SYNDROMES D'EHLERS DANLOS VASCULAIRE
PROJETS EN COURS DANS LE CENTRE DE REFERENCE DES MALADIES VASCULAIRES RARES
ET DANS L' EQUIPE INSERM U970 – EQ3 (RESPONSABLE PR X JEUNEMAITRE)

Introduction à l'attention de l'AFSED

Les projets décrits ci-dessous sont un ensemble de projets de recherche clinique et fondamentale que notre équipe de recherche a initié depuis plusieurs années tant au niveau de l'hôpital (Centre de Référence des Maladies Vasculaires Rares) que de notre équipe INSERM (U970, PARCC). De ce fait, de nombreuses personnes sont associées aux différentes recherches indiquées ci-dessous, et plus particulièrement : Dr Michael Frank, Dr Salma Adham pour les projets de recherche clinique ; Dr Juliette Albuisson, Dr Anne Legrand pour les projets de recherche génétique ; Dr Juliette Albuisson, Dr Khadija Lalhou-Lafotêt, Mr Jean-Michael Mazzella pour les études d'impact psychologique et social ; Mme Irmine Loisel-Ferreira, Mlle Amélie Gianfermi, Mme Erika Fontaine, Dr Ophélie Said, Mlle Hélène Servas, pour les projets de recherche expérimentale.
Certains de ces projets sont très aboutis, d'autres viennent juste de démarrer ; certains ont reçu des financements institutionnels (Ministère de la Santé, INSERM, Agence Nationale pour la Recherche), d'autres des financements d'association de patients (AFSED) et de fondations (Fondation pour la Recherche Médicale). Ces financements récurrents ne suffisent cependant pas à couvrir l'ensemble des consommables et des salaires nécessaires et nous devons opérer des choix, afficher des priorités sur tel ou tel aspect. Un financement supplémentaire venant de l'AFSED serait investi de façon prioritaire sur les recherches thérapeutiques expérimentales chez la souris ainsi que sur la recherche de biomarqueurs de sévérité et de gènes modificateurs chez l'homme (voir ci-dessous).
Comme l'ensemble de nos programmes de recherche sont écrits en anglais, nous nous permettons de vous transmettre les principaux qui sont en cours et pour chacun les éléments principaux dans cette langue, ce qui nous évite une traduction de l'ensemble des textes. Nous sommes cependant à votre disposition pour toute précision.

Le 5 février 2017 – Xavier Jeunemaitre

RESEARCH PROJECTSON VASCULAR EHLERS DANLOS SYNDROME

1. Introduction
Vascular Ehlers-Danlos syndrome (vEDS, OMIM #130050) is a rare inherited autosomal dominant disorder with an estimated prevalence of 1/150,000. It is caused by mutations at the COL3A1 gene encoding the pro-alpha 1 chain of type III procollagen (OMIM #130050). Patients are exposed to unpredictable life-threatening complications, typically spontaneous arterial dissections and ruptures, bowel perforations and uterine ruptures (Pope et al. 1996). Clinical events usually occur at early adulthood. Each complication is at high risk of death and consequently, life expectancy is reduced, estimated in the early 2000's to a median 48 years (Pepin, 2000).
Through the National Referral Centre of Rare Vascular Diseases at HEGP, in the last 8 years, we have collected clinical, radiological and biological information, have set up a biobank of cultured fibroblasts from affected patientsand have conducted the only one published randomised

therapeutic trial (Ong, 2010) for this pathology. We have now access to a unique French cohort (>120 families; 250 affected subjects) that will be instrumental for the clinical research programme detailed below. In addition, we have created a KI col3a1 mouse model that reproduces the main features of the pathology, i.e. spontaneous aortic rupture at a young age (roughly 30-50% mortality between 8 and 20 weeks of age). Together with the heterozygous col3a1 KO model that we imported (Liu, 1997) and already studied at the vascular level in our laboratory (Faugeroux, 2013), these mouse models will be used in a translational research seeking for the better therapeutic strategy.

