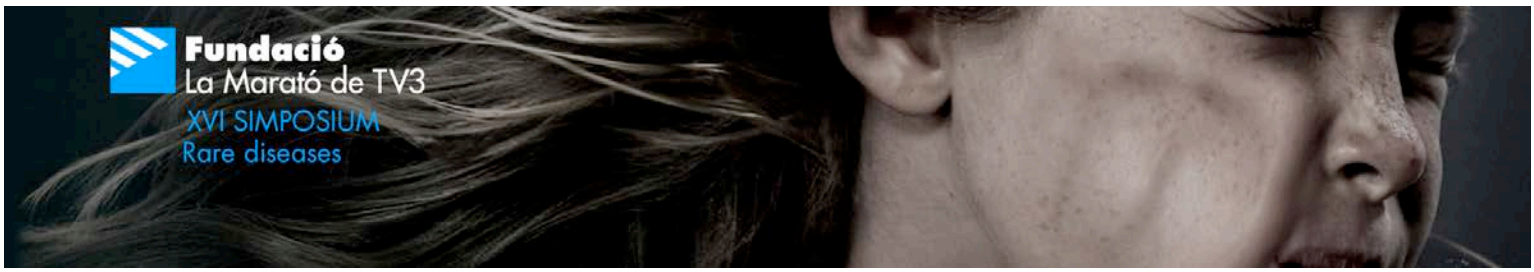

Hepatic and brain mitochondrial cholesterol/sphingolipids and altered metabolism contribute to the pathology of Niemann Pick type C disease and caveolinopathies

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1. Project summary

Niemann Pick type C (NPA) disease is a more complex lysosomal storage diseases that features neurologic alterations (e.g. ataxia, cataplexy, tremors and supranuclear dementia) as well as hepatosplenomegaly and progressive liver disease. In some cases, patients exhibit fatal liver failure before the onset of neurological symptoms. At the molecular level, NPC disease is caused by mutation in the endolysosomal NPC1/NPC2 proteins, which play a key role in the intracellular trafficking of cholesterol/glycosphingolipids (GSLs), which accumulate in lysosomes of affected tissues (liver and brain). Caveolinopathies encompass a number of degenerative diseases whose symptoms are in some cases similar to those of NPC disease, and are determined by impaired function of caveolin-1 (CAV1), a key protein that defines specific domains of membrane bilayers and is involved in the regulation of cholesterol and GSLs. As mitochondrial failure contributes to many neurological and liver diseases and deficiency of NPC1 and CAV1 determined increased mitochondrial cholesterol accumulation, our hypothesis posits that mitochondrial cholesterol loading is a key factor contributing to NPC disease and caveolinopathies. The overall goal of the present project was to test this hypothesis using mouse models of these diseases (NPC1 and CAV1 deficient mice) and human cell lines from patients with NPC disease (fibroblasts and lymphocytes) to determine mitochondrial function and antioxidant defense as well as global analyses of lipid composition and impact hepatocyte and Purkinje cell functions. Moreover, we tested the role of improved antioxidant defense on mitochondrial function, body weight and survival in mouse models of these diseases.

2. Main results

Mitochondrial cholesterol and antioxidant defense in NPC and CAV1 deficient mice

As a lysosomal storage disease, NPC disease is characterized by disrupted lysosomal homeostasis and accumulation of cholesterol and GSL. CAV1 deficiency impacts on intracellular cholesterol homeostasis and trafficking and has been shown to compromise energy demand in regenerating liver, suggesting disrupted mitochondrial function. Although cholesterol and GSLs are integral membrane lipid constituents that determine membrane physical and functional properties, their accumulation in mitochondria not only impairs mitochondrial function by uncoupling oxygen consumption with oxidative phosphorylation but also interferes with the activity of specific mitochondrial carriers, including the mitochondrial glutathione (GSH) transport, resulting in mGSH depletion. To examine the impact of NPC1 and CAV1 deficiency on mitochondrial physiology we examined the status of mitochondrial cholesterol pool, as well as function and antioxidant defense in mitochondria isolated from liver and brain of NPC1 and CAV1 null mice. We focused on the status of GSH as its transport from cytosol is known to be sensitive to changes in mitochondrial membrane dynamics. Our data established the accumulation of unesterified cholesterol in the inner mitochondrial membrane fraction in both types of mice compared to control littermates, which correlated with decreased transport of cytosolic GSH causing mitochondrial GSH (mGSH) depletion, decreased membrane fluidity and altered state 3 vs state 4 respiration leading to lower acceptor control ratio in the presence of ADP, indicative of impaired mitochondrial function. Finally, we have demonstrated, in ER and MAMs purified from mice liver that CAV1 is a resident protein in these domains and that the lack of CAV1 in MAMs significantly increases the cholesterol levels. These domains are metabolically very active and known to regulate the transport of ions and lipids between the ER and mitochondria.

