency has been reported only once in the literature (Arroyave et al., 2018).

Premature osteoarthritis is very frequent in *SRD* with almost half of the mutated patients suffered from osteoarthritis (44/100, Table 1). Interestingly, *SMAD3* polymorphisms have recently been linked to the osteoarthritis risk (Hong et al., 2018). The key role of *SMAD3* and the *TGFβ* pathway in maintaining joint cartilage and preventing osteoarthritis

has been reported several times (Chen, Thuillier, Chin, & Alliston, 2012), especially in mice model where a *SMAD3* knock-out model presents with similar articular damages than in human osteoarthritis (Yang et al., 2001). Other joint diseases can also be found like osteochondritis dissecans that likely happened in one of our patients (patient 3, Data S2).

Osteoporosis has been reported in *TGFβ*vs (Kirmani et al., 2010), as well as in *MFS* (Haïne et al., 2015) and the importance of *TGFβ* pathway on the bone matrix metabolism is well known (Kirmani et al., 2010), the frequency of osteoporosis in *SRD* is not really known but could represent a third of patients (Schepers et al., 2018). Our data underline the high prevalence of decreased bone mass in *SRD* and more generally in *TGFβ*vs (Kirmani et al., 2010) that can become an important issue in patients care.

Although various type of allergies, such as asthma, eczema and allergic conjunctivitis, have been previously described as frequent in *SRD* (Aubart et al., 2014) and are present in most of our patients (10/17, Table 1; Data S2), two of them (patient 10 and 17) made an anaphylactic shock (Data S2) which has not been described yet.

The systemic score of the revised Ghent Nosology (Loeys et al., 2010) shows the phenotypic overlap between *SRD* and *MFS* with 20% of our patients (4/20, Table 1) having positive score (≥ 7). Nevertheless, this score is

Family 3

SMAD3 c.1153A>G
TGFB2 c.470T>C

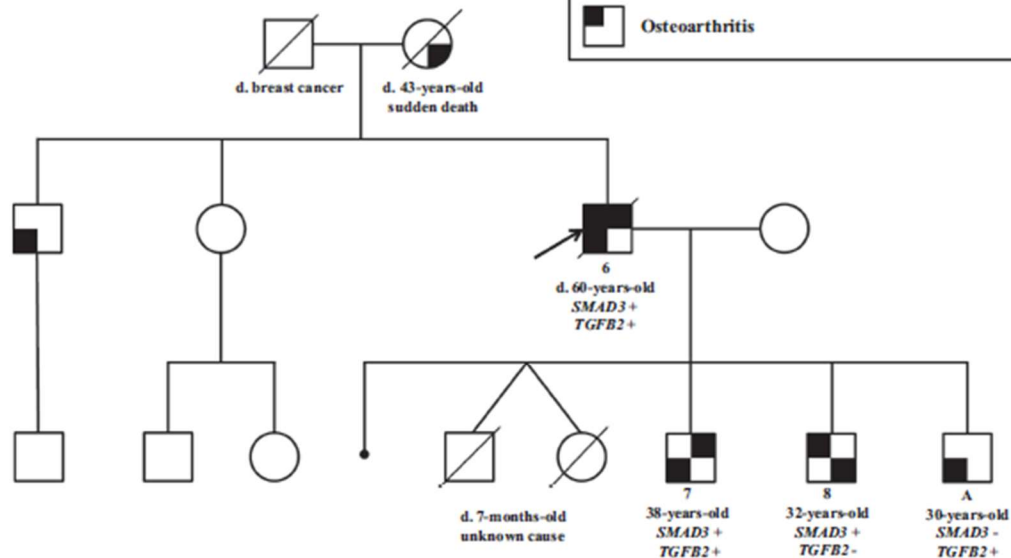


FIGURE 2 Pedigree of Family 3 with the segregation of the two heterozygous variants in *SMAD3* and *TGFB2* in patients 6, 7, 8 and A. The legend indicates the clinical features of the patients. The presence or the absence of the family variants in *SMAD3* and *TGFB2* is indicated by “+” or “-” after the gene name

varying from 0 to 8, illustrating the high clinical variability of SRD and therefore the lack of power of such a score to detect *SMAD3* pathogenic variants as it was designed for MFS and does not include premature osteoarthritis which is more frequent and specific of SRD. Nevertheless, SRD are much less frequent than MFS and the use of multigene panels to diagnose connective tissue disorders with a vascular risk makes the development of a score dedicated to SRD not really helpful in clinical practice.

