axis view showing marked asymmetric LV hypertrophy involving the septum and anterior wall; parasternal short axis and 2-chamber CMR section confirming the marked degree of hypertrophy involving multiple LV segments.

(L) CMR images from a patient with the TNNT2-F110L mutation. From left: 4-chamber and 3-chamber sections showing extensive regions of non-compaction (black arrowheads) in the apical and apico-lateral wall of the LV; 2-chamber section and 4-chamber section showing large areas of late gadolinium enhancement (black arrowheads) in the anterior free wall and in the septum, respectively.

In this specific subset of patients, the rate of lethal or potentially lethal arrhythmic events was significantly higher, suggesting the presence of a severe arrhythmogenic substrate probably linked to this specific molecular alteration. In mutant mouse hearts, the increased Ca^{2+} sensitivity of myofilament induced by thin filament mutations is directly associated with a susceptibility to arrhythmias, which can be observed even in the absence of any detectable cardiac hypertrophy or fibrosis [8].

However, the absolute magnitude of such risk remains low and, consequently, the mere identification of thin filament mutations does not justify aggressive management strategies.

2.1.2 Thin filament HCM as a progressive disease

The analysis of our data depicted a novel and unexpected profile of thin filament disease, which emerged in our cohort as a progressive disease, more than as a purely arrhythmogenic disease, a feature that appears to have been largely underappreciated in the past. The different

architecture of the thin filament HCM was accompanied by substantial functional alterations, regarding both systolic and diastolic function.

The high prevalence of severe diastolic dysfunction, represented by abnormal filling patterns such as restrictive MV inflow or triphasic LV filling, reflected a profound impairment in the compliance of the left ventricle. In particular, the presence of a triphasic pattern, characterized by prominent mid-diastolic flow velocity (L-wave), reveals the reduced LV compliance and elevated filling pressures [Figure 2.1-3]. Moving from bedside to bench, preliminary data of isolated myofibrils with thin filament mutation showed that the "extra" wave in the diastolic pattern could probably be explained by their incapacity to achieve a fast and fully relaxed state during the diastolic phase.

Thin filament HCM were associated with a higher likelihood of major clinical outcomes, as reflected by composite CV death, life-threatening arrhythmia, and progression to NYHA class III-IV. This difference was largely driven by the higher rate of symptoms progression to NYHA Class III/IV, which was related to the development of LV systolic dysfunction [Figure 2.1-4 and 2.1-5].

The prevalence of "end-stage" disease, a morpho-functional state represented by overt systolic dysfunction and/or restrictive LV filling pattern, is around 1% in unselected HCM populations [9, 10]. In our population, adverse LV remodeling and disease progression was found in more than one quarter, with an incidence of end-stage disease close to 3% per year. Furthermore, the considerable prevalence of heart failure-related symptoms and complications are only rarely associated by dynamic LV outflow tract obstruction in thin filament HCM and rather reflect progressive systolic and diastolic LV dysfunction.

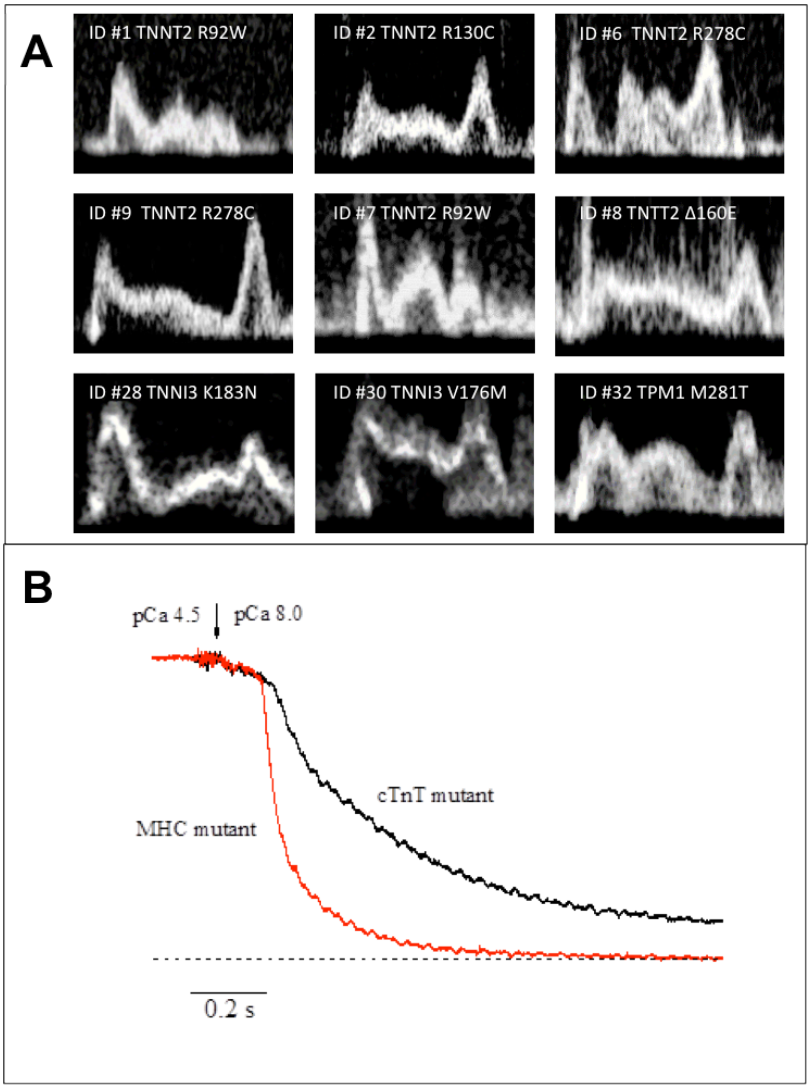

Figure 2.1-3 Morphologic aspects and in-vitro correlates of triphasic LV filling.

(A) Representative examples of transmitral blood flow velocity pattern at Doppler echocardiography from different patients of the thin-filament cohort, showing examples mid-diastolic flow velocity (L-wave).

(B) Comparison of full relaxation from maximal Ca^{2+} activation in isolated myofibrils from an HCM patient with the *MYH7* mutation (red tracing) and a patient with the *TNNT2* mutation (black tracing). In spite of a similar initial rate of tension decay, the early linear isometric phase of relaxation is prolonged and the final exponential phase of relaxation is decelerated in the thin filament mutant myofibril, suggesting impairment of the molecular mechanisms that switch contraction off.

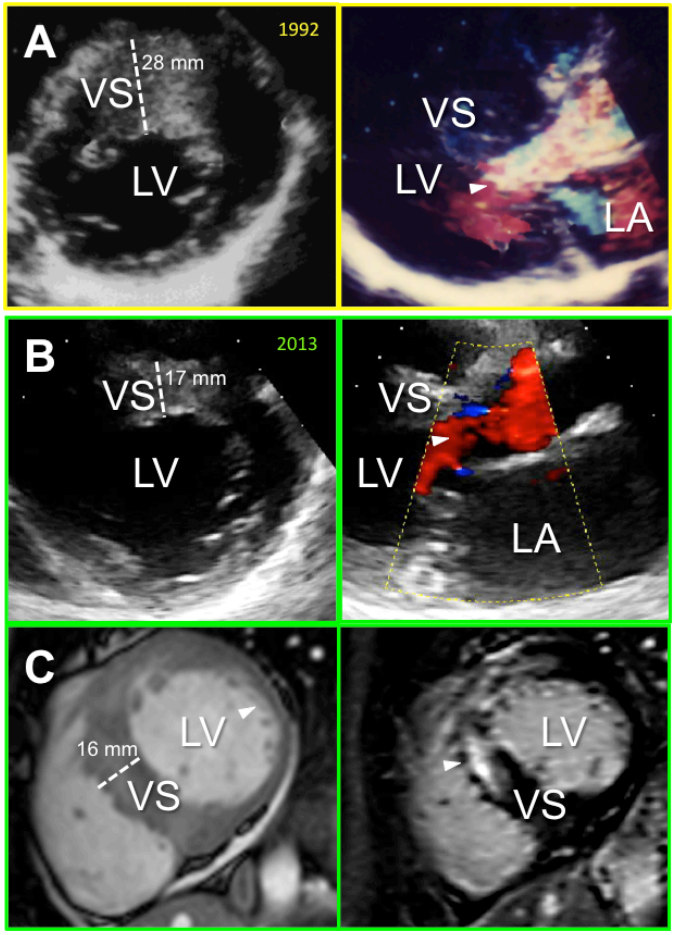

Fig. 2.1-4 Evidence of disease progression in thin-filament HCM.

Echo and CMR images from patient ID#5 carrying the *TNNT2*-F110L mutation.

(A) Echocardiographic images from an early clinical assessment in 1992. Left: parasternal short axis view showing severe asymmetric septal hypertrophy. Right: color-Doppler image displaying turbulent flow in the LV outflow tract (white arrowhead) due to severe dynamic obstruction.

