Mitochondrial microRNAs are dysregulated in patients with Fabry Disease

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List of Abbreviations

ERT: enzyme exogenous administration
FD: Fabry Disease
GAL: alpha-galactosidase A
miRNAs, miRs: microRNA
mitomiRs: mitochondrial related miRNAs
SIGNIFICANCE STATEMENT

We demonstrate for the first time that a specific signature of circulating mitochondrial microRNAs (mitomiRs) is dysregulated in Fabry disease (FD). In our study, we observed that mitomiRs regulating fundamental aspects of mitochondrial homeostasis and fitness, including expression and assembly of the respiratory chain, mitogenesis, antioxidant capacity, and apoptosis are significantly dysregulated in FD patients. Taken together, our new findings introduce mitomiRs as unprecedented biomarkers of FD and point at mitochondrial dysfunction as a novel potential mechanistic target for therapeutic approaches.
Abstract

Fabry disease (FD) is a lysosomal storage disorder caused by mutations in the gene for alpha-galactosidase A (GAL) inducing a progressive accumulation of globotriaosylceramide (Gb3) and its metabolites in different organs and tissues. GB3 deposition does not fully explain the clinical manifestations of FD, and other pathogenetic mechanisms have been proposed requiring the identification of new biomarkers for monitoring FD patients. Previous evidence suggests the involvement of mitochondrial alterations in FD. Here, we propose mitochondrial related microRNA (miRNAs, miRs) as potential biomarkers of mitochondrial involvement in FD. We observed that miRNAs regulating different aspects of mitochondrial homeostasis including expression and assembly of respiratory chain, mitogenesis, antioxidant capacity, and apoptosis are consistently dysregulated in FD patients. Our data unveil a novel non-coding RNA signature of FD patients, indicating mitochondrial related miRNAs (mitomiRs) as new potential pathogenic players and biomarkers in FD.

Key Words: Anderson Fabry Disease, biomarkers, mitochondria, microRNA, mitomiRs
Introduction

Fabry disease (FD) is an X-linked inherited disorder of glycosphingolipid metabolism characterized by reduced or absent lysosomal alpha-galactosidase A (GAL) activity due to mutations of GLA gene (Germain, 2010), resulting in a progressive accumulation of globotriaosylceramide (Gb3) and its metabolites (Germain, 2010; Miller et al., 2020). The clinical presentation of FD patients includes a poor quality of life and a reduced life expectancy due to multi-organ complications (Morand et al., 2019). Cardiovascular disease remains the leading cause of death in these patients (Sorriento and Iaccarino, 2021). At the molecular level, Gb3 deposition represents the key pathogenetic mechanism of this rare pathology and the actual therapies are aimed at correcting the enzymatic deficiency, either by enzyme exogenous administration (ERT) or enzyme stabilization (e.g. using a chaperone, like Migalastat) (Ioannou et al., 2001; Pisani et al., 2012; Germain et al., 2016; Azevedo et al., 2020; Riccio et al., 2020). However, these therapeutic cannot revert FD pathology and its clinical manifestations, thereby suggesting that other pathogenetic mechanisms are involved. This aspect is further supported by the great variability of clinical phenotypes occurring among patients with the same exact genetic mutation (Sorriento and Iaccarino, 2021), maybe depending on different triggering mechanisms. Among them, mitochondrial dysfunction has been proposed in FD. Indeed, fibroblasts from FD patients display altered mitochondrial respiration, even if the identification of its specific pathogenetic role needs more investigations (Das and Naim, 2009; Namdar et al., 2012; Rozenfeld and Feriozzi, 2017). An early diagnosis of FD is decisive for reducing the morbidity and mortality and activate organ-specific therapeutic interventions.

