Galactocerebrosidase deficiency induces an increase of lactosylceramide content: a new hallmark of Krabbe disease?

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ABSTRACT

Galactocerebrosidase (GALC) hydrolyses galactose residues from different substrates including galactosylceramide, psychosine (*i.e.* galactosylsphingosine), and lactosylceramide. Its severe deficiency has been associated with the accumulation of psychosine, a toxic molecule with detergent-like features, that alters membrane structures and signalling pathways, inducing the death of oligodendrocytes and a sequence of events in nervous system that can explain the appearance of many clinical signs typical of Krabbe disease. Nevertheless, new evidence suggests the existence of other possible links among GALC action, myelination and myelin stability, apart from psychosine release. Here, we demonstrated that lactosylceramide metabolism is impaired in fibroblasts isolated by Krabbe disease patients, in absence of psychosine accumulation. This event is responsible for the aberrant and constitutive activation of the AKT/PRAS40 signalling axis which, in turn, induces Bcl-2 over-expression and GSK-3β inhibition. In addition, nuclear factor E2-related factor 2 (NRFFR2) underwent a higher nuclear translocation. Due to the relevance of all these molecular alterations regarding neurodegeneration, lactosylceramide increase should be evaluated as a novel marker of Krabbe disease and because its significant connections with signalling pathways.

1. Introduction

Toxic accumulation of psychosine has been described, for a long time, as the focal event leading to the clinical phenotype of Krabbe disease (KD), also known as globoid cell leukodystrophy (OMIM: #245200) (Miyatake and Suzuki, 1972). Psychosine is formed in a reaction catalysed by ceramide galactosyltransferase mainly in olygodendrocytes and Schwann cells, by linking a beta-D-galactosyl residue to sphingosine (Mitsuo et al., 1989). In addition, psychosine has been demonstrated to originate from the deacylation of galactosylceramide mediated by acid ceramidase (Li et al., 2019). Primary genetic defects inducing a severe deficiency of galactocerebrosidase (GALC) enzymatic activity (less than 10%) make impossible for cells to catabolize and remove psychosine. For many years, it was detected at increased levels in brains of KD deceased patients (Svennerholm et al., 1980). Psychosine is a lysolipid with detergent-like features, thus it strongly affects membrane biophysical and structural properties. Particularly, it has been demonstrated to alter membrane compactness and surface electric charge (Zulueta Díaz et al., 2018), expands the rigidity of plasma membranes domains, induces the shedding of microvesicles also from myelin (D'Auria et al., 2017), and disrupts the conformation of lipid rafts (White et al., 2009). All these events appear to be related to deep toxic effects ascribed to psychosine, including phospholipase A2 activation, the release of pro-inflammatory cytokines, the impairing of the mitochondrial electron chain and membrane potential, alteration of protein folding with the formation of α-synuclein aggregates, apoptosis, astrocytosis, and microgliosis (Graziano and Cardile, 2015). The G protein-coupled receptor, TDAG8 (T-cell death-associated gene 8), has been recognized

