Pathology and Function of Conduction Tissue in Fabry Disease Cardiomyopathy

Running Title: Frustaci et al.; Conduction Tissue in Fabry diseases

Andrea Frustaci, MD¹,²; Emanuela Morgante, PhD¹; Matteo A. Russo, MD³; Fernanda Scopelliti, PhD²; Claudia Grande, PhD²; Romina Verardo, PhD²; Pasquale Franciosa, MD¹; Cristina Chimenti, MD, PhD¹,²

¹University of Rome Sapienza, ²IRCCS L. Spallanzani, ³IRCCS S. Raffaele Pisana, Rome, Italy

Correspondence:
Andrea Frustaci, MD
Department of Cardiovascular, Respiratory, Nephrologic, Anesthesiologic & Geriatric Sciences
Sapienza University
Viale del Policlinico 155
00161, Rome
Italy
Tel: + 39 06 5517 0520
Fax: +39 06 55 170575
E-mail: biocard@inmi.it

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Abstract:

**Background** - Cardiac arrhythmias are common in Fabry disease (FD) and may occur in pre-hypertrophic cardiomyopathy (CM) suggesting an early compromise of conduction tissue (CT). FD X-linked and, CT therefore may be variously involved in male and female FDCM, affecting CT function.

**Methods and Results** - Among 74 pts with endomyocardial biopsy diagnosis of FDCM, 13 (6M, 7F mean age 50.1 ± 13.5 years, maximal wall thickness (MWT) 16.7 ± 3.7 mm) had CT included in histological specimens and 6 also at electron-microscopy. CT glycolipid infiltration was defined as focal, moderate, extensive or massive, if involved ≤30%, ≤50%, >50% or 100% of cells; identified as loosely arranged small myocytes positive to HCN4 immunostaining, supplied by a centrally placed thick-walled arteriole. CT involvement was correlated with age, sex and alpha-Gal gene mutation. CT function was evaluated by electrophysiological study (EPS) and arrhythmias at Holter registration. CT infiltration was focal/moderate in 4 females with no arrhythmias and normal EPS; extensive in 3 females with atrial and/or ventricular arrhythmias and short HV interval; massive in 6 males with atrial fibrillation and/or ventricular arrhythmias and short HV. Short P-R/AH with increased refractoriness was additionally found in 3 pts with extensive/massive CT infiltration. A male with the shortest HV presented infraHissian block during decremental atrial stimulation. There was no correlation with age, MWT and type of gene mutation.

**Conclusions** - CT infiltration in FDCM is constant in male and variable in female due to skewed X-chromosome inactivation; its extensive/massive involvement causes accelerated conduction with prolonged refractoriness and electrical instability.

**Key words**: arrhythmia, conduction, Fabry disease, molecular rehabilitation, conduction tissue
Introduction

Fabry disease (FD) is an X-linked lysosomal storage disorder caused by deficiency of the enzyme alpha-galactosidase A, leading to progressive intracellular deposition of globotriaosylceramide (Gb3) and related neutral glycosphingolipids in multiple organ systems, including skin, kidneys, vascular endothelium, ganglion cells of peripheral nervous system and heart\(^1\). Cardiac involvement is common both in homozygous males and in heterozygous females and contributes substantially to disease-related morbidity and mortality\(^2\)\(^-\)\(^3\)\(^-\)\(^4\). Noteworthy, the heart can be the only organ involved in the so called “cardiac Fabry variant”\(^5\), raising specific diagnostic problems with hypertrophic cardiomyopathy.

Indeed, GB3 accumulates in all cardiac cell types, including microvascular endothelial and smooth muscle cells, fibroblasts and cardiomyocytes, leading to myocardial ischemia, valve abnormalities and myocardial hypertrophy that mimic the morphological and clinical picture of hypertrophic cardiomyopathy\(^5\)\(^-\)\(^6\).