We and others have shown that microRNAs (miRNAs), small non-coding RNAs of ~22 nucleotides, may serve as reliable biomarkers of various human disorders (Keller et al., 2014; Santulli et al., 2014; Sardu et al., 2014; Novak et al., 2015; Santulli, 2015; Santulli, 2016; Cammarata et al., 2018; Slota and Booth, 2019; Dama et al., 2020; Donati et al., 2021; Jankauskas et al., 2021; Kanach et al., 2021; Kansakar et al., 2022). Therefore, the microRNAs profile could be a promising biomarker candidate...
for diagnosis and prognosis of FD. Alterations in over 100 circulating miRNAs were identified in blood samples from FD patients in treatment with ERT compared with untreated patients (Xiao et al., 2019). Increased expression of miRNA miR-29a-3p and miR-200a-3p were detected in urinary extracellular vesicles isolated from patients with FD nephropathy (Levstek et al., 2021) which associates with the progression of kidney damage (Maier et al., 2021); miR-let-7a and let-7d were significantly increased in FD after therapy and have been suggested as potential markers for enzyme activity and inflammation in FD patients (Maier et al., 2021). These data point at miRNA profiling as a powerful diagnostic tool of FD progression and drug testing, as well as indicators of the development of specific FD phenotypes. Furthermore, deciphering a specific FD miRNA signature could help to unveil alternative pathogenetic mechanisms and identifying specific therapeutic targets. Several miRNAs are specifically associated with the mitochondrial phenotype in human diseases (Zhang et al., 2021). Some of them, also known as mitomiRs, are produced by the mitochondrial genome to regulate the mRNA transcription in loco, others can target nuclear encoded mRNAs localized on the mitochondria surface, or are necessary for mitochondrial homeostasis (Latronico and Condorelli, 2012; Song et al., 2019). The mechanisms underlying mitomiRs biogenesis and action sites are still poorly understood, however they are a fascinating class of miRNAs which could allow to track the mitochondrial status and health in pathological conditions (Latronico and Condorelli, 2012). Indeed, differentially expressed mitomiRs have been observed in heart failure (Pinti et al., 2017; Wang et al., 2017), where mitochondrial dysfunction is evident (Santulli et al., 2015; Sties et al., 2018; Del Campo et al., 2021; Guitart-Mampel et al., 2021; Morciano et al., 2021; Yang et al., 2021; Schwemmlein et al., 2022). Despite their potential power in mirroring systemic mitochondrial homeostasis, the assessment of mitomiRs in FD has never been contemplated. Here, we evaluated in FD patients a cluster of mitomiRs and miRNAs with a recognized role in regulating mitochondrial function. Our data provide new insights about mitomiRs as biomarkers and therapeutic targets, adding a new mosaic tile in the delineation of the complex FD pathophysiology, potentially implicating mitochondrial dysfunction.
Materials and methods

Study population

FD patients of both sexes with confirmed FD diagnosis and healthy controls were recruited from the FD Clinic of AOU Federico II in the year from March 2021 to February 2022. FD patients met the following inclusion criteria: Adult ≥ 18 years of age, confirmed diagnosis of classic FD, subscription of informed consent. Specifically, the diagnostic algorithm is based on the measurement of α-Gal A activity, that is recommended in males and optional in females, and on the genetic confirmatory testing, that is mandatory in both genders (Vardarli et al., 2020). The study was officially approved by the Institutional Ethical Committee (protocol n. 181/19). We excluded 11 patients with late-onset or non-classical variants of FD. In the end, we enrolled 63 FD and 14 healthy controls. Table 1 summarizes the main clinical characteristics of our study populations.

Blood collection and circulating miRNAs determination

Peripheral blood was collected from patients in EDTA-tubes and plasma was obtained by centrifugation as previously reported (Gambardella et al., 2020). We extracted microRNAs using the mirVana miRNA isolation kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol; the quality of miRNA was determined using Agilent Small RNA Kit (Matarese et al., 2020; Mone et al., 2021). A custom panel of mitochondrial related miRNAs was quantified as we previously described (Gambardella et al., 2021).

Isolation of PBMCs and western blot analysis

Peripheral blood was collected from both patients and controls in presence of EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described (Santulli et al., 2011; Gambardella et al., 2020; Gambardella et al., 2022). Briefly, PBMCs ring was obtained by blood stratification (diluted 1:1 in PBS) on ficoll gradient using HISTOPAQUE-1077 (Merck, Germany). The PBMCs
ring was collected and lysed in RIPA buffer and the protein concentration was determined spectrophotometrically. Western blot analysis was conducted as previously reported (Gambardella et al., 2020). Briefly, proteins were separated by 4–12% SDS/PAGE gel and transferred to an Immobilon-P nitrocellulose filter (Merck); levels of ETC subunits were determined by using an antibodies cocktail for total OXPHOS (abcam), and purified proteins (ETC complexes) as positive control. Actin levels used as loading control were visualized by a specific primary antibody provided by Santa Cruz Biotechnology (Dallas, TX). Secondary peroxidase-conjugated antibodies (ImmunoReagents, Raleigh, NC) were used to visualize the antigen-antibody complexes on nitrocellulose filter by chemiluminescence. A standard chemiluminescence reaction kit (Thermo Fisher Scientific) was used for autoradiography on film.