as a direct target of psychosine, which could be implicated in cytokinesis block and then in the formation of multinuclear cells (Im et al., 2001; Mitchison, 2001). The alterations induced by psychosine on myelinating cells are well-recognized; recently, also neuronal toxicity has been identified (Lim et al., 2016). Thus, the toxic potential acted by psychosine accumulation is undiscussed and the so-called "psychosine hypothesis" formulated as back as in 1972 (Miyatake and Suzuki, 1972) is still actual. Nevertheless, increasing evidences suggest that psychosine accumulation could not explain all dysfunctions described in KD and that additional mechanisms could be involved. In murine models, it has been noted that psychosine accumulation do not correspond to cell damage and demyelination (Potter et al., 2013). In *Danio rerio*, transient GALC downregulation gave rise to decreased expression of the neuronal marker NeuroD and increased apoptosis in nervous system, indipendently from psychosine accumulation (Zizioli et al., 2014). Moreover, $GALC^{+/-}$ heterozygote mice presented normal myelin levels, but remyelination and microglial phagocytic response are impaired, after cuprizone exposure for 4-weeks, though psychosine is not accumulated (Scott-Hewitt et al., 2017). Also, GALC represents a genetic locus associated with a strong risk to develop another demyelinating disease, that is multiple sclerosis (Giussani et al., 2021). Overall, in sphingolipidoses, it has been demonstrated that the primary deficiency can affect other secondary biochemical pathways, thus inducing cellular injury (Prinetti et al., 2011). Sphingolipid traffic and metabolism machinery can be distorted leading to unexpected quantitative and qualitative alterations of sphingolipids, such as GM2 and GM3, not easily attributable to the primary enzymatic defect (Breiden and Sandhoff, 2020; Prinetti et al., 2011). In KD patients cerebral cortexes and white matters, a decreased content of gangliosides GD1a and GM1 and, in turn, the increase of GD2, GD3, and GM3 have been identified, but the clinical significance of these facets has never been defined (Vanier and Svennerholm, 1975). Thus, it can be hypothesized that additional mecchanisms triggered by GALC deficiency can be implicated. In addition, it is well-known that lipid alterations can affect signalling pathways and cellular processes (Ballabio and Gieselmann, 2009; Fiorenza et al., 2018). Based on these premises, we investigated the presence of sphingolipid and molecular alterations in cell models characterized to GALC deficiency but not showing psychosine accumulation. Our results obtained in fibroblasts isolated from KD affected children demontrated that: a) GALC deficiency induces the increase of lactosylceramide (LacCer) content; b) LacCer is responsible for deep modifications concerning crucial signalling pathways.

2. Materials and methods

2.1. Cell culture

Four different fibroblast cell lines were used, two from healthy donors (L40 and RB1818) and two from KD patients (VA1679, FO86/78). Human skin L40 fibroblast cell line was kindly supplied by Prof M. Aureli (University of Milan, Italy). Human fibroblast cell lines RB1818 and VA1679 were kindly provided by Dr C. Gellera, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy. Fibroblast cell line FFF0861978 (FO86/78) was kindly provided by Cell Line and DNA Biobank from Patients Affected by Genetic Disease (Gaslini Institute, Genova, Italy), member of Telethon Network of Genetic Biobanks (Filocamo et al., 2014).
All cell lines were grown in DMEM high glucose with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and maintained at 37°C and 5% CO_2. All cell reagents were from EuroClone (Gibco, Paisley, UK).

2.2. Psychosine determination through mass spectrometry (MS)

MS analyses were carried out using a LCQDeca ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ionization source, an Xcalibur data system, and a Jasco PU980 Pump HPLC. Separations of extracted psychosine were obtained on a LiChrospher 100 RP8 column (5 μm, 250 × 4 mm; Merck). Elution species was carried out at a flow rate of 0.150 ml/min, with a gradient formed by solvent system A (acetonitrile: water (95:5, v/v)), and solvent system B (water), both containing 5mM ammonium acetate. The injection volume for each sample was 20 μl. A gradient with the following time program was used: t =0 min, 100% solvent A; t = 45 min, 50% solvent A, 50% solvent B; t = 50 min, 50% solvent A, 50% solvent B; t = 60 min, 100% solvent A. Optimum conditions for MS analyses of ganglioside molecular

species included sheath gas flow of 60 arbitrary units, spray voltage of 5 kV, capillary voltage of 45 V, and capillary temperature of 250°C. Mass spectra were acquired over a range of m/z 150–2000.

2.3. Metabolic labeling with [3-^{3}H] Sphingosine

In order to determine the sphingolipid pattern cells were plated in 60 mm dishes at the concentration of $8.4x10^3$ cells/cm^2 and, 24h after plating, were subjected to metabolic labeling as previously described (Papini et al., 2012). Cells were incubated for a 2h-pulse. Then, medium containing [3-^{3}H] sphingosine was collected and cells were incubated in DMEM-high glucose with 10% (v/v) FBS for a 48h-chase in order to reach the metabolic steady state. At the end of chase period medium was removed, cells were harvested by scraping in PBS, lyophilized and subjected to lipid extraction, as previously described (Papini et al., 2012). Radioactive lipids were visualized with a Beta-Imager 2000 (Biospace, Paris, France) and identified by comparison with radiolabeled standards. The radioactivity associated with individual lipids was calculated with the specific β-Vision software (Biospace, Paris, France).
To study LacCer metabolism, cells were pulse-labeled for 2h with [3-^{3}H] sphingosine (0.145 μCi/60 mm dish) as described above. In a different pulse experiment, 10 mM NH_4Cl was used to block lysosomal catabolism. At the end of pulse period the medium was collected, cells were scraped off the plates, lyophilized and subjected to lipid extraction.