About 2/3 of patients have a missense variant (91/155) whereas 1/3 have a truncating variant (58/155) and less than 5% affects the splicing (6/155). Although no hot spot has been identified in *SMAD3* (Schepers et al., 2018), half of the *SMAD3* variants identified in our cohort are supposed to affect the MH2 domain (71/155 patients had a MH2 missense variant, Data S3).

From all these clinical and molecular data, we tried to establish genotype-phenotype correlations, as it is relevant in clinical practice. For example, genotype-phenotype correlations have been made, using a very large cohort of patients, to help the clinical management and the risk stratification in

subjects with MFS (Faivre et al., 2007). Today, there are only few genotype-phenotype correlations in TGF β vasculopathies, made difficult owing to wide inter and intrafamilial phenotypic variability (Aubart et al., 2014; Hostetler et al., 2019; Laar et al., 2012) and often incomplete penetrance at pediatric age. Nevertheless, clinical and molecular characterization of novel patients is important to help the establishment of possible correlations and *in fine* to help clinical management.

In this work, we wanted to explore some aspects of the *SMAD3* phenotypic variability, asking the question of the involvement of specific variants with an “aortic tropism” in patients. Thus, we checked here if a correlation exists between the variant type (truncating vs. missense) and the occurrence of an AP, namely the presence of an aortic event (dissection, rupture or elective repair of the aorta) or aneurysm in the disease course. Moreover, to take in consideration a potential recruitment bias due to generally more severe symptoms in index cases, we also compared the phenotype of index cases only. Nevertheless, we did not find any correlation between the type of variants and the

occurrence of an aortic disease in patients. This data are consistent with the recently published work by Hostetler et al. (2019) as they did not detect a statistical difference in the rate of aortic event in their cohort. Likewise, we did not detect any significant difference in the aortic disease occurrence between MH1 or MH2 missense variants and truncating variants, despite the fact that aortic events have been shown to happen earlier in life in patients with MH2 missense variants than in patients with truncating variants (Hostetler et al., 2019). Aortic dilatation is often asymptomatic, the age of apparition is then difficult to determine and not mentioned in the different reports. Furthermore, there is a bias between index cases (often diagnosed following an aortic event) and their relatives who benefit from a precocious vascular follow up and can therefore have an earlier diagnosis of aortic dilatation. Therefore, in our study, it was not possible to take the age in consideration in genotype-phenotype correlations. Moreover, the lack of difference that we observe herein, could actually be due to a lack of statistical power. However, the p-values that we obtained are very high and the analyzed sample (149 patients) is not so small, so if there is a difference, it is probably not major. Of note, Hostetler's study (Hostetler et al., 2019) recorded an AP solely for 177 of the 212 patients.

As some patients have vascular aneurysms or dissections in small arteries without AP, it would be interesting to investigate the vascular phenotype in SRD. The absence of a full vascular imaging in most of the patients reported in the literature makes the study of vascular phenotype difficult using these data. In addition, the phenotypic variability in the skeletal and joint features, in particular osteoarthritis, would also be interesting to investigate, as it appears as a frequent feature. However, establishment of genotype-phenotype correlations based on these features in patients with *SMAD3* variant is made difficult given osteoarthritis and osteoporosis are common disorder in the general population, often banalized and underdiagnosed in the SRD patients, explaining the lack of data in the literature. Moreover, it appears from this work that aortic insufficiency and severe allergic phenomenon are probably also underestimated, unlike Charcot-Marie-Tooth-like neuropathy, which was not found in our patients.

Thus, to date, there is no genotype-phenotype correlation in the penetrance of the cardiovascular phenotype identified in the *SMAD3* gene. This is in line with the high variability observed in this syndrome, in particular the wide intra-familial variability that is largely illustrated in this work. This also suggests that there are other factors impacting on the phenotype to be discovered and the presence of genetic modifiers can be one of them. Indeed, one of the family reported here (family 3) allows illustrating this phenomenon, with the probable effect of a variant in a genetic modifier influencing on the phenotypic expression and severity, as reported in MFS (Luyckx et al., 2019). Thus, the identification

of genetic modifiers that could reveal, reduce or increase the *SMAD3*-related phenotype seems to be an interesting and challenging area of research, especially as the use of NGS technology spreads out in routine genetic testing.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Automatic determination of aortic compliance With Cine-Magnetic Resonance Imaging

An Application of Fuzzy Logic Theory

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Lalande A, van Kien PK, Salve N, et al. Automatic determination of aortic compliance with cine-magnetic resonance imaging: An application of fuzzy logic theory. *Invest Radiol* 2002;37:00-00.