Effect of GSH ethyl ester in mitochondrial GSH, susceptibility to oxidative stress and mitochondrial function in hepatocytes and Purkinje cells.

GSH regulates many cellular functions, particularly the antioxidant defense, principally by acting as a component of the GSH redox cycle. To examine the consequences of mGSH in oxidative stress susceptibility we first established

strategies that allowed the restoration of mGSH pool despite increase cholesterol levels. Moreover, we also examined in greater detail the impact of mGSH depletion in mitochondrial function using high-resolution respirometry (HRR). Treatment with GSH ethyl ester (GSHee) significantly restored the mGSH pool in liver and brain tissues from NPC-/- mice. This recovery was also observed in human skin fibroblasts from NPC-/- patients and in NPC-/- primary mouse hepatocytes (PMHs). In contrast, N-acetyl-cysteine (NAC) did not significantly increase mGSH in liver or in PMHs

HRR analysis in liver homogenates of $NPC^{+/+}$ (control mice), $NPC^{-/-}$ and $NPC^{-/-}$ GSHee treated mice determining the oxygen consumption at the leak state (no ADP present, state 2 respiration) in the presence of malate and glutamate (complex I substrates) a significant reduction in oxygen consumption in the $NPC^{-/-}$ mice was observed compared to NPC+/+ mice. GSHee treatment of $NPC^{-/-}$ mice did not improve respiration under the leak state conditions. Subsequently, ADP was added to the respirometry chamber to assess oxidative phosphorylation capacity through complex I; the differences in respiratory capacity observed at the leak state are maintained among groups. Thus, liver oxidative phosphorylation capacity is significantly reduced in $NPC^{-/-}$ mice compared to $NPC^{+/+}$ mice without improvement following GSHee treatment. A similar picture was obtained in liver when respiratory capacity was evaluated by the use of rotenone (complex I inhibitor) and succinate (complex II substrate). Oxidative phosphorylation capacity through complex II was significantly reduced in $NPC^{-/-}$ and $NPC^{-/-}$ GSHee-treated mice compared to $NPC^{+/+}$ mice. A similar pattern was also observed when coupled respiration was calculated after the addition of the ATP synthase inhibitor oligomycin. Finally, the protonophore FCCP was added to obtain maximal electron transfer system (ETS) capacity under these experimental conditions. Again, a significant reduction in ETS capacity was observed in livers from NPC-/- mice when compared to the $NPC^{+/+}$ mice; and GSHee treatment was unable to reverse the decrease in respiratory potential.

HRR studies were also performed in cerebellum homogenates from the three experimental groups. $NPC^{+/+}$, $NPC^{-/-}$ and $NPC^{-/-}$ GSHee treated mice. In contrast to the results observed in liver we do see a clear effect of GSHee treatment in the different respiratory states evaluated. Leak respiration under complex I substrates do not show any significant differences among the groups. Oxidative phosphorylation by complex I substrates tends to be decreased in $NPC^{-/-}$ mice when compared to NPC+/+, control, mice. GSHee treatment of NPC-/- mice improves complex I oxidative phosphorylation significantly when compared to untreated $NPC^{-/-}$ mice. When oxygen consumption was assessed after rotenone and succinate additions a non-significant decrease in complex II oxidative phosphorylation, couple respiration, and ETS capacity was observed. Interestingly NPC-/- treated mice significantly improve oxygen consumption rates when compared to untreated NPC-/- mice at the three respiratory states CII, coupled CII and ETS CII.

To evaluate if the alterations observed in mitochondrial respiration were caused by abnormal amounts of different complexes we studied the pattern of the different mitochondrial complexes in liver and brain samples from the different groups. Mitochondrial liver complexes of $NPC^{-/-}$ mice were significantly decreased, in particular CIV (cytochrome *c* oxidase) and CV complexes that were not recovered with GSHee treatment. In contrast, no complexes alterations were observed in brain of NPC-/- mice compared to NPC+/+ littermates.