Conduction tissue (CT) is believed to be specifically affected as well as supraventricular and ventricular arrhythmias are common and may manifest even in the pre-hypertrophic phase of FD cardiomyopathy (FDCM)\(^7\)\(^-\)\(^8\).

Pathology of CT in FDCM is poorly understood and may vary with sex of patients, due to the X-chromosome localization of alpha-Gal gene and the skewed inactivation of X-chromosome in female and type of gene mutation resulting in isolated cardiac or systemic manifestation. Severity of CT infiltration by Gb3 may reflect on CT function and arrhythmic profile of FD subjects requiring in the advanced stage of disease major therapeutic interventions including pacemaker and/or ICD implantation.

In the present report, pathology of CT included in endomyocardial biopsy specimens
from patients with FDCM, is described. Severity of CT involvement is correlated with patients’ age, sex and mutation (classical or variant) of alpha-Gal gene. The effects of CT infiltration have been evaluated by electrophysiological study and Holter monitoring.

Materials and Methods

Patient Population

From 1998 to 2013, 74 patients with clinical phenotype of hypertrophic cardiomyopathy (idiopathic left ventricular hypertrophy with maximal wall thickening (MWT) ≥ 15 mm), unexplained left ventricular (LV) hypertrophy (MWT between 11 and 14 mm), micro vascular angina, or cryptogenic arrhythmias received histological diagnosis of FDCM at endomyocardial biopsy. Thirteen/74 (6M, 7F, mean age 50.1 ± 13.5, MWT 16.7 ± 3.7 mm) had CT included in at least 1 specimen. In six patients, CT was also detectable at electron-microscopy. These 13 patients, who presented with FDCM and CT inclusion, are our study population. None of them was on enzyme replacement therapy at the time of the study initiation. The study was approved by the Ethical Committee of our Institute and written informed consent was obtained from each patient before study entry.

Cardiac Studies

Extensive clinical examination, including assessment of FD systemic manifestations, non-invasive (resting ECG, Holter monitoring, echocardiography with Tissue Doppler analysis, cardiac magnetic resonance) and invasive cardiac studies were performed in all patients. Invasive cardiac exams were performed and including cardiac catheterization, selective coronary angiography, LV angiography and biventricular or LV endomyocardial biopsy. Endomyocardial biopsies (four to five each ventricular chamber) were performed in the septal-apical region of left or both ventricles. Myocardial samples were processed for routine histological and histochemical
analysis and for transmission electron microscopy.

**Histology and electronmicroscopy**

For histological analysis the endomyocardial samples were fixed in 10% buffered formalin and paraffin embedded. Five micron thick sections were stained with hematoxylin & eosin, Masson thricrome and Miller’ Elastic Van Gieson. Histochemistry with PAS and Sudan black stains was obtained in frozen sections to evaluate the presence of intracellular glycolipid material. A quantitative evaluation of the severity of CT infiltration was assessed counting the percent of infiltrated cells on total number of CT cells in at least 3 serial sections. The infiltration was defined as focal in presence of < 30% of affected cells, moderate with <50%, extensive with >50%, massive in presence of 100% of cell affected.

For electronmicroscopy (TEM), additional samples were fixed in 2% glutaraldehyde in a 0.1 M phosphate buffer, at pH 7.3, post fixed in osmium tetroxide and processed following a standard schedule for embedding in Epon resin. Ultrathin sections were stained with uranyl acetate and lead hydroxyde. A Philips CM-10 TEM was used for observation and photographic analysis.