RATIONALE AND OBJECTIVES. Aortic compliance is defined as the relative change in aortic cross-sectional area divided by the change in arterial pressure. Magnetic resonance imaging (MRI) is a useful imaging modality for the noninvasive evaluation of aortic compliance. However, manual tracing of the aortic contour is subject to important interobserver variations. To estimate the aortic compliance from cine-MRI, a method based on fuzzy logic theory was elaborated.

MATERIALS AND METHODS. Seven healthy volunteers and eight patients with Marfan syndrome were examined using an ECG gated cine-MRI sequence. The aorta was imaged in the transverse plane at the level of the pulmonary trunk. A method based on fuzzy logic was developed to automatically detect the aortic contour.

RESULTS. Through our robust automatic contouring method, the calculation of aortic cross-sectional areas allows an estimation of the aortic compliance.

CONCLUSION. The aortic compliance can be obtained from a fuzzy logic based automatic contouring method, thereby avoiding the important interobserver variation often associated with manual tracing.

KEY WORDS. Aortic compliance; cine-MRI; automatic contour detection; fuzzy logic.

THE CONDUIT arterial system serves as an elastic chamber (windkessel) that smoothes out the pulsatile arterial flow and delivers the blood in a more continuous fashion into the capillary beds.¹ This effect is in part accomplished by the compliance of the arterial system, which allows expansion of the arteries during systole and release of the stored blood in diastole to maintain diastolic flow, thus creating a nearly continuous peripheral blood flow. A normally compliant system can store a considerable volume of blood in the aorta and the large arteries during systole. Abnormalities in compliance can greatly affect cardiovascular function, in particular the systolic blood pressure, the left ventricle load and the cardiac output. Decreased compliance results in an increase in systolic pressure, and a decrease in diastolic pressure. Moreover, the rigidity of the thoracic aorta can modify coronary flow and aortic function can be altered, even if the structure of the aorta appears normal.

Analysis of diastolic pressure decay is one method to estimate arterial compliance. The direct measurement of the arterial compliance remains difficult and particularly invasive, and it must therefore be determined indirectly. Several techniques use a mathematical model describing pulse wave

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propagation and arterial mechanical properties. This model considers the arterial system as a chamber of a finite length and infinite pulse wave velocity and assumes that the arterial tree is an elastic chamber (windkessel model) in which the diastolic pressure decays exponentially with a time constant that is determined by total arterial resistance and compliance (two element windkessel model).²⁻⁵ This model allows an easy calculation of the compliance, based on the relationship between changes in aortic area and pressure.⁵⁻²¹ It assumes that there is a negligible axial vessel movement during pulse pressure, and that the volume changes depend of the expansion of the vessel wall along its radius. Another method is based on the pulse wave velocity, by measuring the flow wave propagation in the aorta.^{8,22-24} The method proposed by Liu et al^{25,26} uses the area under the diastolic pressure waveform.^{12,14,23,27} However, this method requires continuous pressure data recordings during the cardiac cycle.

The aortic compliance has been measured by magnetic resonance imaging (MRI) from a manually traced contour of the aorta over a whole cardiac cycle.^{8,9,16,23,28} However, manual tracing of contours on cine MR images is subject to an important interobserver variation. From our own experience, for example, Figure 1 shows cross-sectional area variations of the descending aorta obtained by two experienced observers on the same examination. These curves differ considerably. To counter this limitation, this work presents a method toward the automatic determination of the aortic compliance from cine-MRI. The method consists of the automatic detection of the contour of the aorta on transversal cine-FISP images. Although the aortic contour is usually well defined, the proximity of pulmonary trunk and flow artifacts can render its definition ambiguous. The principal information in the image is the pixel gray level value. Although the gray level is a precise data, it is uncertain, because it depends on image acquisition quality. The use of

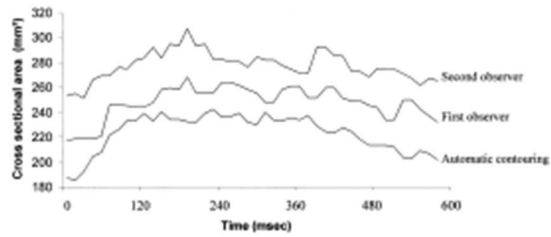


Figure 1. Manual tracing by two experienced observers and automatic contouring performed on the same descending aorta. Note the differences between the three curves, particularly the maximum and minimum surfaces, as well as the shape of the curve.

fuzzy logic²⁹⁻³¹ allows us to deal with these uncertainties. The method is based on fuzzy set theory and dynamic programming with graph searching. Using the automatically traced aortic contour and the arterial pressure, an estimation of the arterial compliance can be made.