**Statistical analysis**

All experiments were performed at least in triplicate by blinded investigators; ANOVA with Bonferroni post hoc test or unpaired t test were performed as appropriate, where applicable. A significance level of P<0.05 was assumed for all statistical evaluations. Statistics were computed with GraphPad Prism (v. 9.0) software (San Diego, CA).

**Results**

**Alterations of several mitochondrial related miRNAs in FD patients**

Circulating miRNAs were assessed on plasma from FD patients compared with age-matched healthy controls. Specifically, the analysis was focused on a custom panel of key mitomiRs. Overall, a considerable dysregulation of mitomiRs was detected, with FD patients showing an emphatic clusterization (Fig.1). Indeed, for most of the analyzed mitomiRs, we observed a significant difference in the relative expression among the two groups.

The mitomiRs affected in FD population regulate several aspects of mitochondria health and biology.
In FD patients, we found the upregulation of miR-181c, miR-338, miR-130 and miR-210, which cause the down-regulation of mitochondrial electron transport chain (ETC) expression and assembly (Fig. 2A). Indeed, the expression of ETC complexes was reduced in cells from FD patients (Fig. 2B).

The class of miRNAs regulating mitochondrial dependent metabolic pathways was also affected. Levels of miR-15a and miR-21-5p, regulating UCP-2 expression and lipid content and peroxidation (Li et al., 2012; Nasci et al., 2019), were significantly higher in FD patients compared to healthy controls (Fig. 3A).

Mitochondrial biogenesis and renewal are essential for a proper mitochondrial function and are therefore strictly regulated at both transcriptional and post-transcriptional levels (Jornayvaz and Shulman, 2010). miR-696 regulates mitochondrial biogenesis by targeting PGC-1α (Aoi et al., 2010), while miR-1 is reported to support the transcription of mitochondrial genome (Zhang et al., 2014). Interestingly, miR-696 was upregulated while miR-1 was downregulated in FD population (Fig. 3B).

Additionally, the antioxidant capacity and the mitochondrial proapoptotic pathway can be modulated by miRNAs: miR-17 and miR-23b are able to suppress critical primary mitochondrial antioxidant enzymes (Schmidt, 1990; Xu et al., 2010); miR-486 may regulate apoptosis via the p53-BCL-2 mediated mitochondrial apoptotic pathway (Sun et al., 2017). In our analysis, miR-17, miR-23b and miR-486 were consistently upregulated in FD patients (Fig. 3C), further supporting the occurrence of mitochondrial stress and damage in FD.

**Effect of treatment on mitomiRs levels**

We then dichotomized FD population in naïve and treated patients to test the effect of treatment on the levels of those mitomiRs that were significantly different between FD and controls (miR-181c, miR-338, miR-130a, miR-210, miR-15a, miR-21-5p, miR-696, miR-1, miR-17, miR-486, miR-23b). Treatment influenced miR-1: in fact, this mitomiR is downregulated in untreated FD patients, and
restored by treatment to the levels observed in control subject (Fig. 4 and Table 2). Opposite, no effect of treatment was observed for all the other explored mitomiRs, which remained statistically different from control subjects (Table 2).

**Discussion**

Our data unveil a dysregulation of mitomiRs in FD. In particular, miRNAs regulating different aspects of mitochondrial biology including oxidative phosphorylation capacity and energetic metabolism, mitochondrial biogenesis, mitochondrial oxidative damage, and apoptosis, were consistently affected. Particularly interesting is the upregulation of miRNAs targeting ETC complexes, thus inhibiting their expression and or assembly. Indeed, miR-181c has been reported to directly bind the 3’UTR of mitochondrial respiratory chain complex 1, determining its downregulation (Das et al., 2012). Similarly, miR-338 induces the downregulation of mitochondrial transport chain complex IV, and its overexpression decreases mitochondrial activity (Aschrafi et al., 2008). A potential interaction between miR-130 and electron transport chain complex III was described (Kren et al., 2009) as well. Additionally, the protein involved in the complex IV assembly, COX10, is the target of miR-210 (Colleoni et al., 2013).

Consistently, we observed the downregulation of ETC complexes in cells derived from FD patients compared to controls. According with the reduced mitochondrial respiration recorded in fibroblasts from FD patients (Lucke et al., 2004), our data indicate that the oxidative phosphorylation capacity could be affected in FD cells and indicate mitomiR dysregulation as possible underlying mechanism.