**Immunohistochemistry for HCN4**

Immunohistochemistry was performed on formalin/PFA-fixed paraffin embedded sections of endomyocardial samples to identify HCN4 positive conduction tissue. To expose target proteins heat induced antigen retrieval was performed using 10mM sodium citrate (pH6.0) buffer microwaved for 15 minutes. Following antigen retrieval tissues were blocked in 3% BSA-PBS for 30 minutes at room temperature. Tissues were then probed at a dilution of 1:20 with a rat monoclonal antibody recognizing HCN4 (Pierce Antibody Products, Thermo Fisher Scientific Inc.) or without primary antibody (negative control) overnight at 4°C in a humidified chamber.
Tissues were washed extensively with PBS and detection was performed using a biotin-conjugated secondary antibody and SA-HRP (UCS Diagnostics S.r.l.) at room temperature for 10 minutes. Samples were washed and incubated with a colorimetric detection using 3,3' - Diaminobenzidine (DAB, Dako), counterstained with hematoxylin and mount with a quick-hardening mounting medium (Eukitt, Bio-Optica S.p.A).

Electrophysiological Study

For EPS two catheters were introduced through the right femoral vein. The first was a steerable decapolar catheter (Bard, Inc Dynamic, Deca, MA) and was positioned in the CS, the second was a quadripolar steerable catheter with 4 mm tip electrode (Biosense Webster, Inc Diamond Bar, Ca) and was applied, in turn, in the right ventricular and His region. EPS evaluated the duration of AH and HV intervals basically and after atrial and ventricular stimulation. If short PQ was revealed, presence of an anomalous pathway was investigated.

Statistics

Normal distribution of variables was assessed with Kolmogorov–Smirnov test. Quantitative measurements were expressed as mean ±SD. Difference between two groups was determined by unpaired t-test for continuous variables and Fisher's exact test for categorical data. Correlations were analyzed by Pearson's correlation coefficient. A 2-tailed P value <0.05 was considered statistically significant.

Results

Main characteristics of single patients are summarized in table 1.

Cardiac Studies

ECG showed increased QRS voltages with alterations of ST segment and/or T wave in 11 of 13 patients. Short PR (Fig 1 and 3 panel A) was identified in 3 patients (patient 2, 3 and 10). In 11
patients 2D-echocardiography revealed a diffuse myocardial hypertrophy more pronounced in the LV (LVMWT 16.7 ± 3.7 mm) but extended also to the right ventricle where the free wall was between 8 and 12 mm. In 2 patients the exam was normal. Right Ventricle (RV) and LV hypertrophy was usually symmetric but in 1 subject (patient 9) was localized in the LV apex being paralleled by giant negative T wave in the V3-V5 electrocardiographic leads, mimicking the clinical phenotype of apical HCM. CMR confirmed magnitude and distribution of myocardial hypertrophy and in addition showed a subepicardial delayed enhancement in the infero lateral LV wall, after Gadolinium infusion, in 7/13 FDCM subjects, particularly evident in those with a MWT > 15 mm. At cardiac catheterization, RV and LV end-diastolic pressure were elevated (>8 and >12 mmHg respectively). Coronary angiography showed a normal network whit slow flow in 6 patients. No major complications related to cardiac catheterization and biopsy have been observed: in 1 patient a transient left bundle branch block following LV biopsy was reported. Holter monitoring (24 hours for at least 2 registrations) revealed no significant ventricular arrhythmias in 4 female FDCM (Lown class 1-2) and atrial fibrillation and/or significant ventricular arrhythmias (Lown class 3-4A) in 3 female and all male (Lown class 3-4B) (table 1).

We did not find statistical significant correlation between severity of ventricular arrhythmias (Lown class) and age (p=0.18, r²=0.15) or severity of cardiac hypertrophy (p=0.34, r²=0.08).

Occurrence of atrial fibrillation was not associated with age (p=0.65) or severity of cardiac hypertrophy (p=0.09). Patients with cardiac variant (N215S and R227Q mutations) did not show increase in the severity of ventricular arrhythmias (p=0.06) or in the occurrence of atrial fibrillation (p=0.39) compared with patients with classical mutation.
Pathology of Myocardium and Conduction Tissue