Materials and Methods

Study Population

The method was tested in eight patients with Marfan syndrome³² (age: 22 ± 10 years; range 12-38 years). Seven healthy volunteers free of cardiovascular risk factors were also studied (age: 37 ± 12 years; range 18-49 years). The study was conducted in accordance with the recommendations of the local ethics committee, and informed consent was obtained from each subject. The data from Marfan syndrome patients were included in the study to increase the range of aortic cross-sectional areas.

Magnetic Resonance Imaging

Magnetic resonance imaging was performed on a 1.5 T magnetic resonance whole body imager (Siemens Magnetom Vision, Siemens GmbH, Erlangen, Germany). The data

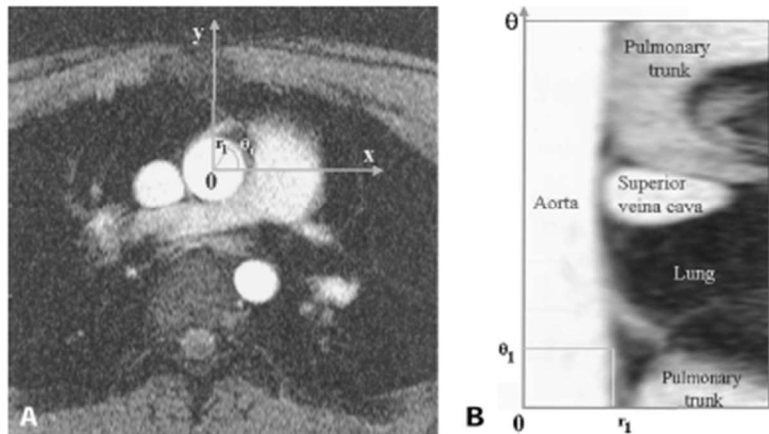


Figure 2. Transformation into polar coordinate system. (A) Cartesian coordinates. (B) Polar coordinates.

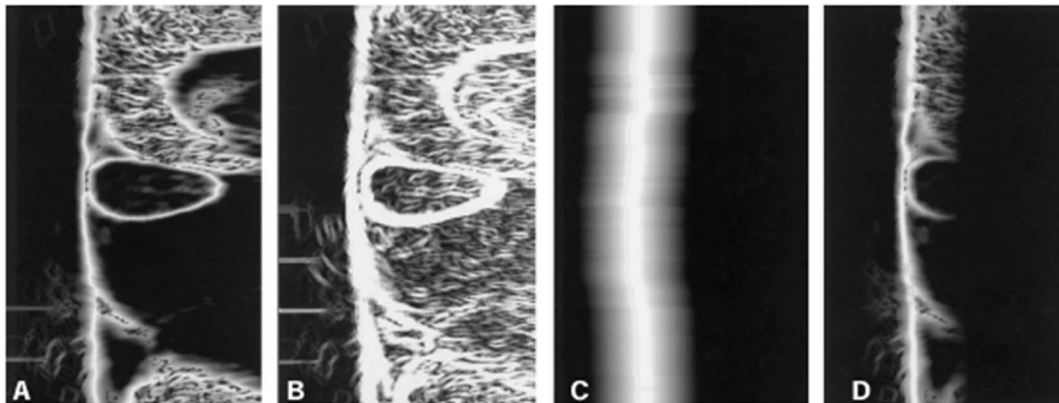


Figure 3. Fuzzy set of the aorta wall points determined on the initial image transposed into the polar coordinate system. (A) Matrix corresponding to the fuzzy set associated with the pixel gray level. (B) Matrix corresponding to the fuzzy set associated with the presence of edges. (C) Matrix corresponding to the fuzzy set associated with the region of interest. (D) Matrix associated with the fuzzy set of the aorta wall points, corresponding to the intersection of the three previous matrices.