2.4. RNA extraction and real time RT-PCR

Total RNA was extracted from fibroblast cell lines using the RNeasy mini kit (Qiagen, Milan, Italy), according to the manufacturer' s protocol. The iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Segrate, Milan, Italy) was employed to reverse-transcribe 0.8 μg of RNA. Real-Time PCR was performed as previously reported (Tringali et al., 2007). The fold change in expression of the different genes in KD fibroblasts compared with normal fibroblasts was normalized to the expression of GAPDH and was calculated by the equation $2^{-\Delta\Delta Ct}$. The accuracy was monitored by the analysis of the melting curves. The sequence of primers was: GAPDH (forward 5'-AGGGCTGCTTTTAACTCTGG-3'; reverse 5'-CATGGGTGGAATCATATTGG-3'), GALT5 (forward 5'-CTGGAACAGAGTACAGAATG-3'; reverse 5'-TACCTTCCAAGAAACTGGAC-3'), GALT6 (forward 5'-GATGATGACCTTTGGAACAG-3'; reverse 5'-CTATGGTGATGAGGAATTGAC-3'), ST3GAL5 (forward 5'-CCCTGAACCAGTTCGATGTT-3'; reverse 5'-CATTGCTTGAAGCCAGTTGA-3').

2.5. Analysis of intracellular distribution of fluorescently labelled LacCer

Fibroblasts were seeded on a glass coverslip in six-well plates (approximately 4.5 x 10^4 cells per well). 48 h after plating, cells were washed once with KRH buffer (25 mM HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4 1.2 mM $MgSO_4$, 2 mM $CaCl_2$ and 6 mM glucose) and incubated with 5 μM BODIPY™ FL C5-Lactosylceramide (as 1:1 complex with fatty acid free BSA) (ThermoFisher Scientific, Waltham, MA, USA) in KRH buffer at 4 °C for 30 min. After washing twice with KRH buffer, cells were incubated with 0.34 mg/ml fatty acid-free BSA in DMEM supplemented with 10% FBS for 45 min at 37 °C. Cells were then washed with PBS with calcium and magnesium and fixed with 4% (w/v) paraformaldehyde in PBS (Merck, Darmstadt, Germany). Coverslips were mounted on glass slides and images were immediately acquired using a fluorescence microscope (Olympus BX-50, Olympus Europe, Hamburg, Germany) equipped with a fast high resolution charge-coupled device camera (Colorview 12) and a software for image analysis (analySIS from Soft Imaging System GmbH).

2.6. Total cell extract preparation and nuclear and cytoplasmic proteins extraction

Cells were washed with PBS once and then lysed for 10 min at 4°C in lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1% v/v NP40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A). Insoluble material was removed by centrifugation at 14000g for 10 min at 4°C, supernatants were collected, assayed for protein concentration using Coomassie Protein Assay (ThermoFisher Scientific) and analyzed by immunoblotting.

Nuclear and cytoplasmic extracts were prepared according to Cattaneo et al. (Cattaneo et al., 2011).