2 Research programme

2.1 Establishing the first national prospective cohort :RaDiCo SEDVASC (Funds INSERM)

RaDiCo (Rare Disease Cohorts) is financed by the Ministry of Research until December 2019 in the amount of € 10,072,118 in the context of the 'Cohorts' program of *Investissementsd'Avenir* (Investments for the Future) managed by the National Research Agency (ANR). The program is designed to equip France with major epidemiological instruments enabling enhanced elucidation of health determinants, optimization of medical practices and optimization of public health policies related to patients with rare disease.

The principal objective of RaDiCo is prospective collection, in the field of rare diseases (RD), of extensive phenotype data with a view to clinical and epidemiological research in liaison with translational and basic research. The data may be diverse: anatomical (medical imaging), biochemical, molecular, etc.

The Centre des Reference des Maladies VasculairesRares(CRMVR) has applied successfully in 2014 for the establishment of a national cohort on vascular Ehlers Danlos syndrome. It one of the 16 cohorts selected amongst >50 proposals.Through the creation of a prospective database, accessible via the Web for both the CRMVR and the 14 competence centers, with items describing clinical, biological, radiological and therapeutic characteristics of each patient, our cohort RaDiCo SEDVASC has the following objectives:

i) the establishment of a national database of the pathology, having satisfied all technical and regulatory requirements and for which variables are internationally exchangeable , and that will interface with the minimum data set of the national Rare Disease Dataset (BAMARA).
ii) an estimation of the health cost of this rare but severe disease by recording the number of outpatients and inpatients visits including imaging examinations, hospitalizations for complications, morbidity and mortality.
iii) the study of the precise natural history of the disease and in particular the relationships between major and minor signs of the disease, different types of complications (arterial, digestive, lung, uterine), and prospective genotype -phenotype relationships.
iv) the prospective assessment – in an open blind fashion - of the beneficial effect of celiprolol especially on the occurrence of new sites of arterial dissection/rupture and the corresponding morbidity and mortality
v) the facilitation of patients recruitment to future national and even international trials

The e-CRF of the cohort has been built on a REDCAP platform by the RaDiCo team together with members of the CRMVR. The study has obtained in 2016 all the technical an legal requirements (Ethical committees, CCTIRS, CNIL). The kick-off meeting with all the centres involved took place in

November 2016. After information and appropriate informed consent, it is expected that the collection of retrospective and prospective data should start on February 2017.

2.2. Retentissement Professionel, Psychologique, et de l'ERrance diagnostique dans le Syndrome d'Ehlers-Danlos vasculaire (Etude REPERE, Financement AFSED)

OBJECTIFS

1. Identifier et caractériser les difficultés dans les domaines psychologique, familial, et socioprofessionnel des patients dans le contexte spécifique de cette pathologie et de l'errance diagnostique qui y est associée,
2. Evaluer les conséquences de l'annonce dans ces différents domaines après l'établissement du diagnostic,
3. Proposer des recommandations en réponse à ces difficultés.

METHODES

Nous proposons une approcheinterdisciplinaireassociant l'analyseet l'expertised'un groupe d'équipes travaillant avec ou dans le CRMVR, dans le domaine de la génétique médicale et de la médecine vasculaire (Equipe 1), de la psychiatrie de liaison (Equipe 2), de la santé au travail (équipe 3) et des associations de patients(Equipe4). Cette étude quantitative sera basée sur l'utilisation d'un questionnaire portant sur les domaines précédemment cités.

1. Partie recherche (18 mois) **:** Après accords des Comités d'Ethique et de la CNIL, un auto-questionnaire construit en trois volets (socioprofessionnel, psychométrique, axé sur l'évaluation de l'errance diagnostique) sera envoyé à la majorité des patients de la population suivie par le CRMVR, les CC et les services de médecine vasculaire locaux, soit environ 200 patients (cas-index et apparentés). Il aura pour objectif de déterminer les caractéristiques démographiques et socioprofessionnelles des patients, certains éléments de leur parcours médical, ainsi que leurs difficultés psychologiques, familiales, et d'intégration sociale et professionnelle. Sa distribution sera précédée d'une phase de sensibilisation par l'Association Française du Syndrome d'Ehlers-Danlos (AFSED).