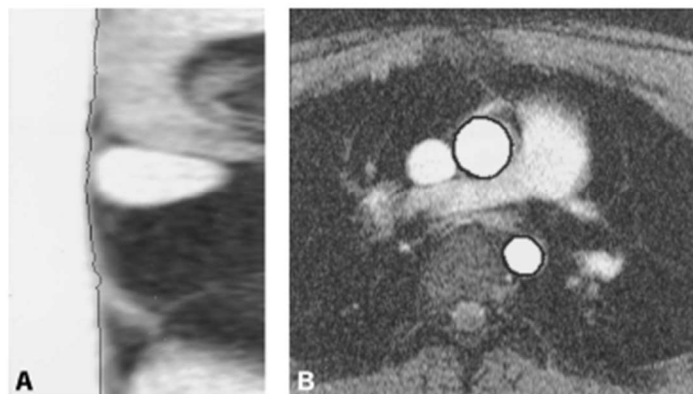
were acquired using an ECG gated cine-FISP sequence. The acquisition parameters of this sequence were: repetition time (TR) = 15 milliseconds; echo time (TE) = 7 milliseconds; pulse flip angle = 20°; number of acquisitions = 2. The slice thickness was 7 mm. A 512² matrix and a 350 mm FOV were used to achieve a nominal 0.7 mm pixel size. Forty to sixty images of a single slice were acquired, thereby covering the whole cardiac cycle. The aorta was imaged in the transverse plane at the level of the bifurcation of the pulmonary trunk. Hence the ascending and descending aortas can be studied simultaneously. The arterial diastolic and systolic pressures were estimated in the brachial artery by using a sphygmomanometer.

Automatic Detection of the Aortic Contours

On the MR images, the contours of the ascending and descending aortas are detected from the first image acquired

just after the R-wave through to the image corresponding to the end of the cardiac cycle. Each image was processed individually and the processing of the ascending and descending aortas was identical. The coordinates of the center of the aorta are the starting point for the ensuing contour detection. On the first image following the R-wave, this point is determined manually. For the subsequent images, it is approximated by the center of gravity of the aorta; this being defined by the aortic contour detected on the previous image. To process the data it is convenient to transpose the image from its original Cartesian coordinates (Fig. 2A) into the polar coordinate system (Fig. 2B). By choosing the center of the aorta as the origin of the polar coordinate system, the roughly circular form of the aortic contour produces approximately vertical lines after coordinate transposition.^{33,34} Then, three parameters are assigned to each

Figure 4. Automatic detection of the ascending aortic wall from the fuzzy set of the aorta wall points. (A) Ascending aorta wall detection via dynamic programming in polar coordinates. (B) Final result with ascending and descending aorta walls in Cartesian coordinates.