Cardiomyocytes were regularly arranged and enlarged with clear perinuclear and cytoplasmic vacuoles that in the advanced disease occupied > 50% of cell surface. These vacuoles were PAS and Sudan Black positive at histochemistry of frozen sections, suggesting an accumulation of glycolipid material. The interstitium was widened due to intercellular, perivascular and replacement fibrosis. Myocardial arterioles presented a lumen narrowing with thickened wall due to hypertrophy and hyperplasia of smooth muscle cells showing perinuclear vacuoles suggesting a vascular Gb3 infiltration. Semithin sections from epon-embedded samples showed massive accumulation of glycolipid bodies in vessel, myocytes and CT (Fig 1 panel D) at ultrastructural examination cell vacuoles consisted of large lysosomes containing myelin bodies (Fig 1 panel E).

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Cardiac conduction tissue (CT) was observed mostly in LV biopsy fragments (specifically in 2 samples from RV and 9 from LV). CT was identified at histology as loosely arranged small myocytes, positive to HCN4 immunostaining (see insert in panel C of Fig 1) supplied by a centrally placed thickened wall arteriole, circumscribed by a fibrous membrane in a fascicle configuration (Monckeberg and Aschoff criteria) (Fig 1 panel C and D). At ultrastructural examination CT appeared as small myocytes containing sparse myofibrils, a large number of endocytic vescicles, and particularly rich lateral gap junctions (Fig 1 panel E and detail).

CT cells were variably vacuolated in relation with patients’-sex. In particular, in 3 females less than 30% of cells were affected and vacuoles were confined to perinuclear area (Table 1, Fig 2 panel C and D). In 1 female, number of affected and unaffected cells was balanced (around 50% in pt 8). In 3 females vacuoles involved > 50% of cells and expanded toward the cytoplasm (Fig 1 panel C/D/E). In the 6 males, 100% CT cells were homogeneously
infiltrated and vacuoles occupied nearly the entire cell surface (Fig 3 panel C and D). Severity of CT infiltration in females failed to correlate with age (p=0.16, r²=0.08) and with severity of LV hypertrophy (p=0.96, r²=0.00). Female patients with cardiac variant (N215S and R227Q mutations) did not have a more severe CT infiltration compared with non cardiac variant (p=0.16). CT arterioles in males and females with extensive CT infiltration were affected with thickened walls and hypertrophied smooth muscle cells containing glycolipid accumulation (Fig 1 and 3 panel C and D).

**Electrophysiological Study**

Four females (patients 4, 7, 8 and 11 of table 1) had normal PR (> 120 msec) and normal basal intervals (AH 90 ± 20 msec; HV 45 ± 10 msec) with normal functional intervals (Wenckebach point 350 ± 50 msec; effective refractory period of AV node was 600/400 ± 20 msec and of right atrial 600/240 ± 20 msec. Ventricular stimulation was decremental and concentric. This cohort had no palpitation and/or arrhythmias at Holter monitoring.

Three females (patient 2, 9 and 10) had a short (< 35 msec) HV interval and two patients (2 and 10) also a short AH (< 90 msec). This group presented an abnormal (> 400 msec) Wenckeback point and a nodal AV increased refractory period (> 600/400 msec). These patients manifested supra-ventricular as well as ventricular arrhythmias.

The six male patient had short HV and patient 3 also had a short PR and AH. This last patient presented the shortest HV interval (20 msec) and an infraHissian block at 390 msec cycle during decremental atrial stimulation (Fig 3 panel B). This patient cohort manifested supraventricular and/or ventricular arrhythmias and patient 1 needed an ICD implantation due to an episode of sustained ventricular tachycardia.

Presence of accessory pathway was ruled out because of concentric retroconduction.
during decremental stimulation from RV apex with V-A dissociation at elevated intervals.

**Correlation between EPS and CT Pathology**

EPS was normal in 3 females with focal (< 30%) and 1 female with moderate CT cell involvement. It showed an accelerated conduction with short HV interval in the 3 females with extensive infiltration and in all male FDCM. Additionally, a short PR (< 120 209 ms) at ECG and short AH interval at EPS were observed in two females with extensive (patient 2 and 10) and in one male patient (3) with massive CT infiltration.