(B) Same views from last evaluation in 2013, showing marked reduction in septal thickness and spontaneous loss of dynamic obstruction, with laminar flow in the LV outflow (white arrowhead); the left atrium is markedly enlarged.

(C) Left: CMR scan from 2013 showing mild LV hypertrophy and areas of non-compaction in the lateral wall (arrow). Right: progressive septal thinning observed over the years is associated with extensive transmural fibrous substitution, as indicated by the large areas of late gadolinium enhancement (white arrowhead).

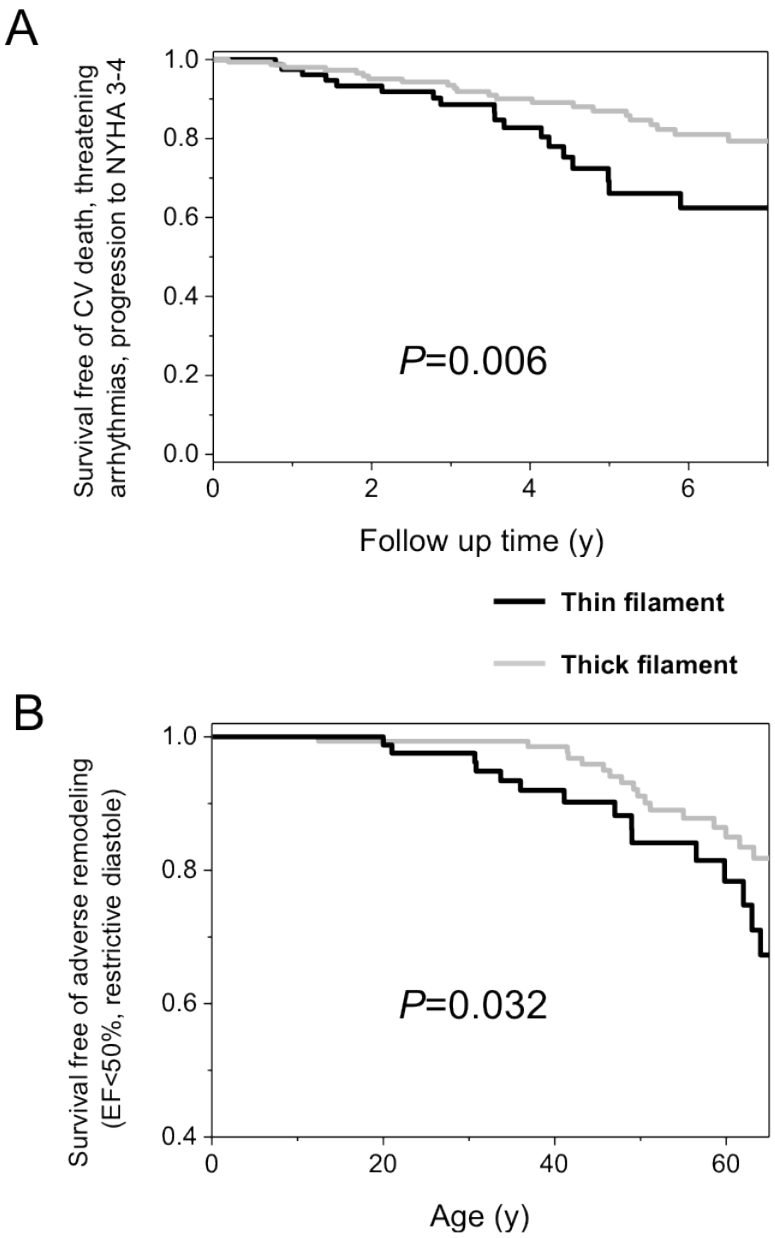

Figure 2.1-5. **Outcomes of thin- versus thick-filament HCM**

Kaplan Meier survival curves showing: (A) cumulative incidence of the *primary end-point* (combination of cardiovascular death, resuscitated cardiac arrest or appropriate ICD shock, non-fatal stroke and progression to NYHA class III or IV) during follow-up; (B) lifelong occurrence of the *secondary end-point*, assessing functional changes in LV function due to adverse remodeling, based on the echocardiographic detection of LV systolic dysfunction (ejection fraction <50%) and/or restrictive LV filling (mitral ratio of peak early to late diastolic filling velocity ≥2 in conjunction with deceleration time of 150 ms or less or, in patients with atrial fibrillation, a transmitral deceleration time <120 ms).

Notably, a purely restrictive evolution with preserved LV systolic function was observed in 11% of patients with thin-filament HCM, consistent with prior reports emphasizing the prevalence of isolated, severe diastolic dysfunction in patients with troponin mutations, with particular regard to troponin I.

Both the development of LV systolic dysfunction and restrictive filling, bear major adverse prognostic implications in HCM patients [9, 11]. Life-threatening arrhythmic events remain an important feature of thin filament HCM, as previously emphasized, but their absolute prevalence is low. Preclinical studies on animal models support the view that HCM due to thin filament mutations is a progressive disease, and suggest that the same molecular mechanisms may underlie both arrhythmogenesis and LV dysfunction [2]. Skinned tissue from patient samples and animal models have consistently shown marked increases in myofilament Ca^{2+} sensitivity in the presence of thin filament mutations [12, 13], closely related with abnormalities of cardiac relaxation [14, 15], diastolic dysfunction and an early increase in susceptibility to arrhythmias.

These elements, consistent with a recent report based on a large group of HCM patients with *TNNT2* mutations, depict a novel and unexpected profile of thin filament disease [7], potentially relevant to risk stratification and management. Specifically, follow-up strategies should not be limited to risk stratification for potentially lethal ventricular arrhythmias, but also focus on the early detection of adverse LV remodeling, significant increases in myocardial fibrosis and decline in LV function. Present options and future advances in antiremodeling therapy may prove useful, if introduced in a timely fashion, to prevent disease progression in this challenging HCM subset.

Furthermore, thin filament mutations can alter cardiac function by increasing the energy cost of contraction [15, 16]. While several of these abnormalities are in common with other HCM-related genes [17], their extent is generally greater in thin filament mutant samples. The constellation of early impairment in excitation-contraction coupling, myocardial energetic derangement and intrinsic abnormalities of sarcomeric relaxation constituting direct consequences of thin filament mutation, all drive an aggressive remodeling process at the level of the single cardiomyocyte [18]. The ultimate result of these abnormalities is a reduction in contractile capacity both at the molecular and cellular level, which precedes structural changes and clinically evident LV dysfunction.

Of note, thin filament-HCM transgenic mouse lines display a remarkable tendency towards disease progression by developing restrictive diastolic patterns and systolic dysfunction over time [19], consistent with the severe diastolic impairment observed in our patients. One of the most intriguing findings in the present cohort was the elevated prevalence of triphasic LV filling pattern in the thin-filament subset compared to the thick-filament subgroup. Trans-mitral diastolic blood flow assessed by pulsed wave Doppler classically comprises two velocity peaks, the E and A waves, occurring during early rapid filling and atrial contraction. However, a triphasic LV filling pattern may be seen when an additional velocity peak of at least 0.2m/s, the L wave, is present during diastasis [20]. The presence of the L-wave has been associated with severe diastolic dysfunction and extensive septal scarring in HCM patients. Interestingly, the presence of the L wave in thin filament-HCM patients was independent of the overall pattern of LV filling, suggesting that patients with various degrees of diastolic dysfunction, and not only those with most severe impairment, may display such abnormality [21, 22].

These data therefore support the view that triphasic filling may represent the clinical counterpart of intrinsic molecular abnormalities associated with thin-filament mutations, although features such as severe microvascular ischemia and myocardial fibrosis are likely to concur, as shown in transgenic TNNT2 mutant mice [23]. When relaxation kinetics were comparable in myofibrils from HCM patients carrying mutations in TNNT2 and MYH7, overall relaxation upon Ca^{2+} removal was clearly prolonged in the former, but not in the latter. In agreement with available evidence from the literature, these findings suggest important differences in the mechanisms leading to diastolic dysfunction for the two forms of HCM. Indeed, while diastolic abnormalities in MYH7-mutant HCM hearts are likely related to secondary changes in energetics and E-C coupling of HCM [24, 25], thin filament mutations appear to directly impair cardiac relaxation. This idea is supported by the finding that, at variance with thick filament mutant myofibrils, resting tension is higher in thin filament mutant myofibrils compared to controls. If confirmed by ongoing studies, these preliminary findings may help identify novel, gene-specific therapeutic targets in thin filament HCM.

In conclusions, HCM associated with thin filament mutations is characterized by significant differences in phenotype and clinical course compared to the more prevalent thick-filament HCM, including increased likelihood of LV dysfunction and progressive heart failure. Management strategies, classically focusing on arrhythmic prophylaxis in this subset, should be reconsidered to include adequate surveillance for adverse LV remodeling and worsening systolic and diastolic function, pointing to specific molecular mechanisms leading to diastolic impairment in this challenging HCM subgroup.