2. Partie analyse et intervention (6 mois) **:**

Cette seconde partie permettra la mise en place du plan d'analyse statistique, le dépouillement et la saisie des données, l'analyse et la synthèse de l'étude quantitative. Cette étape finale de synthèse fera l'objet d'une approche multidisciplinaire en regroupant tous les acteurs de tous les partenaires du projet, impliquant fortement l'association de patients.

PERTINENCES DU PROJET, RESULTATS ATTENDUS, IMPACT

L'errance diagnostique et les difficultés d'insertion des patients n'ont jamais fait l'objet d'étude pour le SEDv, pathologie de l'adulte jeune particulière par la bénignité de son phénotype avant la survenue de complications graves.

L'analyse des données permettra de préciser l'influence de la maladie, de sa morbidité, et de son délai diagnostique sur l'état psychologique, les difficultés familiales, le parcours scolaire, l'insertion professionnelle de ces personnes. Cette analyse permettra la rédaction et la diffusion de recommandations, adaptées aux réalités médicales et sociales, et en accord avec les attentes des patients. Les pratiques médicales mises en place secondairement à ces recommandations feront l'objet d'études prospectives ultérieures.

2.3. Plasma and urine biomarkers associated withphenotypic severity (Funds INSERM, FRM)
One of the main features of vEDS is the unpredictability of the occurrence of major complications, especially arterial ruptures usually not preceeded by the presence of aneurysms. Our aim is thus to identify plasma or urine biomarkers that could reflect the diffusion and the severity of the disease and possibly be predictive of sudden arterial events. Plasma and urine collectionis currently conducted in a translational clinical investigation that compares 50 adult vEDS patients, 50 patients with other arterial disease and 100 controls (Clinical Research Protocol already approved, CRC – MEDIC 2011). Positive results will be replicated in a second set of 50 vEDS patients prospectively followed every 6 months for three years with evaluation of clinical and angiographic outcomes in the context of the clinical research programme. In addition, they will be measured in our two mouse models of the pathology

Three maintypes of biomarkers will be tested:

1) Alteration of the degradation products of the extracellular matrix (ECM)
The collagen network is a metabolically active structure, with a turnover (3-4 months) that is the result of a balance between synthesis by fibroblasts and myofibroblasts and degradation by metalloproteinases (Laurent, 1987). Research on circulating biomarkers of collagen metabolism has identified two categories: those related to the synthesis of new collagen molecules and those that reflect their degradation (Lopez, 2010). The carboxy- terminal propeptide of procollagen I (PCIP) reflects the synthesis and maturation of procollagen type I. There is a stoichiometric ratio of 1/1 between the number of molecules of type 1 collagen produced and the amount of PCP released in plasma. Similarly, the carboxy -terminal propeptides and amino -terminal collagen type III (PCIIIP, PNIIIP) reflect in part the synthesis of procollagen III. The markers of collagen degradation, metalloprotease deviation (MMP- 2 and MMP- 9 in particular to collagen type III) and inhibitors of metalloproteases (TIMP-1 to 4) are also of major importance in the metabolism of ECM proteins and have been extensively studied in cardiovascular diseases(Ketelhuth, 2011). Our hypothesis is that the synthesis and turnover of collagen type III should be strongly altered and may be also that of other fibrillar collagens, collagen type I in particular with which collagen type III can form heterotrimers.
The measurements of these proteins and peptides will be performed i) on plasma for which these assays are now standardized using ELISA kits available on the market, ii) on urine by mass spectrometry by our collaborator (JP Schanstra, Proteomic platform, I2MC, Toulouse). Indeed, CE-MS provides a robust and reproducible analyticalplatform providing exceptional resolution to separate several thousand low molecular weight proteins/peptides in less than 1 h. Interestingly, collagen I and collagen III fragments are amongst the most abundant urinary peptides (Siwy, 2011) and are likely to reflect the turnover of the extracellular matrix (synthesis and degradation by proteases). Plasma and urine dosages will be performed on the collection of patients and controls described in the introductory paragraph as well as in our two col3a1 KO and KI mouse models (Zurbig P, 2009).