Fundamental miRNAs regulating mitochondrial biogenesis were also affected in FD patients. Indeed, miR-696 is able to target and downregulate PGC-1α, the master regulator of mitochondrial biogenesis (Aoi et al., 2010), whereas miR-1 is able to modulate the expression of transcripts encoded by the mitochondrial genome (Zhang et al., 2014). Specifically, miR-1 has been reported to enter the mitochondria, where it can stimulate, rather than repress, the translation of specific mitochondrial
transcripts, playing a protective role for mitochondria. Consistently, in our analysis, miR-696 was upregulated in FD patients while miR-1 was downregulated, supporting the hypothesis that both mitochondrial and nuclear factors orchestrating the synthesis of mitochondrial components could be compromised in FD patients.

Altogether, our data suggest the implication of mitochondrial dysfunction in FD and shed light on mitomiRs as potential functional players in contributing to aberrant mitochondrial homeostasis. Mitochondrial related miRNAs dysregulation could represent a new signature of FD underling the alterations of mitochondria homeostasis in FD (Fig. 5).

Consistent with our results, alterations of mitochondrial function and regulation are common in lysosome storage disorders (de la Mata et al., 2016) but they have not been fully investigated in FD. Moreover, our findings are in agreement with a recent investigation indicating that miR-184 levels are altered in FD patients (Salamon et al., 2021); indeed, miR-184 is known to target another gene that is crucial for mitochondrial function, namely Slc25a22 (Morita et al., 2013), a mitochondrial carrier that transports glutamate (Goubert et al., 2017) and asymmetric dimethyl L-arginine (Porcelli et al., 2016). Our results call for future investigations in this direction, unveiling the essential role of mitochondria as a new frontier in FD research.

Generally, the diagnosis of FD is made by detecting Gb3 in urine and plasma and is confirmed by genetic analysis especially in females. However, GB3 levels and genetic information often do not provide a reliable prognostic value, probably because other GB3 independent mechanisms could contribute to organ damage and dysfunction in FD. Several alternative strategies have been proposed in the last years to monitor FD patients (Simonetta et al., 2020). Albeit requiring further investigations, mitomiRs could represent a new class of biomarkers in FD. Our data show that most mitomiRs seem to be not sensitive to FD therapies (ERT, chaperone-based therapy) suggesting that their dysregulation is probably an intimate mechanism of FD, probably independent from GB3
accumulation (Weidemann et al., 2013; Braun et al., 2019), which opposite is reduced by treatment. In this view, their levels could be extremely useful to monitor the onset of GB3 independent damage, highlighting once again the urgent need for new therapeutic targets, beyond current therapies. Intriguingly, miR-1 levels were instead significantly different between untreated and treated patients. In particular, the treatment seems to restore miR-1 levels in FD patients, indicating this miRNA as valuable candidate marker of FD, useful also to monitor the response to therapies. Our research has several limitations. The effect of treatment, for instance, was tested in different patients, as our study did not have a before and after design. It is likely that our observation are limited to the Caucasian population, since no other ethnicities were investigated. Also, the relative abundance of mitomiRs should be confirmed in a larger population of FD patients and controls.

In conclusion, our results support the involvement of mitochondria in FD. In this view, mitomiRs could have a pathogenic role to induce the mitochondrial alterations described in FD, and therefore could as well represent a potential therapeutic target. Also, miR-1 could be a useful biomarker for the response to treatment in FD.

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Author Contributions
Participated in research design: Gambardella, Santulli, and Iaccarino.
Conducted experiments and contributed new reagents or analytic tools: Gambardella, Fiordelisi, Sorrentino, Cerasuolo, Buonaiuto, Avvisato, Pisani, Varzideh, Riccio.
Performed data analysis: Gambardella, Fiordelisi, Santulli, and Iaccarino.
Wrote or contributed to the writing of the manuscript: Gambardella, Santulli, Iaccarino, Sorrento, Pisani. All authors contributed to the article and approved the submitted version.
References


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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Figure legends

Figure 1. Clusterization analysis of circulating mitomiRs and mitochondrial related miRNAs.
Heat map showing the profile of mitomiRs relative abundance between healthy controls and patients with Fabry disease (FD).