2.2 Early cardiac phenotype in patients with Anderson-Fabry disease.

Those who are familiar with Anderson Fabry disease could find this simple title very intriguing. For those who are not, we should take a step back.

Anderson-Fabry disease (AFD) (OMIM#301500) was named after two dermatologists, W.Anderson and J. Fabry, who separately described two cases of patients with *angiocheratoma corporis diffusum* in 1898 [26, 27]. Almost a century later AFD was showed to be a rare X-linked multisystemic lysosomal storage disorder of glycosphingolipids catabolism, due to the deficiency of the lysosomal enzyme a galactosidase A (a-Gal A) [28, 29]. As a consequence of α-gal A deficiency, progressive accumulation of globotriaosylceramide (Gb3) and its deacylated form, the globotriaosylsphingosine (lyso-Gb3), occurs in various tissues throughout the body, within the lysosomes and in the plasma [30].

AFD is considered a rare disease, with a reported prevalence in the general population of about 1:117.000 [31]. Recently however, newborn screening initiatives have found an unexpectedly high prevalence, as high as 1 in ~3900 newborns [32], suggesting that prior figures may represent a substantial underestimate. While the classic phenotype of AFD is actually rare, these data show that milder variant with late-onset phenotype may be much more frequent than expected [33, 34]. The real strength of these newborn-screening initiatives goes well beyond the simple data itself. They shifted a historic paradigm of a rare disease, with multisystemic involvement in young males, to a more common disease, with a wide phenotype, sometimes with disease manifestation confined

to one organ system [Fig 2.2-1]. Furthermore, screening for AFD among selected groups of patients, i.e. patients with juvenile cryptogenic stroke, renal failure or left ventricular hypertrophy, suggests that many patients are misdiagnosed.

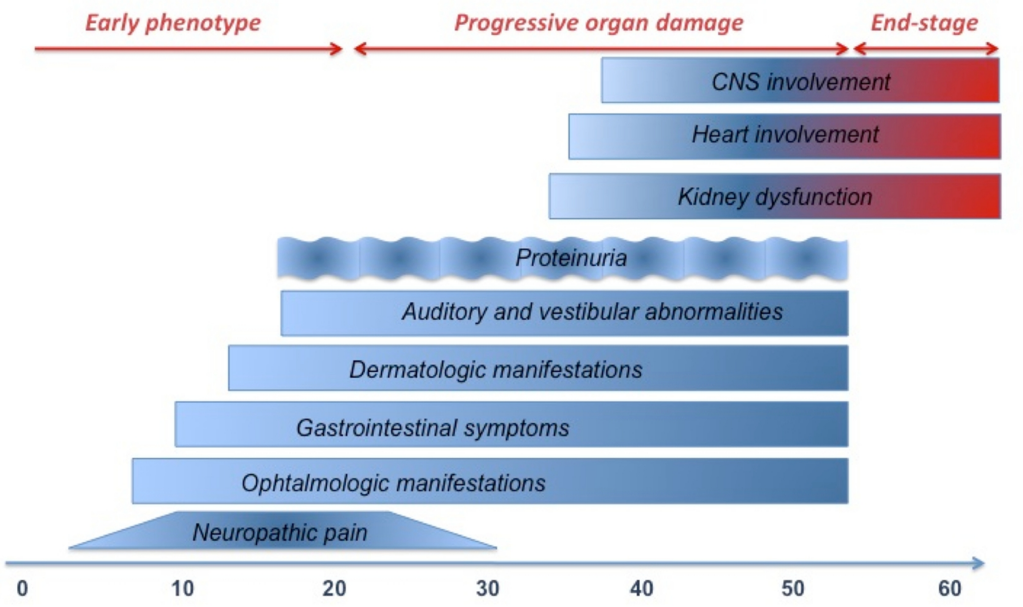

Fig 2.2-1: Symptoms onset and disease progression in classic Anderson-Fabry disease (AFD).

Signs and symptoms of AFD progressively increase with age. In infancy and adolescence neuropathic pain, gastrointestinal manifestations and angiokeratomas are the most frequent manifestations. Proteinuria, the first sign of kidney involvement, is also present in young affected males. Major organ involvement usually develops slowly with age.

Although having an X-linked pattern of inheritance, the disease may affect males and females, the latter with an average 10 years delay [36]. Metabolites storage leads to progressive cellular and multiorgan dysfunction, with either early and late onset variable clinical manifestations that usually reduce quality of life and life expectancy [37].

Heart and kidney failure, stroke and sudden death are the most devastating complications.

A specific therapy for AFD, enzyme replacement therapy (ERT), is available since 2001[38]. New treatment approaches, such as chemical chaperone therapy, alone or in combination with ERT, are currently under investigation [39, 40]. While long-term efficacy of ERT in advanced stage is still debated, increasing evidence shows greater efficacy of early treatment initiation [41].

2.2.1 Hypertrophic cardiomyopathy and Anderson Fabry disease: the linking point

The referral center for cardiomyopathies in Florence has been historically known as primary committed to the management of patients with hypertrophic cardiomyopathy. However, in 2004 genetic analysis of one patient with an established diagnosis of HCM revealed a mutation in the GLA gene. Later, he was proved to have a late-onset variant of AFD, with manifestation confined to the heart. The fact that he was misdiagnosed for almost 4 years, spurred our curiosity and we started to study AFD in its genetic and clinical aspects. To present, almost 70 patients with AFD have been evaluated in our center and more than 50 are in regular follow-up.

Given the expertise our center had with the issue of microvascular dysfunction in hypertrophic cardiomyopathy, in 2006 we decided to study the presence, prevalence and clinical significance of cardiac microvascular dysfunction (CMD) in our cohort of AFD patients.

The linking point between AFD cardiomyopathy and HCM is the myocardium: both these conditions affect primary the heart muscle.

In AFD patients, cardiomyopathy represents one of the features of a multisystemic condition, which affects not only the heart, but also the kidney and the peripheral and central nervous system [29]. Cardiac involvement has been described in both genders and is mainly characterized by variable degrees of hypertrophy and interstitial fibrosis. Although commonly classified as a storage disease of the heart, AFD cardiomyopathy is characterized by true hypertrophy, as Gb3 accumulation only accounts for a very limited proportion of cardiac mass increase. In hypertrophic cardiomyopathy, a genetic mutation in one of the gene encoding for sarcomeric proteins leads to variable degrees of hypertrophy [45]. Tissue architecture in HCM appears overturned, with cardiomyocytes derangement (the so-called “disarray”) and interstitial fibrosis [46].

The structure and function of coronary microcirculation are also altered in both these conditions [Fig 2.2-2]. Narrowing of the arterioles lumen is caused by endothelial Gb3 storage in AFD [47] and hypertrophy of the media wall in HCM [46], and it is accompanied by functional alterations. Therefore, blood supply of the myocardium may be normal at rest but it is usually impaired during exercise and may lead to myocardial ischemia, cardiomyocytes death and cardiac dysfunction.

Our interest in the issue of microvascular function arises from the fact that an impaired response of myocardial blood flow to dipyridamole has been proved to represent a strong and independent predictor of clinical deterioration and death and is associated with poor prognosis in patients with HCM and idiopathic dilated cardiomyopathy [48]. Thus,

since microvascular dysfunction may represent a common pathway leading to disease progression in different cardiomyopathies, we thought to investigate the presence, characteristics and clinical severity of microvascular function also in patients with AFD.

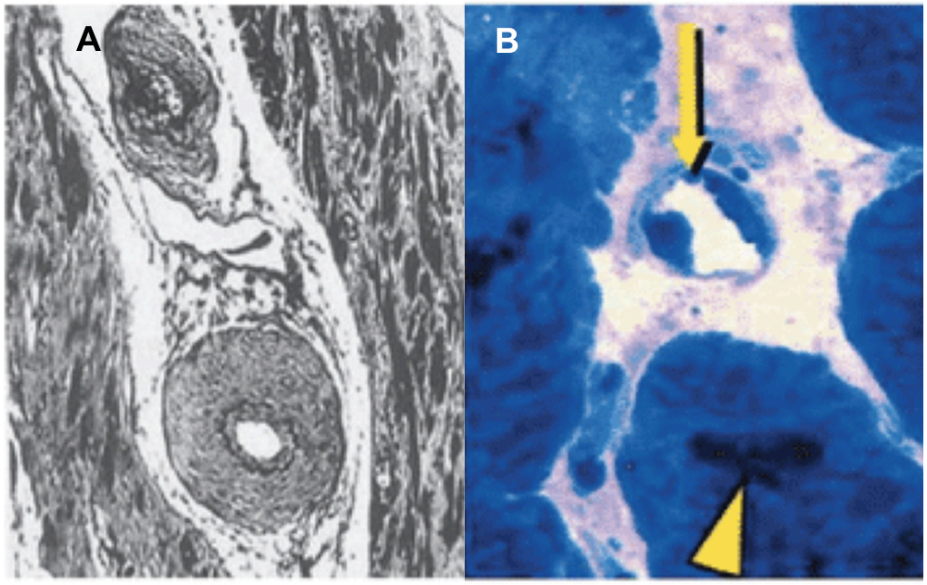

Fig 2.2-2: Gb3 inclusions in the endothelium and cardiomyocytes.