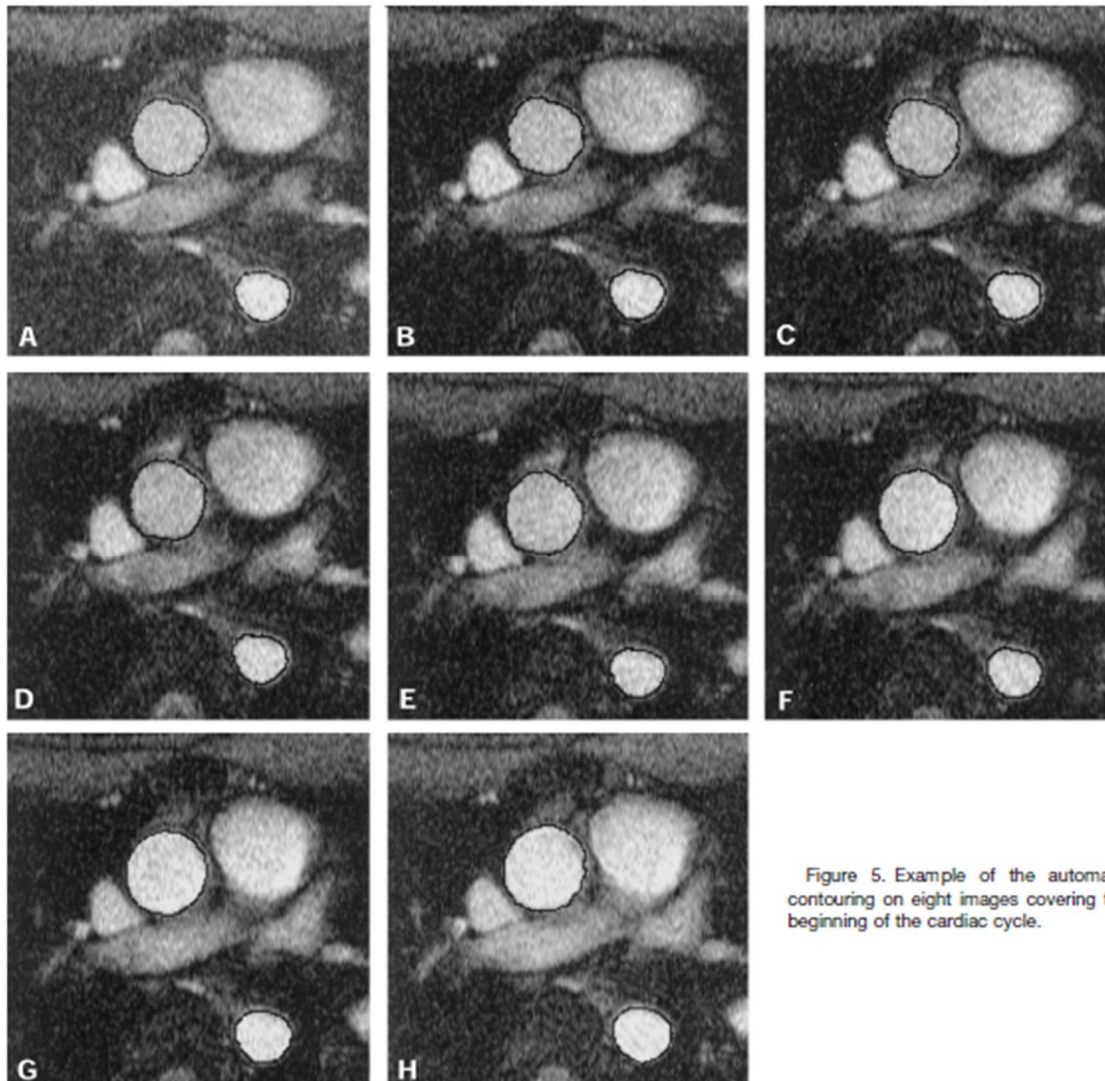


Figure 5. Example of the automatic contouring on eight images covering the beginning of the cardiac cycle.

pixel. The processing in the polar coordinate system allows a parameter assignment performed line by line. The first parameter depends on the pixel gray level value (Fig. 3A), the second on the presence of an edge (Fig. 3B), and the third on the information retrieved on the previous image (Fig. 3C). The third parameter defines a region of interest. From each parameter, a fuzzy set is created. The definitions of the membership function associated with the fuzzy sets are similar to those presented in a previous publication.³³ The use of the theory of fuzzy logic allows one to deal with the uncertainty related to the data.²⁹⁻³¹ The aortic contour points are represented by a class of points that is deduced

from the conjunction of the three above pixel-based parameters. Indeed, the pixels of the aortic contour have a specific pixel gray level value, belong to an edge, and are enclosed within the region of interest. This class is a fuzzy set whose membership function is given by the minimum of the membership functions of the three parameters. The calculation of the membership degree to this fuzzy set for each pixel involves the creation of a matrix of membership degrees (Fig. 3D). Finally a dynamic programming technique³⁴⁻³⁷ called graph searching is applied on this matrix to detect the aortic contour (Fig. 4A). It is a nonfuzzy contour detection method particularly suited to the detection of straight lines,

F4

TABLE 1. Aortic Parameters

	Ascending aorta			Descending aorta		
	Smax (mm ²)	ΔS (mm ²)	Aortic compliance (mm ² /mmHg)	Smax (mm ²)	ΔS (mm ²)	Aortic compliance (mm ² /mmHg)
Patients with Marfan syndrome	744 ± 166	141 ± 51*	2.41 ± 0.69*	373 ± 86	70 ± 40	1.20 ± 0.58
Control subjects	642 ± 189	80 ± 24	1.68 ± 0.42	338 ± 108	62 ± 16	1.32 ± 0.39

* Statistical significance between Marfan syndrome patients and control subjects: $P < 0.05$.
Smax: Maximum cross-sectional area. ΔS: Maximum amplitude.

like the aortic contour in polar coordinates. The aim of graph searching is to detect the best path between two sets of points in a matrix. If these two sets of points are the same, this method then provides a closed contour. The definition of this path is based on nearest neighbor maxima, rather than a series of local maxima. Thus, this method provides a continuous contour, and deals with local and global information. Once the contour is detected, the aortic contour points are transposed back into the Cartesian coordinate system on the initial image (Fig. 4B). If an aberrant contour appears on one image, it is usually propagated on the following images. In that case, the automatic contour detection is restarted at the image with the aberrant contour after taking a manually traced contour as the starting point.