2.7. Western Blotting analysis

Nuclear, cytoplasmic, and total proteins were separated on 10% SDS-PAGE and blotted onto Hybond PVDF membranes (Amersham, GE Healthcare GmbH, Solingen, Germany). Membranes were incubated overnight at 4°C with following primary antibodies: anti-phospho-Akt (Ser473) (D9E), anti-Akt (pan) (C67E7), anti-phospho-EGF receptor (Tyr1068) (D7A5), anti-EGF receptor (D38B1), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-p44/42 MAPK (Erk1/2), anti-phospho-GSK-3β (Ser9) (D85E12), anti-GSK-3β (D5C5Z), anti-phospho-PRAS40 (Thr246) (D4D2), anti-PRAS40 (D23C7) (Cell Signaling Technology, Danvers, MA, USA); anti-Bcl-2 (Bcl-2-100) (Sigma–Aldrich, St Louis, MO, USA); anti-Active-β-Catenin (anti ABC, clone 8E7) (Merck Millipore); anti-HSP 70 (3A3), anti-calregulin (A-9), anti-NRF~~f~~2 (C-20), anti-GRP78 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A mouse monoclonal anti-GAPDH antibody (6C5) (Immunological Sciences, Roma, Italy) was used as loading control for whole cell lysate and cytoplasmic proteins and a rabbit monoclonal anti-Histone H3 antibody (D1H2) (Cell Signaling Technology) was employed as loading control for nuclear proteins. Then PVDF membranes were washed and incubated at room temperature for 1h. After the incubation with the appropriate secondary antibody conjugate with horseradish peroxidase, immunoreactive protein bands were visualized by chemiluminescence using different luminol-based enhanced chemiluminescence HRP substrates (Cyanagen, Bologna, Italy; Bio-Rad Laboratories, Richmond, VA, USA). Images were acquired using the Alliance MINI HD9 system (Uvitec, Cambridge, UK) and quantified through the NineAlliance software.

2.8. L40 fibroblast treatment with LacCer

L40 fibroblasts were seeded in 35 mm dishes at the density of 1.8 x10^4 cells/cm^2. Treatments were performed 48h after plating. Cells were pre-incubated with DMEM with 4% (v/v) FBS for 30 min and then treated with 20 μM LacCer (Santa Cruz Biotechnology) dissolved in DMSO for 0, 5 or 10 min. Control cells were incubated with 0.1% (v/v) DMSO for 10 min. After treatment cells were harvested, total cell extracts were prepared as described in *2.6* and analyzed for AKT phosphorylation.

2.9. Statistical analysis

Data are expressed as means ± standard deviations (SD) of independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests to determine significant differences. $p<0.05$ was considered statistically significant. Pairwise comparisons of KD fibroblasts versus control fibroblasts are shown in black, conversely comparisons intra controls or intra KD fibroblasts are in grey color. All statistical analyses were performed using GraphPad Prism™ 8 (San Diego, California, USA).

3. Results

3.1. The catabolism of LacCer is impaired in KD fibroblasts

Because GALC is responsible for sphingolipid metabolism, we investigated cellular profiles in L40 and RB1818 fibroblasts isolated from children not affected by KD (control fibroblasts) and in VA1679 and FO86/78 fibroblasts isolated from children suffered KD (KD fibroblasts). We ascertained by mass spectrometry that there was not psychosine accumulation in KD fibroblasts. To increase the sensitivity of the assay, we put together the lipid extracts from L40 cells with those of RB1818 cells and the lipid extracts of VA1679 cells with those of FO86/78 cells, respectively, and we also performed the assay after adding psychosine to lipid extracts of control cells (Fig.1, panels A, B, C, respectively). As showed, we were able to exclude psychosine accumulation in KD fibroblasts. There were no m/z ions corresponding to psychosine at the retention time characteristic of psychosine itself.

Cellular sphingolipid profiles were obtained by a metabolic labelling with [3-H^3] sphingosine, at steady-state, and a partition of lipid extracts which allowed to separate gangliosides from neutral glycolipids. No significant changes related to KD phenotype were revealed in cellular ganglioside profiles (Fig.1 D, E). Instead, among neutral glycolipids and sphingomyelin, the most significant alteration was the increase of LacCer in VA1679 (+167% compared to RB1818 cells) and FO86/78 (+138% compared to RB1818 cells) fibroblasts, in comparison to both control fibroblasts (Fig.1 F, G). According to other studies (Calvano et al., 2019; Sala et al., 2002), LacCer was barely present in control fibroblast (less than 2% of neutral sphingolipids). Gb3 was increased in KD fibroblasts only if compared to RB1818 cells (Fig.1 F, G). This difference between control fibroblasts could be ascribed to the existence of a lipid heterogeneity among fibroblasts isolated from different donators. Thus, the unique change really associated with the KD phenotype was the more consistent appearance of LacCer.