Panel A shows an intramural coronary artery in a patient with hypertrophic cardiomyopathy. Wall thickening is due primarily to severe thickening of medial wall, while the intima is mildly thickened.

Panel B shows interstitial capillaries in the myocardium of a patient with Anderson-Fabry disease. Glycosphingolipid inclusions are present in the endothelium (arrow) and cardiomyocytes (arrowhead)

At that time, the knowledge of coronary microcirculation in Fabry disease was very limited [47]. CMD had been described only in male patients with hypertrophy and advanced stage of disease [49, 50]. Despite the absence of available data about this issue, we believed that CMD

could be a feature of AFD cardiomyopathy, independently of the presence of LV hypertrophy and other instrumental signs of cardiac involvement. What made our hypothesis plausible was the fact that in patients with sarcomeric hypertrophic cardiomyopathy, CMD was known to be a diffuse phenomenon within the LV, involving even non-hypertrophied LV walls, and it could possibly precede LVH development.

This data spurred our curiosity: was CMD also present in AFD female patients, generally exhibiting more subtle cardiac manifestation than men? Was CMD present in patients without any instrumental sign of cardiac involvement? Thus, the present study was undertaken to investigate coronary microvascular function in a cohort of male and female AFD patients at different stages of disease, with and without evidence of LVH, in order to assess whether CMD may occur independent of LVH and in the absence of any other sign of cardiomyopathy.

2.2.2 Coronary microvascular dysfunction as a universal manifestation of Fabry cardiomyopathy.

Thirty patients with an established diagnosis of AFD were selected and underwent a thorough cardiac evaluation by clinical examination, resting ECG, 24-hours Holter monitoring and echocardiography. The same study protocol was employed in 24 healthy controls, who were investigated for exclusion of heart disease, and were comparable to patients in terms of gender and age. Two-third of our patients showed increased LV wall thickness values, ranging from 13 to 27 mm. In contrast to patients with HCM, the distribution of LV hypertrophy was mostly concentric, with milder degree of hypertrophy [Fig 2.2-3 and 2.2-4]. The remaining 10 patients had no evidence of cardiac involvement, with

normal LV wall thickness values and normal Tissue Doppler Imaging mitral septal annulus velocities at echocardiography.

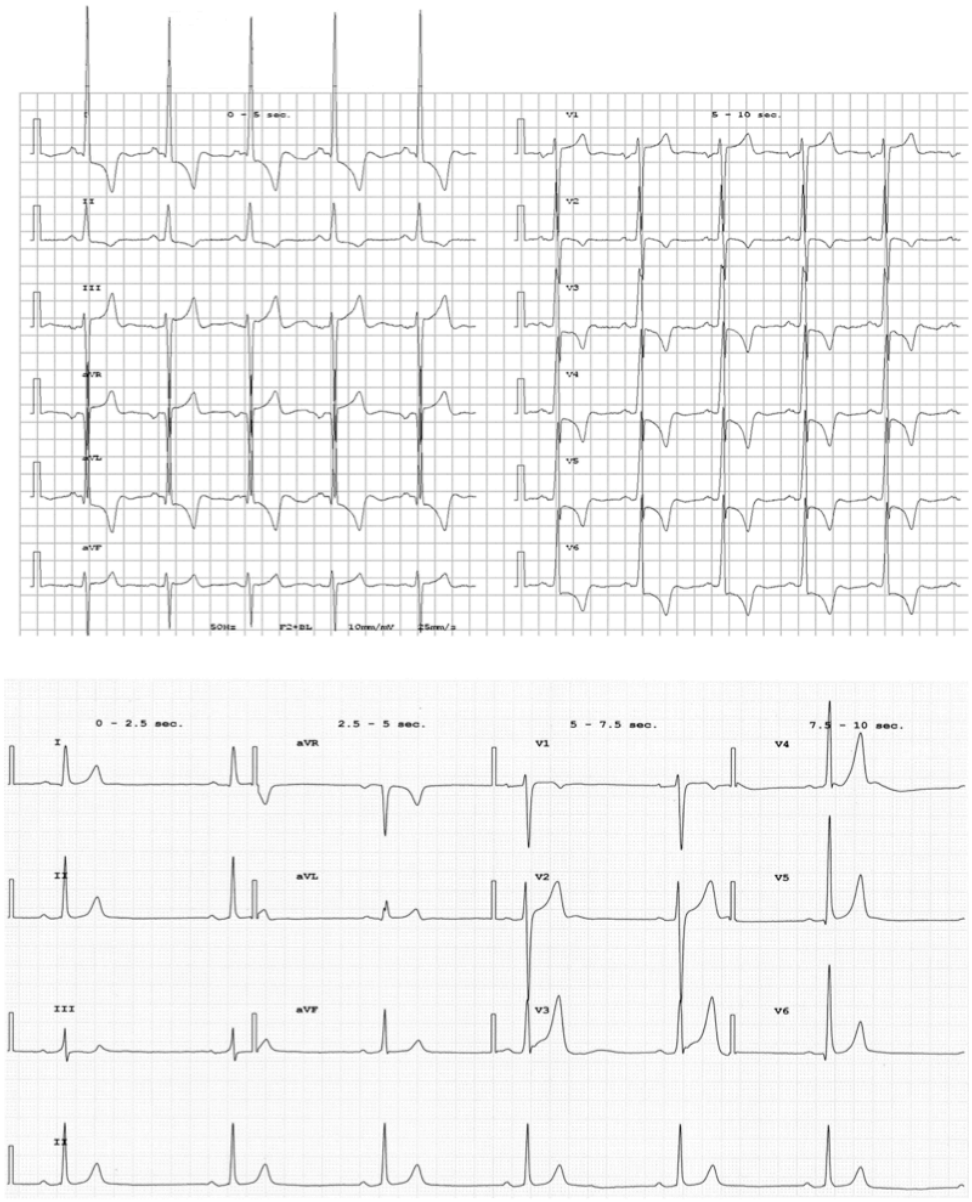

Fig 2.2-3: ECG of patients with Anderson–Fabry disease (AFD).

The ECG on the top shows signs of atrial enlargement, LVH and marked repolarization abnormalities in a 45 year-old man with a late onset variant.

The ECG on the bottom shows sinus bradycardia in a 23 year-old male with classical phenotype.

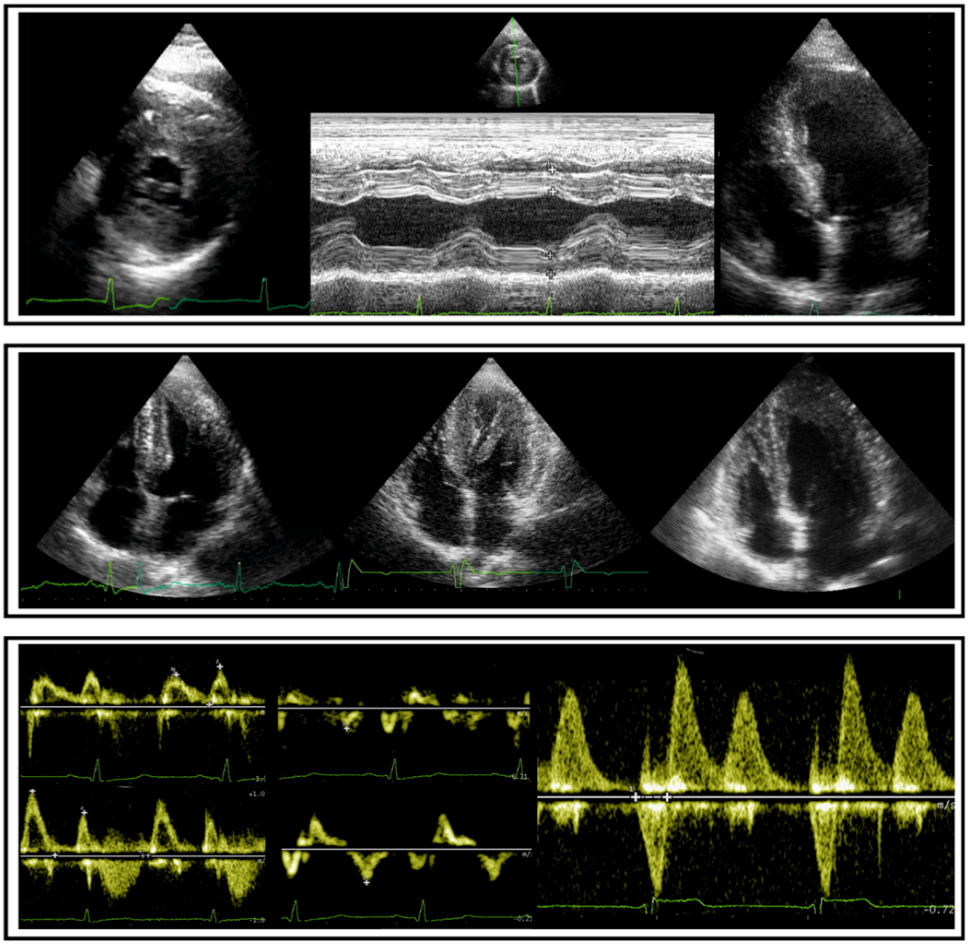

Fig 2.2-4: Echocardiography images of a patient with Anderson–Fabry disease (AFD), carrying the N215S mutation.