Figure 2. Analysis of miRNAs associated with mitochondrial health and function.
Significant dysregulation in FD patients of miRNAs related to the expression and assembly of mitochondrial respiratory chain (A); in the violin plots, median (dashed line) and quartiles (dotted lines) are indicated. Western blot analysis of mitochondrial ETC revealed a significant downregulation of I-II-IV and ATP-Synthase complexes; actin was used as loading control; data are mean±SE (B). CTRL+: Purified proteins provided by the supplier, used as molecular weight referrals; *p <0.05, **p <0.005, ***p <0.0005, ****p <0.0001, Fabry vs CTRL

Figure 3. Analysis of miRNAs associated with mitochondrial health and function.
Significant dysregulation in FD patients of miRNAs regulating mitochondrial metabolism (A), mitochondrial biogenesis and renewal (B) or mitochondrial oxidative damage and apoptosis (C). In the violin plots, median (dashed line) and quartiles (dotted lines) are indicated. * p <0.05, **p <0.005, ***p <0.0005, ****p <0.0001.

Figure 4. Effect of treatment on mitochondrial related miRNAs in FD.
Evaluation of mitomiRs in untreated and treated FD patients. Untreated FD patients show a reduction in circulating miR-1; treatment restores miR-1 levels in FD patients to the levels observed in control subjects. Data are mean±SE; *p <0.05 vs Controls; # p<0.05 vs untreated FD patients.

Figure 5. Mitochondrial related miRNAs dysregulation as signature of FD.
Mitochondrial miRNAs are significantly dysregulated in FD. The altered miRNAs are involved in fundamental mechanisms required for the maintenance of mitochondrial homeostasis, including ETC expression and assembly, mitochondrial metabolism, mitogenesis, mitochondrial oxidative damage and apoptosis. This emphatic pattern could represent a new FD footprint.
Table 1

Characteristics of FD and control populations

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>14</td>
<td>63</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36 ± 13.4</td>
<td>37 ± 13.5</td>
</tr>
<tr>
<td>Gender (male, %)</td>
<td>45.4%</td>
<td>45.6%</td>
</tr>
<tr>
<td>Caucasian race (%)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>FD treatment</td>
<td>N/A</td>
<td>54.2 % (43.7 %, 56.3%)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD or percent. FD: Fabry Disease; N/A: Not applicable. Data were analyzed by t test. A significance level of P<0.05 was assumed for all statistical evaluations.
**Table 2**

*Effect of treatment on miRs levels*

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Untreated FD</th>
<th>Treated FD</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-1</td>
<td>9.81±0.926</td>
<td>4.62±0.568*</td>
<td>7.42±0.872#</td>
</tr>
<tr>
<td>miR-181c</td>
<td>9.49±0.887</td>
<td>18.98±2.167*</td>
<td>18.89±1.920*</td>
</tr>
<tr>
<td>miR-338</td>
<td>9.86±1.436</td>
<td>19.18±1.586*</td>
<td>23.07±1.966*</td>
</tr>
<tr>
<td>miR-130a</td>
<td>6.81±0.872</td>
<td>14.42±1.802*</td>
<td>15.06±1.563*</td>
</tr>
<tr>
<td>miR-210</td>
<td>9.05±0.788</td>
<td>18.86±2.216*</td>
<td>14.88±1.457*</td>
</tr>
<tr>
<td>miR-15a</td>
<td>10.29±0.986</td>
<td>20.23±2.021*</td>
<td>19.49±1.954*</td>
</tr>
<tr>
<td>miR-21-5-p</td>
<td>8.51±1.208</td>
<td>18.97±1.871*</td>
<td>16.21±1.777*</td>
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<tr>
<td>miR-696</td>
<td>7.67±1.123</td>
<td>17.92±2.152*</td>
<td>18.80±2.040*</td>
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<tr>
<td>miR-17</td>
<td>9.44±1.025</td>
<td>19.06±2.258*</td>
<td>20.21±2.069*</td>
</tr>
<tr>
<td>miR-486</td>
<td>6.10±1.256</td>
<td>20.48±2.271*</td>
<td>18.02±2.250*</td>
</tr>
<tr>
<td>miR-23b</td>
<td>13.41±1.116</td>
<td>22.82±2.476*</td>
<td>19.54±1.769*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE. FD: Fabry Disease; Data were analyzed by ANOVA with Bonferroni post hoc test. *p <0.05 vs Controls, #p <0.05 vs untreated
Figure 3

A

miR-15a (copies/μl) vs CTRL and Fabry

miR-21-5p (copies/μl) vs CTRL and Fabry

B

miR-696 (copies/μl) vs CTRL and Fabry

miR-1 (copies/μl) vs CTRL and Fabry

C

miR-17 (copies/μl) vs CTRL and Fabry

miR-486 (copies/μl) vs CTRL and Fabry

miR-23-b (copies/μl) vs CTRL and Fabry

** indicates statistical significance at p < 0.01.

*** indicates statistical significance at p < 0.001.