To better appreciate if LacCer increase was due to a catabolic impairment or to different anabolic flows, we performed a 2h pulse with [3-H^3] sphingosine, not followed by the chase. We also executed the same assay in the presence of NH_4Cl, able to neutralizing lysosomal pH, reducing catabolism. In these conditions, about 40% of radioactivity associated with the organic phase was due to ceramide, without significant differences among the cells, demonstrating that cells entered into a biosynthetic phase at similar degree (Fig.2 A, B). In the absence of NH_4Cl, the most significant modification verified in both KD fibroblasts, compared to both controls, was the increase of LacCer (+335% in VA1679 cells; + 250% in FO86/78 cells compared to RB1818 cells) (Fig.2 A, B). LacCer levels were reduced in all cells, compared to values recorded performing the experiment at the steady-state (Fig.1 F, G); this because the cells were still involved in the first steps of biosynthetic pathways leading to complex sphingolipids, as demonstrated by increased levels of Cer, as above outlined, and GlcCer. As known, the synthetic pathway foresees Cer, GlcCer, and then LacCer formation. To gain a conclusion about the cause leading to LacCer increase in KD fibroblasts, we added NH_4Cl to strongly reduce lysosomal catabolism in all fibroblasts during the assay. In these conditions, LacCer levels were almost the same among the four cell lines (Fig.2 C, D).

We explored the expression of enzymes involved in LacCer synthesis (GALT5 and GALT6) or in its conversion into ganglioside GM3 (ST3GAL5), but we did not appreciate any significant changes but a GALT6 increase in VA1679 cells (Fig2 E). To this end, it should be underlined that the main enzyme responsible for LacCer synthesis is considered GALT5 that is expressed in foetal and adult tissues, and, if lacking, impairs development. GALT6 has been demonstrated to be significantly expressed only in human brain (Chatterjee et al., 2021). Moreover, GALT6 knock-out in mice did not lead to significant changes in sphingolipids (Tokuda et al., 2013). Also in Real Time PCR assay performed on our cells, GALT5 showed mean Ct values significantly lesser than GALT6. Thus, it is plausible that in VA1679 cells the increase in GALT6 expression could not really have an impact on LacCer synthesis.

We tried to further frame this finding, administrating BODIPY FL C_5 LacCer to fibroblasts. As shown in Fig.3 A, in both KD fibroblasts fluorescence was enhanced, compared to controls, to demonstrate a metabolic impairing of the molecule. Moreover, at the highest enlargement (Fig.3 B), KD fibroblasts presented a punctate pattern spread along the entire cell, that could be consistent with a lysosomal staining (Chen et al., 1999), whereas, a more diffuse and less granular pattern was observable in control fibroblasts.

3.2. AKT signalling pathway is upregulated in KD fibroblasts

The analysis of the molecular signature of KD fibroblasts identified significant modifications in comparison to control fibroblasts, concerning key signalling pathways. Firstly, AKT was significantly upregulated in both KD fibroblasts compared to both control, though also between L40 and RB1818 cells we revealed a dissimilarity (Fig.4 A, B). The difference in AKT activation could be related to molecular heterogeneity normally present in fibroblasts isolated from different donators (Lynch and Watt, 2018); however, AKT upregulation observed in KD fibroblasts is significant compared to both control fibroblasts, therefore it can be considered a feature of the pathologic phenotype. Accordingly, the prolin-rich AKT substrate of 40 kDa (PRAS40) and glycogen

synthase kinase 3 beta (GSK3β) were significantly more phosphorylated on sites recognized by AKT in both KD fibroblasts (Fig.4 A, B). This event has been referred to the activation of PRAS40 and the inhibition of GSK3β. The axis epidermal growth factor receptor (EGFR)/ERK appeared to be not altered in KD fibroblasts (Fig.4 A, B).
Only VA1679 presented a higher content of cytosolic active β catenin compared to all other fibroblast lines (Fig.4 A, B).