Aortic Parameters

On each image, the surface delineated by the aortic wall is calculated. By calculating this surface at different phases of the cardiac cycle, aortic area-time curves can be obtained. For each series of images, the maximum cross-sectional area (Smax), the minimum cross-sectional area (Smin) and the amplitude (ΔS) were calculated for the ascending and descending aortas. Compliance of the aorta was calculated as:

$$C = \frac{S_{max} - S_{min}}{P_s - P_d} = \frac{\Delta S}{\Delta P}$$

Ps is the systolic pressure, Pd the diastolic pressure and ΔP = Ps-Pd. Ideally aortic compliance is calculated from the slope of the aortic area-pressure relation. However Lang et al²⁰ have shown that the calculation of aortic compliance using ΔS/ΔP provides similar results.

To compare the automatic detection approach with a manual one, the ascending and descending aorta contours were manually traced on three patients with Marfan syndrome and on three healthy volunteers. Inter- and intraobserver variabilities were tested on the same examinations.

Statistical Analysis

All results were expressed as mean ± SD. Nonparametric Kruskal-Wallis one-way analysis of variance was adopted to compare aortic parameters. A P value <0.05 was considered significant.

For inter and intraobserver variability analysis, as well as manual versus automatic contouring, a comparison of surfaces was also made using the Bland-Altman method.³⁸ For each surface, the values used for the intraobserver variability were averaged for the interobserver variability study.

Results

An example of automatic contouring is shown in Figure 5 on eight images covering the beginning of the cardiac cycle, including the images corresponding to Smax and Smin. Table 1 shows the results for the ascending and descending aortas obtained with automatic processing. The amplitude and the compliance of the ascending aorta are significantly greater for the patients with Marfan syndrome than for the control group. For the descending aorta, the results do not reveal any significant difference between the two groups. Figure 6 depicts the variation of the aortic cross-sectional area during a cardiac cycle of the ascending aorta for a patient with Marfan syndrome and a healthy volunteer, respectively.

For the intraobserver variability, a good correlation was obtained for the surface determination ($r = 0.997$ for the ascending aorta, and $r = 0.994$ for the descending aorta).

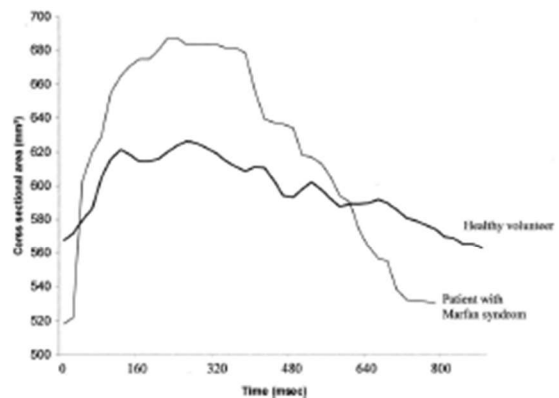


Figure 6. Ascending aorta cross-sectional area versus time within the cardiac cycle in a patient with Marfan syndrome and a healthy volunteer.

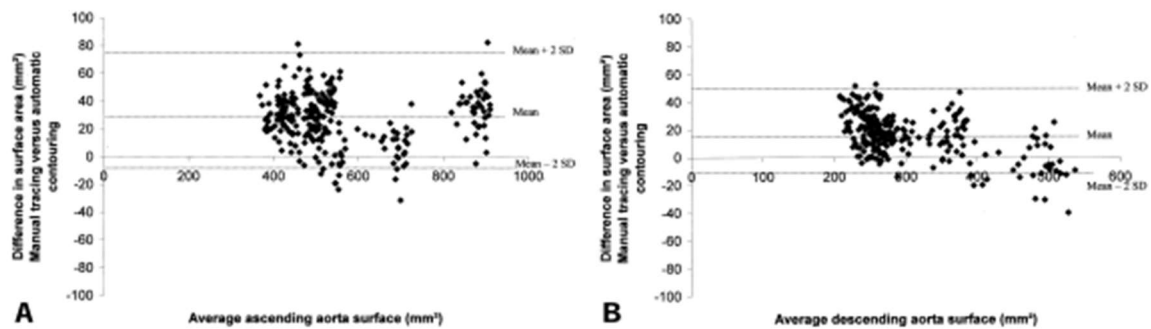


Figure 7. Bland-Altman plot of the (A) ascending aorta surface and (B) descending aorta surface derived from manual tracing and automatic contouring.