2) The second hypothesis concerns the plasma concentration of microRNAs (miRs)
Several miRs play a key role in vascular development or have been associated with various cardiovascular diseases, such as myocardial infarction, hypertrophic cardiomyopathy, or thoracic aortic aneurysms(**Creemers, 2012**). Regarding the ECM and the collagen family of proteins, the mir-29 family has been shown to regulate a series of targets including several collagens (**Kriegel ,**

2012). Plasma miR-29a concentration has recently been associated with both hypertrophy and fibrosis in patients with hypertrophic cardiomyopathy (**Roncarati, 2014**), identifying it as a potential biomarker for myocardial remodeling assessment in HCM.

Our hypothesis is that the plasma concentration of a limited set of miRs could be used as diagnostic and/or prognostic biomarkers in vEDS patients. We have conducted a pilot-study on 13 vEDS patients and 13 age- and sex-matched controls. The results were verified by a replication study comparing 25vEDS patients, 43 healthy controls and 28 MFS. Three miRs, miR-483-5p, miR-335 and miR-133a were reproducibly increased in the two series of vEDS patients to controls Interestingly, we also found that plasma concentration of 10 miRs, especially miR-483-5p, miR-155 and mir-335, was different betweenvEDSand MFS patients. We will extend this analysis to a third set of vEDS patients. In order to test the specificity of these findings, we will measure miR expression in aortas of our col3a1 mouse models compared to littermates (n=10 in each group). *We are currently (February 2017) replicating the analysis of a set of 15 circulating miRs in measuring all the patients (n=50) and controls (n=100) of the MEDIC study. We should get final results before june 2017. Ms A Gianfermi (Engineer is currently working on this project).*

3) A third set of biomarkers will be tested corresponding to the TGF-□ pathway and a set of biomarkers of hemostasis and fibrinolysis. A first study performed on 35 vEDS patients compared to 75 age and sex matched controls, showed an association with circulating cytokines among them pro-inflammatory markers such as TGF-□1, TGF-□2, C-reactive protein, ICAM-1, ICAM-2 (Morissette, 2014).Analyses on a larger cohort as ours are warranted to confirm these findings. This analysis will be performed using the Luminex bead-based multiplex assays – human 25-Plex panel (Life technologies) and specific immunoassays for the TGF-□1, TGF-□2 concentrations in collaboration with Pr E Tartour's team inour research centre (PARCC, U970).

2.4. Gene modifiers explaining phenotypic heterogeneity (Partly funded by FRM)

As indicated above, vEDS might be a life-threatening condition but with a large phenotypic heterogeneity. In our series, we found that 50% of patients have experienced at least one major complication at the age of 28 years, but with a very large variability (extremes : 8-55 years). The type of mutations explain only part of this variability, (see Pepin 2014, Frank 2015). However, it does not explain the major part of the phenotypic inter and intra-familial variability observed in our cohort. As it has been demonstrated for other Mendelian inherited diseases such as inherited cardiomyopathies (Roncarati et al. 2013), we rather hypothesize the role modifier genes, in our case additional rare or more common variants in genes expressed in the extracellular matrix (ECM), belonging to the TGFbeta pathway or coding for collagen receptors or products regulating its expression and production.