3.3. Bcl-2 expression is upregulated in KD fibroblasts

Because dysfunctions concerning the PI3K/AKT axis and its substrates were related to neurodegeneration (Rai et al., 2019; Xu et al., 2020), we explored some potential molecular cues involved in the pathway, potentially important for KD clinical phenotype. Lysosomal storage disorders, including KD, are characterized by endoplasmic reticulum (ER) stress, molecular effects caused by misfolded proteins, and cell death (Ingemann and Kirkegaard, 2014). Thus, we investigated some markers of ER stress and/or related to protein folding. The glucose-regulated protein 78 (GRP78), that is an ER stressor involved in protein folding (Ibrahim et al., 2019) was significantly increased only in VA1679 fibroblasts (Fig.5 A). It might be interpreted as a possible response dependent on the type of mutation. Calreticulin that among other functions acts as a chaperone and regulates Ca^{++} storage in ER (Wang et al., 2012) and HSP70, involved in protein refolding and stabilization, cell survival, and lysosomal catabolism as reviewed by Ingemann L et al (Ingemann and Kirkegaard, 2014), levels were expressed at similar levels among the four fibroblast lines (Fig.5 B, C).
B cell lymphoma 2 (Bcl-2) expression is modulated by AKT (Pugazhenthi et al., 2000). It plays a key role in several processes including apoptosis, autophagy, Ca^{++} stores maintenance (Swerdlow and Distelhorst, 2007). Bcl-2 levels were significantly higher in both KD fibroblasts, (about 3-fold compared to healthy fibroblasts) (Fig.5 D).
At last, we monitored the transcriptional factor NRF2 (nuclear factor E2-related factor 2) which is responsive of oxidative stress and can be activated also by the AKT signalling (Bryan et al., 2013). Despite the total cellular levels of NRF2 were almost the same in all fibroblasts (Fig.5 E), KD cells showed a higher nuclear translocation (Fig.5 F).

3.4. LacCer increase is responsible for AKT upregulation in KD fibroblasts

To understand if the alterations observed in signalling pathways were due to LacCer increase, we treated L40 fibroblasts with 20 μM LacCer for different times. LacCer treatment gave rise to an increased phosphorylation of (3.6-fold) (Fig.6), becoming significant above all 10 min after the addition of the sphingolipid.

4. Discussion

Recent papers identified and underlined that the dynamic modulation of GALC could play a key role in different aspects associated with neurodegeneration and demyelination, as reviewed by Feltri et al (Feltri et al., 2021). Knockdown of GALC co-orthologs in zebrafish embryos strongly impaired CNS development, without inducing psychosine accumulation (Zizioli et al., 2014). Employing conditional Galc floxed murine models, GALC has been demonstrated to be strongly expressed in brainstem neurons at the early perinatal period and to be crucial for postnatal neuronal differentiation and brainstem development (Weinstock et al., 2020). On the other hand, GALC deficiency has been indicated as an independent cause of neuron death in KD (Lim et al., 2016). Demyelination that is a hallmark of KD has been referred to oligodendrocyte death induced by psychosine accumulation (Giri et al., 2006); however, the GALC SNP rs74796499 has been strongly associated with the risk of developing a widespread demyelinating disorders of adults, multiple sclerosis (Giussani et al., 2021; Sawcer et al., 2011), whereas $GALC^{+/-}$ mice have been demonstrated to difficulty repair demyelination caused by cuprizone, in contrast to wt mice, and to have a reduced expression of Trem2 on

microglia that could be responsible for a decreased capability of removing myelin debris accumulated after the demyelinating insult (Scott-Hewitt et al., 2017; Scott-Hewitt et al., 2018). Thus, these evidences suggest that, despite psychosine accumulation, defects in neurons and in myelination could be directly attributable to GALC deficiency or impairment. In this perspective, identifying other metabolic substrates that could be altered after GALC deficiency is relevant.