Using the Bland-Altman method, we observed a mean difference of -1.3 mm^2 for the ascending aorta and 0.4 mm^2 for the descending aorta. For the interobserver variability, there is also an excellent correlation between the two observers ($r = 0.998$ for the ascending aorta, and $r = 0.960$ for the descending aorta), although for the ascending and descending aorta, mean differences of -10.3 mm^2 and -0.3 mm^2 were observed with the Bland-Altman method. For the comparison between automatic contouring and manual tracing, there is an excellent correlation between the two approaches ($r = 0.990$ for the ascending aorta, and $r = 0.977$ for the descending aorta). However, there is an important overestimation of the aortic surface using manual tracing. This overestimation is illustrated by a Bland-Altman figure (Fig. 7). The mean differences were equal to 27.1 mm^2 for the ascending aorta and 16.9 mm^2 for the descending aorta.

Discussion

This study uses MR imaging and automatic postprocessing to determine aortic compliance of the ascending and descending aortas. The cine-FISP is a dynamic sequence adapted to the study of large vessels. This sequence has some advantages over other techniques. It enhances the signal of flowing blood. The excellent temporal resolution is well adapted to a study of the variation of the aortic area during the cardiac cycle. The postprocessing is performed on a desktop computer equipped with a 1.2 GHz micro-processor and 256 RAM, and the automatic detection of the contours of the ascending and descending aortas from a series of 60 images does not exceed 30 minutes. In comparison, manual tracing on the same series takes roughly the same time.

From one image to the next, manual tracing of the aortic wall can produce important and misleading errors. Consider, for example, an aorta with a radius of 15 mm (ie, a surface area of approximately 700 mm^2), a difference of 1 pixel in the manual tracing (ie, a difference of at least 0.7 mm) will generate an error in the surface calculation close to 70 mm^2 (ie, nearly 10% of the surface). If this error is

committed randomly within a series of images during manual tracing, the shape of the curve can vary substantially. Moreover, in our study we have observed that in practice the difference in the aortic area from one image to another rarely exceeds 40 mm^2 for the ascending aorta, and 30 mm^2 for the descending aorta. An automatic contouring method may significantly reduce this important variation because of subjective manual tracing. An insufficient image contrast can render the detection of the aortic contour difficult. In such case, the use of fuzzy logic allows one to take into account these blurred contours.

The choice of a plane perpendicular to the aorta at the level of the pulmonary trunk allows the evaluation of the surface of the aortic lumen for the ascending and descending aortas. However, at this level the ascending aorta and the pulmonary trunk are juxtaposed and the arterial walls are not always clear-cut. Thus, the separation between aorta and pulmonary trunk may be ambiguous on some images. Most of the automatic detection errors appear on the ascending aorta because of this lack of a clear separation between the vascular structures on the image. The use of a region of interest limits these errors by restricting the number of candidate pixels.

This technique has been tested on two sub-groups: the first was composed of control subjects and the second of patients with Marfan syndrome. It is obvious that this study does not claim to propose gold-standard values for different populations, but simply shows that cine-MRI associated with automatic fuzzy logic-based postprocessing can distinguish groups with different aortic compliance. The patients with Marfan syndrome are known for their higher aortic compliance. These preliminary results show the feasibility of our method. Moreover, these results are close to those already published using a cine gradient echo sequence. Forbat et al⁹ found a mean aortic compliance of $1.48 \text{ mm}^2/\text{mm Hg}$ (we obtained $1.68 \pm 0.42 \text{ mm}^2/\text{mm Hg}$ for the ascending aorta, and $1.32 \pm 0.39 \text{ mm}^2/\text{mm Hg}$ for the descending aorta). The aortic compliance

is higher in the ascending aorta than in the descending aorta, as Mohiaddin et al¹⁶ have already demonstrated with a spin echo sequence. Fattori et al¹⁹ found a maximum ascending aortic area equal to $7.3 \pm 2.3 \text{ cm}^2$ for Marfan patients (we obtained $744 \pm 166 \text{ mm}^2$) and $6.1 \pm 0.6 \text{ cm}^2$ for healthy volunteers (we obtained $642 \pm 189 \text{ mm}^2$). Kupari et al²¹ found a greater systolic luminal area for the ascending area ($7.8 \pm 2.3 \text{ cm}^2$ in an interstudy reproducibility study, and $8.6 \pm 2.5 \text{ cm}^2$ in an interanalysis reproducibility study), with a pulsatile area change close to 1 cm^2 ($80 \pm 24 \text{ mm}^2$ in the present study).

The described technique provides a measurement of aortic compliance with high spatial and temporal resolution. It is easy to implement on most scanners and can be easily performed in patients suspected of an abnormal aortic compliance and scheduled for a MR examination. The availability of an automatic processing method may make aortic compliance measurement part of a routine MR examination of the aorta.

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