Upper and middle panel show different echocardiographic images with LV hypertrophy, which is concentric. Right ventricle and papillary muscles are also hypertrophied. The lower panel shows diastolic dysfunction (from left to right: pulse-waved Doppler of mitral inflow showing mild diastolic dysfunction; TDI images with reduced velocities and pulmonary vein flow with prominent A reverse wave).

Coronary microvascular function was assessed by Positron emission tomography (PET), a technique that allows a direct and precise quantification of microvascular function, through the measurement of maximal global and regional myocardial blood flow after dipyridamole (Dip-MBF) [51]. The measurement of myocardial blood flow in conditions

of near-maximal vasodilatation, obtained by intravenous infusion of dipyridamole, permits calculation of maximum MBF. In the absence of obstructive epicardial coronary disease, flow resistance is primarily determined by the microvasculature (>90% of coronary resistance resides in arterioles <300 um in diameter), and therefore a reduced MBF is a marker of coronary microvascular dysfunction. In normal subjects, maximal myocardial blood flow values >3 ml/min/g after dipyridamole infusion are considered normal. Such values reflect the ability of increase myocardial perfusion to match increased demand.

All AFD patients enrolled in this study exhibited a blunted coronary microvascular response to dipyridamole, as compared to control subjects, with a mean Dip-MBF of only 2.0±0.4 mL/min/g, compared to 3.2±0.5 in normal controls, i.e. a 60% reduction in coronary flow [Fig. 2.2-5].

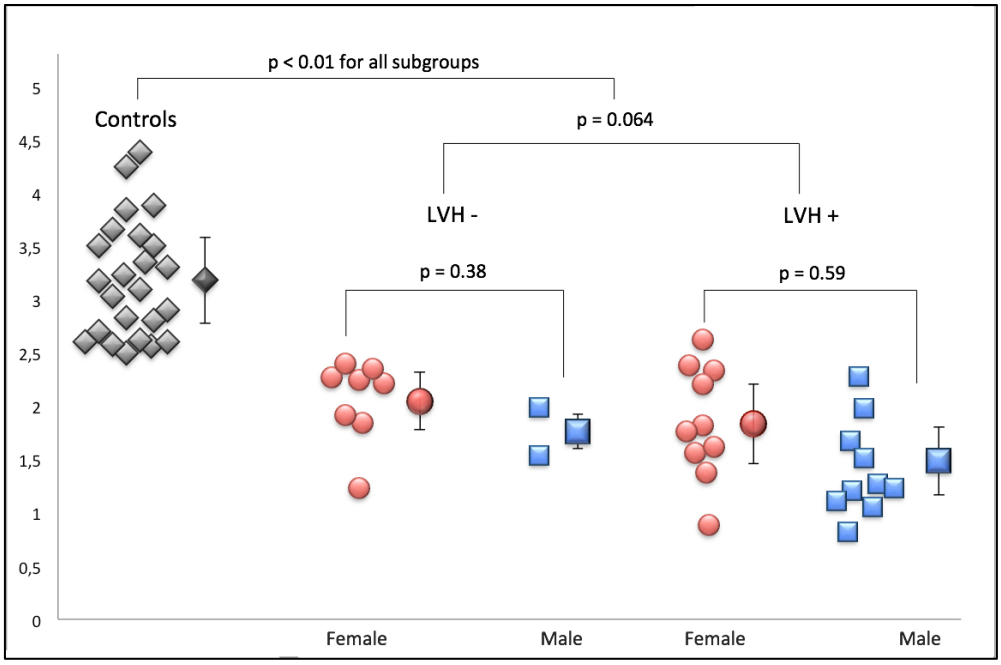

Fig 2.2-5: Coronary microvascular dysfunction in patients with Anderson–Fabry disease.

Myocardial blood flow following dipyridamole infusion is markedly impaired in AFD patients compared with control subjects.

As expected, patients with LVH invariably showed marked degrees of CMD, suggesting that the profound remodeling of the heart structure was invariably accompanied by changes in the architecture of small vessels. However, even patients without LVH showed a blunted response to dipyridamole, with mean myocardial blood values ranging from 2.4 to as low as 1.2 mL/min/g. As a result, CMD in patients without LVH was on average milder, but not statistically different, from those observed in patients with LVH.

Altogether, these findings point to CMD as a constant manifestation of AFD, which may be present independent of other instrumental evidence of cardiac involvement [Fig. 2.2-6]. Moreover, AFD patients without LVH and CMD generally had none of the other features that are considered very early disease manifestations, such as reduced diastolic mitral annulus velocities or presence of LGE [52, 53]. Therefore, this observation implies that myocardial flow abnormalities can indeed be considered to represent initial cardiac involvement, possibly followed by overt cardiomyopathy over time. This novel concept is suggested by the young age of patients with CMD in the absence of LVH.

Although myocardial blood flow was globally impaired in AFD hearts, there was a marked regional heterogeneity of myocardial flow, with prevalent hypoperfusion of the apical region. Such regional nature of cardiac involvement may appear counterintuitive in a systemic storage disease. However, the novel idea of a non-homogeneous cardiac involvement in AFD is in line with prior observations in a number of cardiomyopathies, and particularly to those related to storage or infiltrative disorders, such as for example amyloidosis [54]. The mechanisms accounting for such regionally heterogeneous manifestations remain unresolved.

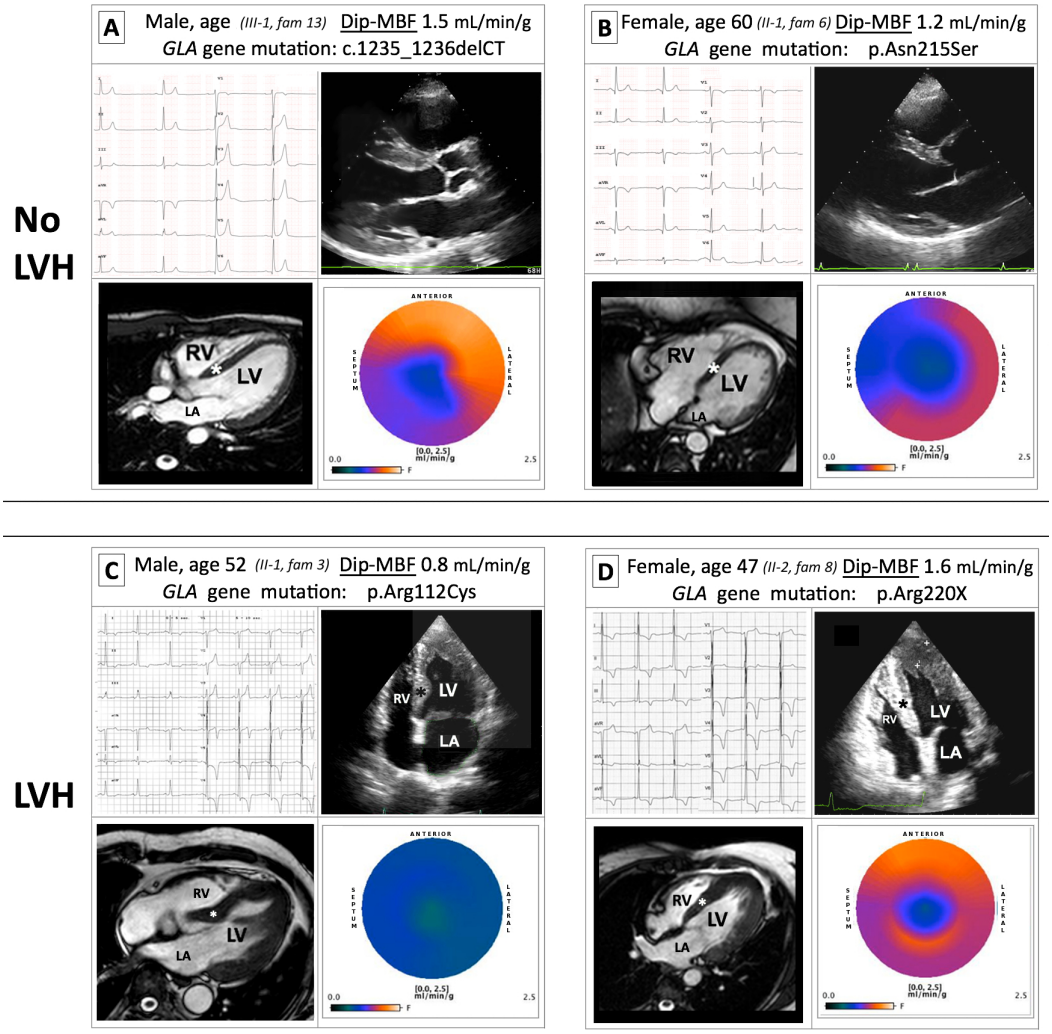

Fig 2.2-6: Relationship of coronary microvasculature dysfunction(CMD) to phenotype in individual patients with Anderson–Fabry disease (AFD).