Our study demonstrates that LacCer levels are increased in KD fibroblasts. In control fibroblasts, LacCer is scarcely present; therefore, this change is even more appreciable in KD fibroblasts and possibly its consequences. Recently, Corado et al proved the presence of high levels of LacCer in cerebrospinal fluid of KD canine models (Corado et al., 2020). Previously, it has been described the reduced capability of tissue lysates, prepared from brain or liver or fibroblasts isolated from KD patients, to catabolize LacCer (Wenger et al., 1974) and also the impaired leukocyte activity toward this substrate in KD (Svennerholm et al., 1981). Based on our results, the cause of LacCer increase in KD fibroblasts appeared to be a lysosomal catabolic impairment. On the other hand, LacCer is hydrolysed by GALC, even if not exclusively; thus it is realistic that LacCer increase is due to GALC deficiency. LacCer is recognized as a bioactive lipid: it induces the release of reactive oxygen species (ROS) through NADPH oxidase, activates pathways involved in inflammation and neuroinflammation, including cytosolic phospholipase A2 (cPLA2), pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), phosphoinositide 3-kinase (PI3 kinase), Ras, AKT, MAPK, and nuclear factor-KB (NF-KB) (Chatterjee et al., 2021; Chatterjee and Pandey, 2008; Nakamura et al., 2013; Won et al., 2007). In KD fibroblasts, we demonstrated that LacCer increase promotes the up-regulation of AKT which, in turn, phosphorylates PRAS40 and GSK-3β. AKT pathway activation is known to be crucial for neuronal cells survival in neurodegenerative diseases, above all for its anti-apoptotic effects (Xu et al., 2020). However, the role played by AKT and its downstream targets in cellular targets is complex. In particular, PRAS40 acts inhibiting mTORC1 and it is phosphorylated by AKT on Thr246, leading to its dissociation from mTORC1 (Thedieck et al., 2007). Phosphorylation of PRAS40 has been demonstrated to decrease autophagy, promoting activation of mTOR after status epilecticus (Lin et al., 2020). Along this line, Bcl-2 expression which is known to be sustained by AKT (Pugazhenthi et al., 2000), in addition to act as an anti-apoptotic cue, blocks autophagy, by associating with Beclin 1 (Pattingre et al., 2005). Significantly, autophagy dysfunctions are a hallmark of lysosomal storage diseases, including KD (Del Grosso et al., 2019; Del Grosso et al., 2016; Settembre et al., 2008).

Referring to nervous system, also other AKT substrates identified to be altered in our study deserve consideration. GSK-3β inhibition in murine models has been proved to cause cognitive and behavioural alterations, similarly to some neurological diseases (Jaworski et al., 2019), and it has been largely identified the role of this kinase in nervous system development (Kim and Snider, 2011). GSK-3β inhibition is also interrelated to the presence of active β catenin that we identified in VA1679 fibroblasts. Notably, GSK-3β modulates many signalling pathways such as Wnt/β catenin, Notch, Sonic Hedgehog that are crucial for nervous system development (Kim and Snider, 2011).

We did not reveal significant alterations of NRF2 expression, at least not in both KD fibroblasts, but its nuclear translocation was considerably enhanced. Commonly, this is a condition related to the presence of a stress, such as oxidative stress, and it is unusual that persists for a long time because multiplex mechanisms concur to regulate NRF2 expression and nuclear translocation. NRF2 has a short half-life, within 30 min (Tonelli et al., 2018). AKT activation and subsequent GSK-3β inhibition have been demonstrated to induce NRF2 stabilization, as reviewed by Bryan et al (Bryan et al., 2013). In addition to promote antioxidant response, NRF2 is also involved in other processes such as unfolded protein response and mitochondrial metabolism; moreover, the impairment of autophagy causes its stabilization, through the proteolysis of Keap1 (Tonelli et al., 2018). Significantly, other indicators of cellular stress were not altered in KD fibroblasts, regardless of the marker of ER stress, GRP78 that was up-regulated only in VA1679 cells, suggesting the possibility of an unfolded protein response related to mutations specifically carried by this cell line. Regarding these molecular effects, some differences were identified between KD fibroblasts. In particular, as previously underlined, GRP78 and active β catenin were upregulated only in VA1679 cells. It should be evidenced that

the mutations carried by the two KD fibroblasts were different and it is plausible that some effects could be specifically related to this. It was demonstrated that unfolded protein response is differently activated in KD, based on the mutation type (Irahara-Miyana et al., 2018). Accordingly, we detected it as a GRP78 increase only in VA1679. GRP78 has been previously demonstrated to activate also β catenin (Li et al., 2014; Xiong et al., 2019).