Noteworthy, a prolonged refractory period with an abnormal Wenckebach point was documented in a female FDCM patient with extensive CT infiltration. Infiltration of CT showed a significant correlation with shortness HV value ($p<0.01; r=-0.6576$).

**Discussion**

Cardiac arrhythmias are common in FD and may occur in the pre-hypertrophic phase of FDCM, in the absence of myocardial fibrosis, suggesting an early compromise of CT. Indeed lone atrial fibrillation, cryptogenic ventricular arrhythmias and sudden death have been reported as a first manifestation of FDCM in absence of clinical (systemic symptoms), electrocardiographic (high QRS voltages and/or ST segment/T wave changes) and cardiac imaging (2D-echo, CMR) abnormalities. A recent report on endomyocardial biopsy sections of CT, has shown that lone ventricular arrhythmias are associated with a prominent infiltration of CT in comparison with working myocytes. The reason for this pathological discrepancy is actually unclear although a higher energy metabolism in CT cells with a lower availability of the lysosomal enzyme, alpha-galactosidase A and a degradation of subcellular ultrastructure, have been hypothesized. Overall, CT involvement seems to play a major role in the generation of cardiac arrhythmias in FDCM. However, pathology of CT is, poorly understood as systematic postmortem studies are lacking.
and inclusion of CT in endomyocardial biopsies is rare. In the present study we report the histological examination of CT in 13 patients with FDCM, identified by Monckeberg and Aschoff morphological criteria and positive immunostaining for the molecular marker HCN4\textsuperscript{11}. The relatively high prevalence of CT inclusion in endomiocardial biopsy specimens (17\% of 74 FDCM subjects) may be due to the bi-ventricular approach with withdrawal of 8-10 fragments per patient. In addition, CT was mostly found in biopsies from the LV, a common site of investigation in our lab for patients with cardiomyopathies\textsuperscript{12} allowing an easier approach of the interventricular septum where CT branches are expected to be more represented in comparison with cardiac apex as well as RV and LV free wall. As far as CT involvement in FDCM is concerned, it might be assumed that its compromise would vary with age, sex, severity of LV hypertrophy as well as type (classical or variant) of alpha-Gal gene mutation. Our study shows that CT is indeed variably affected in FDCM and that the extent of its involvement is essentially related to patients’ sex. In particular, while male subjects had all CT cells affected (Fig 3 C and D), females with FDCM presented focal (< 30\%, Fig 2 C and D), moderate (around 50\%, pt 8) or extensive (> 50\% Fig 1 C/D/E) cell infiltration as a consequence of a skewed inactivation of X-chromosome. On the other hand, CT infiltration in female patients was not correlated with age, severity of LV hypertrophy and type of gene mutation (classical vs cardiac variant). This is exemplified in two sisters aged 58 and 60 years (pt 10 and 11 of Table 1) with a classical gene mutation and similar MWT. CT was extensively infiltrated in the younger subject while it was focally affected in the older one. Finally, in our study, degree of CT infiltration appeared to influence atrio-ventricular conduction, refractoriness and severity of cardiac arrhythmias. In fact, while patients with focal/moderate CT involvement had a normal EPS and no arrhythmias, patients with extensive or massive CT infiltration manifested a short HV interval (in 3 patients...
also a short PR/AH) with supraventricular and/or ventricular arrhythmias requiring an ICD implantation in a male with sustained ventricular tachycardia.

The pathophysiological basis of accelerated atrioventricular conduction in FDCM is still speculative and is essentially correlated with the physico-chemical properties of glycosphingolipids. Indeed, the latter are implicated in the impulse transmission in neurons, and we speculate may cause the short PR and increased QRS voltages in FDCM. In particular, short PR firstly indicated by Roudebush in 1973 as a characteristic of FD, is reported with a prevalence of around 15% in large series. In the absence of accessory pathways, it is attributed to Gb3 infiltration of the AV node. In our report 3 of 13 FDCM (23%) had short PR.