(A) A 28-year-old male with extreme sinus bradycardia (36 b.p.m.) and normal interventricular septal thickness values (*echocardiographic parasternal long axis and cardiac magnetic resonance four-chamber views). Colour-coded polar map obtained by positron emission tomography (PET) after dipyridamole infusion showing regional CMD, with relatively preserved flow in the antero-lateral region.

(B) A 60-year-old female with normal ECG, without normal LV wall thickness values. The polar map shows severe CMD, despite normal LV wall thickness values.

(C) A 52-year-old male with marked ECG abnormalities, severe LV hypertrophy (LVH) (*echocardiographic apical four-chamber view and

cardiac magnetic resonance four-chamber view). The polar map shows severe CMD.

(D) A 47-year-old female with markedly abnormal ECG, and severe LVH. The polar map shows relatively preserved microvascular function, despite the LVH. Dip-MBF, myocardial blood flow following dipyridamole infusion,

2.2.3. Cardiac involvement in females with AFD and influence of genotype

Although AFD is an X-linked disease, female patients are well known to develop the clinical manifestations of the disease, which can be severe and determine outcome [55]. Cardiomyopathy is not an exception to this rule, and has been described in women; however, cardiac manifestations generally have a later onset and are less marked than those occurring in men [56, 57]. Thus, we were expecting milder degree of microvascular dysfunction in females. However, women with and without LVH had considerable degrees of CMD, although to a less sever degree than males. Of note, in females without LVH, a reduced MBF represented the only detectable evidence of cardiac involvement. As a result, heightened awareness should be considered for cardiac involvement in female patients with AFD, even in the absence of an echocardiographic phenotype.

With regard to the genotype of patients, we had the opportunity to study a recurrent mutation in Fabry disease. The c.644A>G *GLA*-gene mutation, leading to the p.Asn215Ser amino acid substitution was present in three index patients and seven family members. This mutation is known to be related to the so-called “cardiac variant” of the disease [58]. Patients harboring this mutation usually have a predominant cardiac phenotype, characterized by moderate to severe degree of hypertrophy,

with an asymmetrical distribution, which is rather uncommon in AFD. Disease onset is usually late, with males developing signs of cardiac involvement during their thirty. Extracardiac involvement, such as renal or cerebrovascular, is uncommon although not impossible.

In our cohort, the p.Asn215Ser group showed similar degree of hypertrophy compared to patients with other genotype. Nevertheless, myocardial blood flow was markedly lower when compared to other genotypes [Fig 2.2-7], suggesting a severe impairment in the microvascular function. Furthermore, at logistic regression analysis, only the mutation p.Asn215Ser proved to be an independent determinant of severe CMD (hazard ratio for Dip-MBF<1.25 ml/g/min 9.0; 95% confidence intervals: 1.3-61.1; p=0.03).

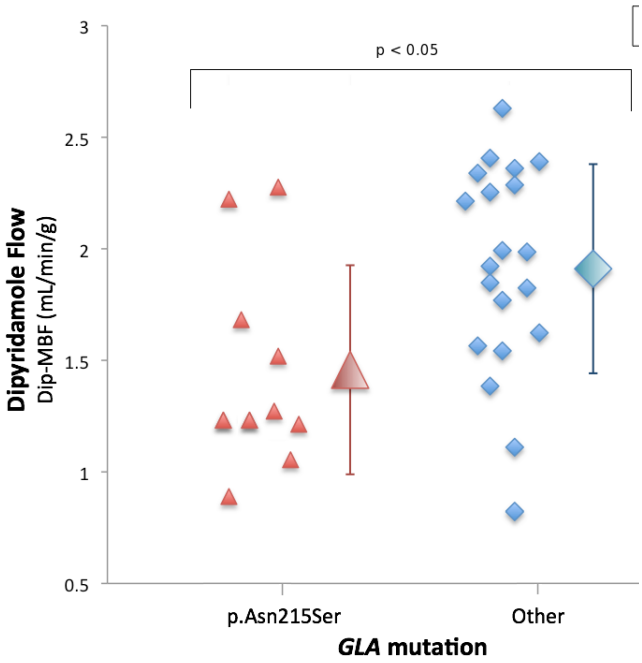

Figure 2.2-7: Genotype influence on microvascular function

Despite similar degree of hypertrophy, patients with the p.Ans215Ser mutation showed a more severe impairment of the myocardial perfusion when compared to other patients.

The consistent and severe blunting of MBF in patients with the p. Ans215Ser GLA gene mutation may suggest a role of genetic status in determining the severity of MBF. Furthermore, the concept of the possible influence of genetic status to microvascular function has been proved in other more common genetic cardiomyopathy (i.e. hypertrophic cardiomyopathy) [59]. Such variability suggests selective microvascular involvement activated by specific gene mutations, and is consistent with the phenotypic variability of clinical manifestations in AFD. In the light of these findings, the molecular links between GLA gene mutations and coronary microvascular remodeling need to be further investigated.

2.2.4. Potential mechanisms of coronary microvascular dysfunction

The potential pathophysiological mechanisms underlying coronary blood flow impairment in AFD are several, and include those mediated by LVH (reduced capillary density, extravascular compression forces) as well as those directly affecting the microvasculature (endothelial dysfunction due to GB3 storage, nitric oxide pathway dysregulation, microvascular remodeling) [60]. However, in patients without LVH, only the latter are present, suggesting that LVH per se is indeed likely a contributor to CMD, rather than a cause.

Despite considerable advances in our understanding of AFD, the mechanisms leading to LVH are not well understood. Storage of Gb3 within cardiac myocytes accounts for a small amount of the whole LV mass, which is mainly represented by true myocardial hypertrophy [44]. One hypothesis is that intracellular accumulation of Gb3 may disturb cardiac energetic metabolism, representing an early trigger for the activation of the intracellular signaling pathways leading to hypertrophy,

fibrosis, apoptosis and necrosis [61]. Furthermore, microvascular dysfunction and chronic hypoperfusion, due to Gb3 accumulation in endothelial cells, may also play a crucial role in the activation and perpetuation of these pathophysiological processes, even at early stages.

An unavoidable limitation of the present study lies in the small sample size and heterogeneity of our study population. Selecting a more homogeneous study cohort, by excluding patients with comorbidities or cardiovascular risk-factors, would not have been feasible in a rare and multisystemic disorder such as AFD. Thus, myocardial blood flow may have been affected in our cohort by the interplay of comorbidities such as hypertension, smoking or dyslipidaemia, as well as ERT itself. This may have potentially led to overestimation of CMD in our patients with regard to the healthy controls, in whom none of the conventional cardiovascular risk factors were present. While we acknowledge this unavoidable bias, however, MBF values in AFD with no history of smoking, hypertension and dyslipidaemia, ideally representing "pure" AFD patients, were comparable to the rest of the cohort, showing similar degree of MBF impairment. Thus, our data are consistent with a disproportionate microvascular involvement in AFD, overruling the effect of these potential environmental confounders.

2.2.5. Early detection of heart involvement in Fabry disease: a crucial point for patient's management

The issue of early organ damage identification has become critical following the introduction in clinical practice of ERT and novel therapeutic molecules, including pharmacological chaperone therapy [39, 40]. To date, evidence of benefit of ERT in AFD patients with overt signs of

cardiomyopathy and LVH is disappointing, and earlier timing of treatment initiation has been advocated in order to improve efficacy [41].