As a first screening approach, we propose to directly sequence the corresponding genes using targeted NGS sequencing on these candidate genes that we have recently set-up in our laboratory. We will analyse vEDS patients with the two types of genetic COL3A1 variants that are the most frequent in our cohort, i.e. splice-site variants (n=58 patients) and glycine substitutions (n=132 patients). They will be compared on all the relevant clinical and radiological items contained in our database. The interpretation of the analysis will be performed by the usual bioinformatic filtering steps and also by the comparison of the minor and major criteria of the Villefranche nosology within each group of genetic variants. We will pay a particular attention to the analysis of the 60 patients with an age of onset for the first major complications below 25 years (n=30) and above 50 years (n=30) as well to and relatives belonging to the 11 large families

with >5 affected individuals, that could provide arguments of co-segregation between a particular modifier gene and the phenotype (Koboldt DC et al, AJHG 2014).
On January 2017, we have also set up a collaboration with the National Genotyping Centre (CNG, Very)) to perform a genome wide screening of common and rare polymorphisms using an Illumina DNA cheap genotyping roughly 1 million variants. More than 250 vEDS subjects will be genotyped. We will first focus on genes related to the extracellular matrix and to pathways related to collagen synthesis or degradation, before the extension to a genome wide approach with the risk of false positive and negative results.

2.4Establishing a functional cellular assay for COL3A1 mutations
We are lacking in vitro functional assays for establishing the deleterious role of the various COL3A1 genetic variants found in vEDS patients.
- The first approach will consist of the analysis of cultured dermal fibroblasts from patients with different type *COL3A1* mutations. We have now collected such cultured cells from>60 unrelated vEDS patients. We will characterize these fibroblasts (5 cells for each group of genetic variants), based upon their expression profile (microarray of 84 genes of the extracellular matrix), their production of collagen type III and type I (Western-blotting), their capacity of proliferation and migration. This characterization will be facilitated by the use ofa real time cell analysis system – for which we ask funding - a new technological approach that allows the real-time adherent cell measurement of cell adhesion and proliferation capacity (Kiely, 2014). We will also assess the importance of the unfolded protein response and dilation of the endoplasmic reticulum as we recently observed in heterozygous col3a1 KI mice (Fontaine, in preparation).
-The second approach will benefit of the possibilities of the CRISPR/CAS9 technology (Hsu, 2014) and of the experience of our new research engineer specialized in this technology, to create fibroblasts cell lines bearing not only one genetic col3a1 variant, but also another variant found by WES as suspected gene modifier. We will use the well-characterized NIH-3T3 fibroblast cell line, originally established from the primary mouse embryonic fibroblast cells. We will create first a Gly183Ser mutation, equivalent to that engineered in theki col3a1$^{+/G183S}$ mouse just created in our laboratory. Comparison between primary cultures of arterial fibroblasts cells derived from this model and the one engineered in 3T3 cells will be performed. If validated for one given mutation, we could thus test the assay for other types of genetic variants (splice-site, null alleles, ..). We could thus design a functional assay establishing causal links between the suspected genetic variations and a cellular phenotype. This could be performed not only for one COL3A1 genetic variant but for the interaction with another variant either in the COL3A1 gene or in genes found as promising in our search for modifiers genes (see above)
A master student, Dr Ophelie SAID, is currently full-time on this project (January 2017-November 2017)

2.5 Testing new therapeutic possibilities using a new mouse model of the pathology (partly funded by INSERM, FRM; AFSED)

As already mentioned, the prognosis of vEDS is severe hampered by arterial ruptures that account for the majority of morbid or fatal events. Up to now, only a beta-blocker treatment (celiprolol) has been shown to provide a partial protection (Ong, 2010). No comparative study has been conducted to date in view of the rarity of the disease. The creation of a KI mouse model of the pathology offers a unique opportunity to test alternative therapeutic strategies alone or in combination that could then be applied to human vEDS patients. Because its spontaneous

mortality by aortic rupture within the first 20 weeks, the col3a1$^{+/G183S}$ mouse model has the major advantage of being able to test rapidly and comparatively one or several combined treatments. Depending on their bioavailability, they may be in an administration of drinking water or for administration by subcutaneous route.