Likewise, increasing of ECG voltages is paralleled by a progressive myocyte infiltration of Gb3 that may completely replace the myocyte myofibrillar content. The positive relation between Gb3 accumulation and QRS voltages is explained with an enhanced myocardial conduction provided by glycosphingolipids.

Interestingly, FDCM with accelerated atrio-ventricular conduction showed at EPS a prolonged refractory period with a lower Wenckebach point. In addition, 1 male subject with associated short PR and very short HV interval had an abnormal infra-Hissian block at decremental atrial stimulation. This apparent functional contradiction probably reflects, in CT cells, the degradation of myofilaments, the toxic effects of Gb3 on energy metabolism (through oxidative mitochondrial damage), and Gb3 hindrance to creatine-phosphate diffusion with consequent dysfunction of membrane pumps. These last considerations explain the occurrence in the advanced disease of the bradyarrhythmias and the need of pace-maker implantation in some patients.

The impact of enzyme replacement therapy (ERT) on CT dysfunction is actually
controversial. Normalization of A-V conduction with prolongation of PR interval\textsuperscript{18} as well as reduction of QRS duration\textsuperscript{19-20} has been occasionally reported after ERT administration. Further studies are needed to clarify whether early administration and enhanced dosage may make ERT more effective.

**Limitations of the study**

Patient selection in our study reflects essentially CT inclusion in endomyocardial biopsy samples and may not be representative of the general FDCM population. Nevertheless male and female FDCM are equally represented with different age and various degrees of LV hypertrophy as well as in the pre-hypertrophic state covering a wide range of clinical situations.

**Conclusions**

CT infiltration in FDCM is constant in male and variable in female consistent with variable X-chromosome inactivation; its extensive/massive involvement causes accelerated conduction with prolonged refractoriness and electrical instability.

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**Conflict of Interest Disclosures:** None

**References:**


Table 1: Characteristics of 13 Patients with FD-CM and Conduction Tissue Inclusion

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*nmol/h per mg of protein, values are the mean (±SD) results of 3 independent determinations on peripheral blood lymphocytes.
†C indicates Classic, V indicates Variant.
‡S indicates skin; M, muscle; CNS, central nervous system; PNS, peripheral nervous system E, eyes, EA, ears, K, kidney. MWT, maximal wall thickness.
§M, moderate; E, extensive; F, focal.
||Electrophysiological study
Figure Legends:

**Figure 1:** A: ECG showing short PR with ST/T changes. B: EPS showing short AH and HV intervals. C: General view of CT involvement in glycolipid accumulation. Paraffin embedded, H&E stained section. A large number of clear vacuoles is visible. Insert shows positive immunostaining for HCN4 confirming the morphological identification of (CT). D: Epon embedded, toluidine blue stained semithin section. Glycolipid bodies are metachromatically stained by toluidine blue, infiltrate CT, vessel components and myocardiocytes. E: Electron microscopy showing glycolipid infiltration of a small CT myocyte and vessel cellular components. CT = conductive tissue; A = arteriole; M = myocardiocyte.

**Figure 2:** A: Normal ECG. B: EPS showing normal AH and HV intervals. C: Focal glycolipid accumulation in female patients. Paraffin embedded, H&E stained section of CT. Arrows indicate affected cells of CT. D: TEM detail of perinuclear glycolipid body accumulation in a small conductive myocyte.

**Figure 3:** A: ECG showing short PR, LV hypertrophy with abnormal T waves. B: EPS showing short AH and HV intervals associated with infraHissian block (thick arrow). C: Massive accumulation of glycolipid bodies in CT of a male patient. Paraffin embedded, H&E stained section of CT. Arrows indicate affected CT cells. D: TEM detail of a large number of glycolipid bodies mixed to mitochondria and drastically reduced sarcomeric fibrils.
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