Our findings imply that a normal echocardiogram cannot exclude deterioration of microvascular function related to the disease, raising important issues regarding risk stratification and management. Thus, early detection of subclinical cardiac involvement, as allowed by PET studies of microvascular function, may become a critical element in clinical decision making especially in young AFD patients. In particular, CMD may represent a viable treatment target, potentially relevant to prevention of disease progression and outcome. In the future, the advent of cardiac magnetic resonance (CMR), by virtue of its larger availability and more favourable safety profile compared to PET [62], may allow large-scale investigation of CMD in AFD patients. To date, however, quantitative assessment of microvascular flow by CMR is time-consuming and largely limited to research purposes.

Furthermore, is it possible to hypothesize that the severity of CMD may be an important predictor of adverse outcome, as for other genetically determined cardiomyopathies [48].

These findings represent a picture of our AFD population at this time. However, since everything we know, especially in medicine, is destined to change or modify, our present goal is to continue to follow this patients in order to be able to answer many more questions arisen from this work. We are in particular interested in determining whether CMD may predict the development of LVH or may be a predictor of outcome.

Full text of the publication may be found in chapter 7.

2.3 Next Generation Sequencing: new horizons in the field of cardiomyopathies

Cardiomyopathies are a clinically heterogeneous group of heart muscle disorders, largely genetic in nature, defined by the presence of abnormal myocardial structure and/or function in the absence of ischemic heart disease or abnormal loading conditions. Cardiomyopathies have been variously classified over the years based on their phenotypes. The current classifications continue to be based on phenotype defined by clinical evaluation of affected individuals and incorporating genotype when possible (*see chapter 1*)[63]. Contemporary classifications distinguish the dilated (DCM), hypertrophic (HCM) and restrictive forms (RCM), as well as arrhythmogenic right ventricular cardiomyopathy (ARVC) and an unclassified group including isolated left ventricular non-compaction (LVNC).

While the familial nature of cardiomyopathies has been known since their earliest clinical description, the first cardiac disease-causing mutation was identified in a family with hypertrophic cardiomyopathy in 1990 [64]. During the past two decades, numerous genes and genetic variants associated with different cardiomyopathies have been identified. However, the great complexity of genotype is paralleled by huge phenotype variability, thus making a genotype-phenotype correlation almost impossible to achieve.

Many attempts at providing distinct boundaries between these clinical entities have been hindered by the increasing evidence of bi-directional overlap: a) different cardiomyopathies share common genetic background; for example, genes encoding sarcomere protein genes may

be associated with HCM, DCM, RCM and LVNC, and Z-disc or calcium handling protein genes may cause both HCM and DCM; b) the same clinical phenotype may be produced by defects in genes that are profoundly diverse in structure and function, such as the lamin A-C gene, titin or beta-myosin heavy chain in the case of DCM. As a consequence, genetic investigations of patients with cardiomyopathies based on traditional sequencing techniques have grown at a relatively slow pace, as each phenotype could only be tested for a limited amount of candidate genes in clinical practice.

Sanger sequencing, even when performed by automated high-throughput machines, analyzes ~2 million bases per day, a very low performance compared to the collective size of the genes involved in familial heart disease. This has led different institutions and companies over the years to design small panels of 8-20 genes for each condition, with yields ranging from ~60% in HCM to ~20% in DCM. The various panels were often not all available in single institutions, because of costs and time constraints, forcing patients to travel and perform repeated analyses before reaching a diagnosis.

The recent advent of next generation sequencing (NGS) technologies have led to massive increase in throughput, up to ~50 billion sequenced bases per day, and reduction in cost. Therefore, NGS, also described as “second generation” or “massively parallel”, has replaced Sanger sequencing as the primary methodology employed by researchers and clinicians to identify disease variant. The ability to simultaneously analyze multiple or very large genes at a cheaper cost per base makes next generation sequencing an attractive solution for clinical diagnostic testing to identify the disease-causing mutation (or mutations) in patients with genetically heterogeneous disorders. Three different approaches

have become feasible in clinical practice (in increasing order of complexity): a targeted approach (with panels possibly including over 100 genes), whole exome and whole genome. While the last two remain expensive, labor-intensive and mainly used for research purpose, a targeted approach is becoming increasingly popular in referral centers for inherited heart diseases.

2.3.1. NGS in the clinical setting: clinical pictures of familial phenotype and genetic study

At our Institution, genetic screening has been offered to HCM patients since 2000. At that time, denaturing high pressure liquid chromatography (DHPLC) was used as an initial approach, and only the 3 most prevalent genes (MYH7, MYBPC3 and TNNT2) were analyzed, as more comprehensive testing would not have been sustainable in a national health system environment. Furthermore, turn-around times were very long (close to 2 years) because of the complex and lengthy procedures employed. By 2006, only 88 index cases had been completed, with an overall yield of mutations approaching 60%. In 2008, robotic automation allowed an expansion of the standard screening set of genes from 3 to 8 (by including TNNI3, MYL2, MYL3, ACTC1, TPM1), and DHPLC was abandoned in favour of direct Sanger sequencing. Costs and turnaround time decreased significantly (to less than 6 months). At the end of 2010, 672 index cases had been completed, with an overall yield of 64%. Thus, even considering most novel variants as pathogenic (an issue that remains controversial to this day), over one-third of our probands proved negative for the most prevalent sarcomere genes and thus the molecular basis of their disease remains unresolved – a problem shared

by all laboratories involved in HCM screening. However, NGS is rapidly changing the landscape of genetic testing because of its power and sensitivity.

In the present section I would like to illustrate the first experience of our referral center, historically committed to genetic studies of familial cardiomyopathies, with these new genetic technologies. Thirteen families with different form of cardiomyopathy, negative after standard genetic analysis, were selected and analyzed using whole exome analysis and a targeted approach [Table 2.3-1].

For the purpose of this thesis, only the most informative families have been selected and will be described in the next sections.

Table 2.3-1: familial cardiomyopathies studied using NGS

Whole exome analysis	***Targeted approach***
1 family with unexplained juvenile sudden cardiac death and ventricular fibrillation	1 heterogeneous phenotype * (HCM / LVNC cardiomyopathy and supraventricular arrhythmias)
8 families with HCM	
1 family with DCM *	
1 family with ARVC *	
1 family with LV aneurism *	

[* = families described in the text].

2.3.2 Whole exome analysis

A family with idiopathic dilated cardiomyopathy

The proband was 31 years old when he was first seen at our center. He was totally asymptomatic and came to our center because of familiar screening. His 39-year-old brother had died suddenly a couple of months earlier and the post-mortem examination revealed dilated cardiomyopathy. His 63-year-old mother had died 3 years earlier because of refractory heart failure. She had had a diagnosis of dilated cardiomyopathy when she was 45 and required a pacemaker at the age of 53 [Figure 2.3-2]. Despite the early onset of the disease in this lady, who had no major cardiovascular risk factors, the possible familial nature of the disease was missed and none of the doctors advise a family screening.

In our patient, echocardiography revealed a dilatation of the left ventricle, with a mild impairment of the systolic function (ejection fraction 45%). Furthermore, the ECG showed I degree atrio-ventricular block (PR interval 420 msec), while dynamic 24-hours holter showed many episodes of type 2 II degree AV block. He also had elevated CPK level, without any sings of skeletal myopathy. These elements prompted us to consider a diagnosis of cardiolaminopathy as the most plausible [Figure 2.3-2].