Human skin fibroblasts from patients with NPC disease showed a significant reduction in mitochondrial performance at routine, coupled and ETS respiratory states when compared to fibroblast from healthy patients.

Calbindin is an essential determinant of normal motor coordination and sensory integration expressed in Purkinje cells. The absence of calbindin results in a deficit of precision in motor coordination. Cerebellum Purkinje cells from $NPC^{+/+}$, $NPC^{-/-}$ and GSHee-treated $NPC^{-/-}$ mice were stained for calbindin detection. As expected,

calbindin levels were diminished in Purkinje cells from $NPC^{-/-}$ mice but, importantly, GSHee treatment restored them. These results paralleled the findings on the level of nitrotyrosine staining in Purkinje cells from NPC-/- mice after treatment with GSHee.

GSHee restores the alteration of GSLs in brain from NPC null mice.

Elevated mitochondrial cholesterol levels have been reported in the liver and brain of NPC1-/- mice. In accordance with these observations, confocal imaging showed increased mitochondrial free cholesterol in NPC-/- PMHs and human skin fibroblasts. The increase in free cholesterol was also detected by biochemical analyses in liver and brain tissues from NPC-/- mice, and also it was evidenced by filipin staining in liver and cerebellum. Also, an increase in ganglioside GD3 was observed in liver mitochondria by high-performance thin layer chromatography and PMHs from NPC-/- mice. Interestingly, GD3 colocalized with cholesterol. Cholesterol delivery/import to mitochondria is carried out by specialized proteins. To evaluate mitochondrial cholesterol homeostasis we measured mRNA levels of key cholesterol transporters. NPC-/- mice had increased StarD1 levels, which were normalized by GSHee treatment. Interestingly, Mln64 levels decreased in liver of NPC-/- mice and GSHee treatment restored these levels. Caveolin 1 expression was also diminished in liver and brain, but GSHee slightly modified its expression.

Furthermore, the levels of sphingolipid species was determined by mass spectrometry. Liver and brain samples from NPC-/- mice had a marked altered pattern of many sphingolipids like sphingomyelin and glycosphingolipids. Intriguingly, GSHee-treated NPC-/- mice completely recovered the sphingolipid brain pattern, but this was not achieved in the case of the liver.

GSH ethyl ester treatment improves lifespan and motor capacity in NPC null mice

To test the impact of the replenishment of mGSH with GSHee on the overall pathology of NPC disease, NPC1 null mice were treated at p7 with GSHee, which as

shown above increases mGSH. Our findings indicated that GSHee administration to NPC1 null mice at p7 significantly improved the hanging test scores, particularly at 8 weeks of age with a higher score 3.2 vs 2.1 for NPC1 null mice treated with GSH ethyl ester compared to null mice treated with saline. Regarding the beam test, there is a significant improvement in the score for the GSHee treated knockout mice, particularly at 6 weeks of age. In addition, NPC1 null mice treated with saline lost body weight compared to GSHee-treated mice. More importantly, GSHee treatment in vivo extended the lifespan of NPC1 null by 45% from 69 days to 117 days. Treatment of null mice with a GSH precursor, NAC, failed to increase lifespan (76 days) in parallel with its inability to restore mGSH and improved mitochondrial function. Importantly, treatment of mice with 2-hydroxypropylcyclodextrin (CDX) extended lifespan to 118 days, similar to the effect of GSHee, while the combination GSHee plus CDX failed to be more effective with respect to either treatment alone. However, the 50% survival time differed between these groups, being highest in the CDX plus GSHee.

3. Clinical relevance and implications

While existing data established alterations in the intracellular trafficking of cholesterol and GSLs in NPC and diseases with CAV1 mutations and disruption of lysosomal homeostasis, the contribution and role of mitochondrial dysfunction in these diseases have been largely overlooked. Our approach was to trace the distribution of cholesterol and GSLs in mitochondria and their impact in mitochondrial function, mGSH status and oxidative defense as well as overall consequences on neurological symptoms and lifespan in mouse models of these diseases. Our data demonstrate for the first time that in vivo treatment of NPC null mice with GSHee restores mGSH levels in liver and brain mitochondria, resulting in the improvement of mitochondrial function and protection against oxidative stress. These effects are also seen in fibroblasts from patients with NPC disease. GSHee treatment also improves cerebellar function reflected in the recovery of

motor deterioration and, more importantly, increases lifespan of NPC null mice. Overall, as GSHee enters the blood brain barrier, our findings point to GSHee as an alternative treatment of NPC disease and caveolinopathies.

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