The following design will be used : experimental trial in parallel groups and in paired cages, the efficacy of one given drug vs placebo or another active treatment. Power calculations have been made that indicates the need for 50 heterozygous mice per group to be able to observe a 30% difference in the overall mortality. Breeding will be performed between heterozygous col3a1$^{+/G183S}$ mice, allowing the generation of both homozygous col3a1$^{+/+}$, heterozygous col3a1$^{+/G183S}$ and homozygous col3a1$^{G183S/G183S}$. Treatment will be given after weaning (5 weeks) and followed up to 24 weeks. Tail-cuff BP monitoring will be performed every 2 weeks starting 8 weeks of age, and survival and aortic rupture rates will be monitored each day.

Among several antihypertensive agents (Bblockers, hydralazine, amlodipine), the effect of an Ang II receptor blocker (ARB, sartan) will be very interesting to test for several reasons. The first is its lowering blood pressure effect, thus the possibility of reducing a major mechanical arterial stress on the aortic wall. Further specific arguments are i) the beneficial effects of ARBs in Marfan syndrome another rupture-prone inherited arterial disease with aortic dilatation, ii) the increased BP and pulse wave velocity (PWV) index in vEDS patients compared to healthy volunteers (Messas, in preparation); iii) the dramatic increased in aortic rupture rate caused by Ang II administration in a mouse model with col3a1 deficiency (Faugeroux, 2013); iv) the inhibitory effect of ARBs on the TGFbeta pathway that has been shown to be stimulated in vEDS. Preliminary positive data suggest that this could be indeed a very interesting pharmacological approach.
At February 2013, we will submit a paper describing the characterization of the first knock-in col3a1 mouse model of the pathology. Heterozygous mice col3a1 $^{+/G183R}$ are viable but have a 60% mortality at 24 weeks of age (0% in controls). Compared to placebo, survival rate is not modified by propranolol, but considerably improved by losartan (10% mortality at 24 weeks) and significantly worsened by amlodipine. We therefore demonstrate the interest of blood pressure reduction and angiotensin I receptor blockade for reducing the rate of arterial rupture in this mouse model of vEDS.
We will now focus our therapeutic test on the possible benefit of hydralazine another vasodilating agent, with special properties on cellular signalization and elastin production that might be beneficial for preventing aortic rupture. (MrsIrmineLoisel-Ferreira, Research Engineer, is in charge of this program.

2.5 Testing the interest of Angiotensin II type 1 receptor blockade in vEDS patients (funded by Ministry of Health, PHRC-N)

Name of the sponsor: Assistance Publique – Hôpitaux de Paris (AP-HP)
Title of study: ARCADE: ANGIOTENSIN II **R**ECEPTOR BLOCKADE IN V**A**SCULAR EHLERS **D**ANLOS SYNDROM**E**: A DOUBLE BLIND, RANDOMIZED, PLACEBO CONTROLLED, MULTICENTER TRIAL

Coordinator: Pr Xavier JEUNEMAITRE, Referral Center for Rare VascularDiseases, Hôpital

européen Georges-Pompidou, Paris, France
Studycenters: Main center: Clinical Investigation Center, Hôpital européen Georges-Pompidou, Paris, France, **+ 15 centers**in France
Studyperiod: 4 years and 3 months
Study duration for the participant: 2 years± 3 months
Study duration of inclusion: 2 years
Number of participants : n =108

The primary objective is to determine, in patients with molecularly proven vEDS, whether the Ang II receptor blocker, irbesartan, prescribed at an optimally tolerated dose (from 150 to 300 mg o.d.) combined with the reference celiprolol treatment, decreases the 24 months rate of both asymptomatic and symptomatic cardiovascular (CV) events (primary composite end-point) when compared to placebo.

This randomized trial has begun on january 2016. More than 40 vEDS patients have been included up to now (feb 2017). Dr M Frank isespecially in charge of the inclusion of the patients and of the coordination of the study.

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