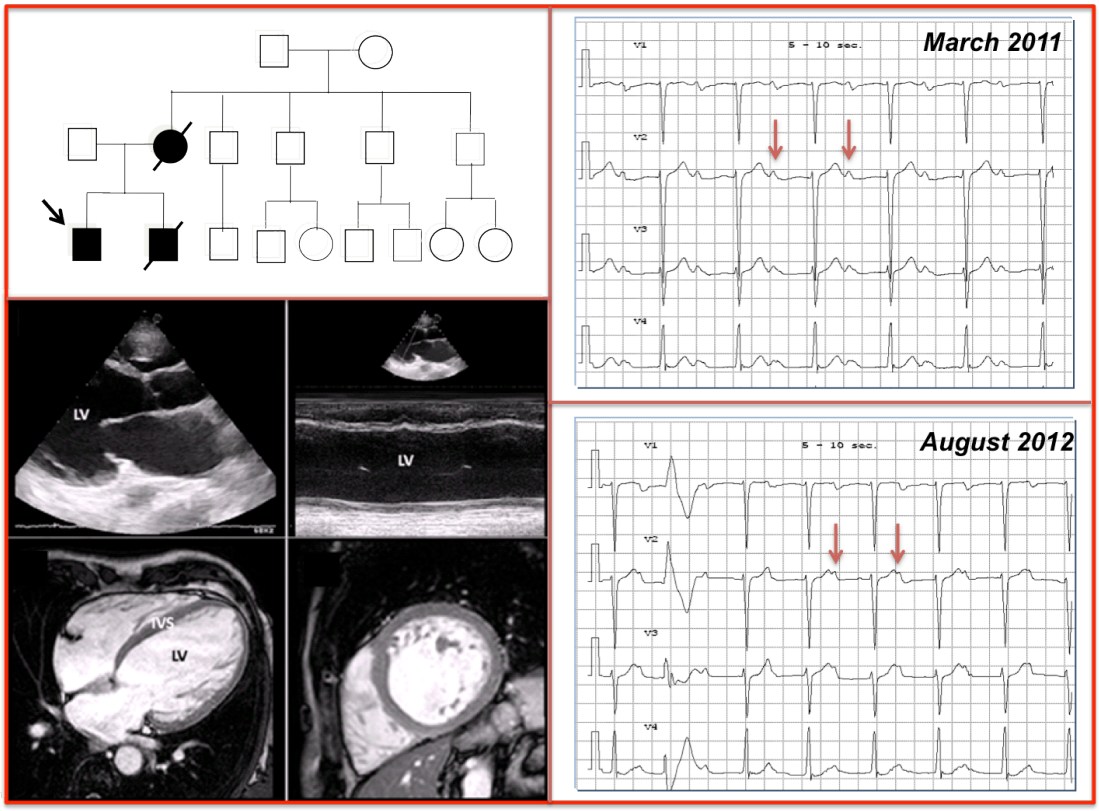

Figure 2.3-2: Clinical features of the proband with DCM

Top right: family pedigree showing a dominant inheritance

Bottom right: echocardiogram and cardiac magnetic resonance images showing dilated LV (EF 45%).

ECG performed during the first examination (*top left*) and during a follow-up visit (*bottom left*): marked first degree atrio-ventricular block was present.

Therefore, after a detailed pre-test counseling with our geneticist, a blood sample was drawn. Since the clinical picture was highly suggestive of a genetic cardiomyopathy related to mutation in the LMNA A/C gene, we choose standard genetic with Sanger sequencing as a first-line diagnostic technique. Surprisingly, genetic analysis came back negative. Filled with curiosity to detect the mutation responsible for such cardiomyopathy, we decided to analyze the whole exome of this subject.

Initial genetic analysis identified almost 55000 variants. The standard bioinformatics analysis (as described in methods) did not allow the identification of the causative mutation.

Sanger sequencing, as well as whole-exome sequencing, have been successfully used to discover the great majority of genomic variants, (i.e common and rare single nucleotide variants (SNVs), small insertions/deletions (indels) and breakpoints of structural variations). However, they are unsuitable for detecting large deletions or duplications because they span one or both primer binding sites. This can be achieved through the analysis of copy number variations (CNVs), a comparative depth of coverage analysis between normal controls and patient samples. In order to do that, the EXCAVATOR software was applied and a huge deletion of 163±22 kb was found. The deleted region, located on the long arm of the 1 chromosome (1q22), comprised the entire LMNA gene.

A family with arrhythmogenic cardiomyopathy

The proband was a 37-year-old woman who was admitted to our hospital after resuscitated cardiac arrest. She had negative cardiological clinical history, without syncope, dyspnea or palpitations. She had been a competitive runner until her thirties and at the age of 35 she gave birth to spontaneous conceived triplets. At the time of the cardiologic evaluation she presented clinical features consistent with a diagnosis of arrhythmogenic cardiomyopathy with biventricular involvement, associated with palmar keratoderma [Figure 2.3-3]. Echocardiography and cardiac magnetic resonance showed a mildly dilated right ventricle with reduced systolic function and multiple areas of bulging of the free wall, associated with reduced left systolic function (EF 45%).

A therapy with ACE-inhibitors, amiodarone and beta-blockers was initiated and an implantable cardioverter-defibrillator was implanted in secondary prevention. She also had family history of juvenile sudden cardiac death: her brother, with a history of syncope, died suddenly at the age of 28 years while playing soccer. He also had scalp dermatitis and woolly hair. A post-mortem evaluation of echocardiograms and electrocardiograms, performed a couple of months before his death, showed clinical findings suggestive of arrhythmogenic right ventricular cardiomyopathy. The grandmother's sister had dilated cardiomyopathy and she also died suddenly at the age of forty. A mild form of ARVC and scalp dermatitis was observed both in the mother and in the uncle of the proband. Clinical features observed were consistent with the diagnosis of Carvajal syndrome [66], although the family's pedigree strongly suggested a dominant model of inheritance [Figure 2.3-4].

Traditional Sanger sequencing failed to identify any mutation in those genes usually associated with ARVC (transforming growth factor beta3, ryanodine receptor 2, desmoplakin, plakophilin2, desmoglein2, desmocollin2 transmembrane protein 43, junction plakoglobin protein).

A whole exome sequencing study was therefore performed in the proband. Data analysis identified 51337 variants, 7517 of which located in coding regions and intron-exon junctions. After frequency filtering (variants with minor allele frequency < 5% in 1000 Genomes Project), 834 variants were selected. Among these, 101 variants were not present in dbSNP Release [Figure 2.3-4].

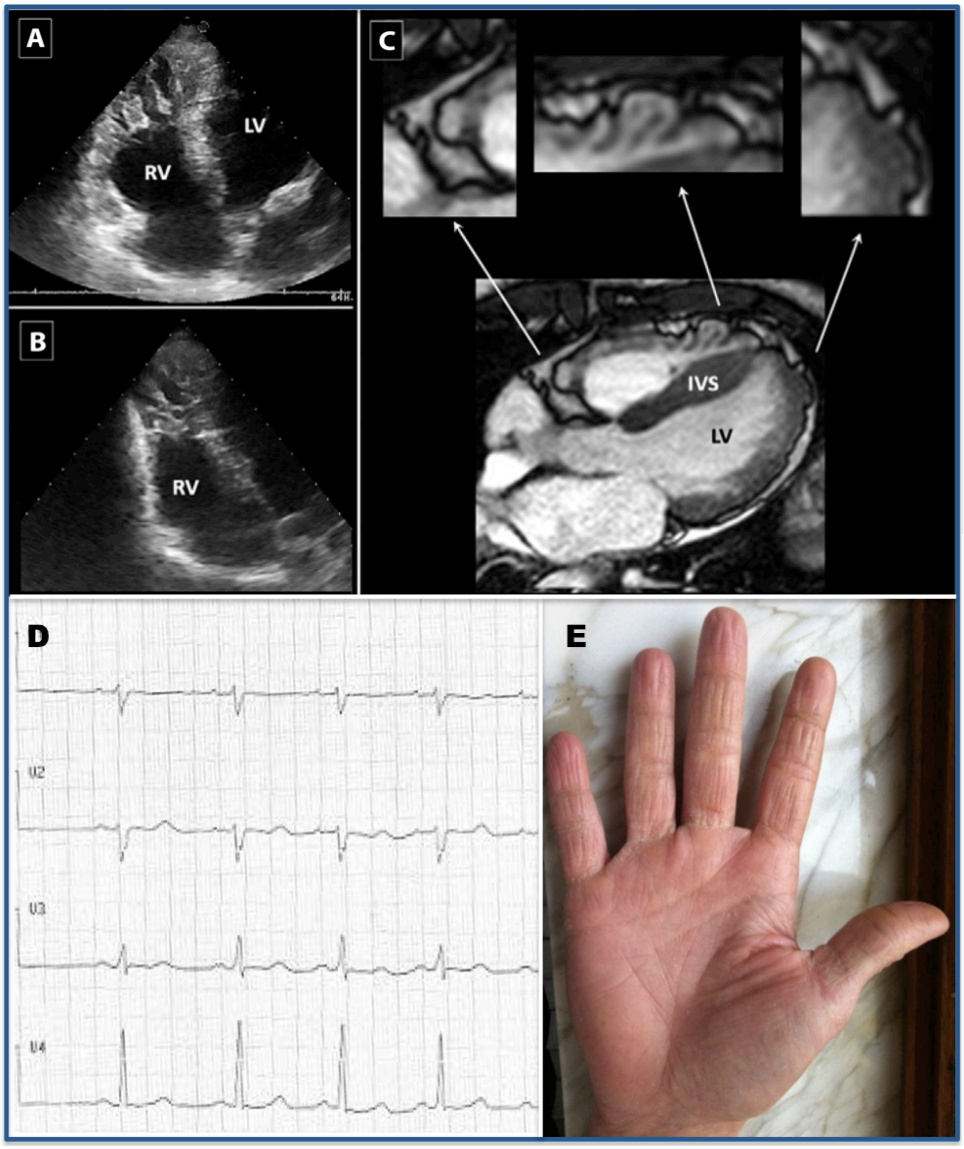

Figure 2.3-3: clinical features of the proband with ARVC

A-C: echocardiogram and cardiac MRI images show wall aneurysms within the so-called ``triangle of dysplasia'' (white arrows: evident systolic bulging in infundibular, apical, and subtricuspid regions of the RV). The LV apex is also involved.

D: ECG of the proband

E: palmar keratosis