Summing up, we identified in KD fibroblasts the increase of LacCer that is associated with multiple alterations in signalling pathways. This finding could deserve consideration to identify the effects on cellular processes and on early defects occurring in nervous system development or in other tissues or cells not accumulating psychosine but involved in KD pathogenesis. Moreover, LacCer could be investigated as a marker for KD screening, as similarly proposed for other sphingolipid-storage disorders (Chen et al., 1999), and in this perspective it can be hypothesized a first-line new-born screening for multiple sphingolipidoses including KD.

Declaration of Competing Interest

The authors report no declarations of interest.

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FIGURE LEGENDS

Fig. 1. Psychosine determination by mass spectrometry and sphingolipid pattern analysis of control and KD fibroblasts. (A) Analysis of the lipid extract of L40 and RB1818 cells (healthy cells). (Left panel) HPLC/MS1 extracted ion chromatogram at m/z 462,3 $[M+H]^+$ and 484,4 $[M+Na]^+$ ions. (Right panel) MS1 spectra associated with the chromatograms in the left panel. (B) Analysis of the lipid extract of VA1679 and FO86/78 cells. (Left panel) HPLC/MS1 extracted ion chromatogram at m/z 462,3 $[M+H]^+$ and 484,4 $[M+Na]^+$ ions. (Right panel) MS1 spectra associated with the chromatograms in the left panel. (C) (left panel) HPLC/MS1 extracted ion chromatogram (*rt* of psychosine: 25 min) at m/z 462,3 $[M+H]^+$ and 484,4 $[M+Na]^+$ ions and (right panel) MS1 spectra associated with the chromatograms in the left panel. In this experiment, psychosine was added to healthy cells. (D) HPTLC separation of gangliosides developed in chloroform/methanol/0.2% aqueous $CaCl_2$ 60:40:9 (v/v). (E) Percentage distribution of ganglioside content; data are expressed as mean ± SD of independent experiments (n=4). (F) HPTLC separation of neutral sphingolipids and sphingomyelin developed in chloroform/methanol/water 55:20:3 (v/v). (G) Percentage distribution of neutral sphingolipid content and sphingomyelin; data are expressed as mean ± SD of independent experiments (n=4). Doubled spots in cellular sphingolipids reflect the heterogeneity of fatty acids in the ceramide moiety. Statistical significance was determined by one-way ANOVA and Tukey's multiple comparisons tests. *: P<0.05; **: P<0.01; ***: P<0.001. SM: sphingomyelin; Gb3: globotriaosylceramide; LacCer: lactosylceramide; GlcCer: glucosylceramide; Cer: ceramide.

Fig. 2. Lactosylceramide metabolism in control and KD fibroblasts. Cells were pulse-labelled with [3-^{3}H] sphingosine in the absence (A and B) and in the presence (C and D) of 10 mM NH_4Cl. (A and C) HPTLC separation of control and KD fibroblast neutral sphingolipids and sphingomyelin; (B and D) relative sphingolipid content. Data are presented as mean ± SD of independent experiments (n=3). Statistical significance was determined by one-way ANOVA and Tukey's multiple comparisons tests; *: P<0.05; **: P<0.01; ***: P<0.001. (E) GALT5, GALT6 and ST3GAL5 mRNA expression by real time PCR in control and KD fibroblasts. mRNA expression was normalized to the level of the housekeeping gene GAPDH and referred to that of RB1818. Data are shown as mean ± SD of independent experiments (n=3; n=4 for ST3GALT5). Statistical significance was determined by one-way ANOVA and Tukey's multiple comparisons tests. *: P<0.05.

Fig. 3. Intracellular distribution of BODIPY™ FL C5-Lactosylceramide in fibroblasts from control individuals and KD patients. Representative images, acquired using Olympus BX-50 fluorescence microscope, are shown. (A) 40x original magnification. (B) 100x original magnification.

Fig. 4. Signalling pathway of fibroblasts from control individuals and KD patients. **(**A) Representative immunoblot images of $pAKT^{Ser473}$, AKT, $pPRAS40^{Thr246}$, PRAS40, $pGSK3\beta^{Ser9}$, GSK3β, $pERK1/2^{Thr202/204}$, ERK1/2, $pEGFR^{Tyr1068}$, EGFR, and active β-catenin proteins. (B) Corresponding densitometric analyses of protein blots. Data are expressed as mean ± SD of independent experiments (n=4 for GSK3β and EGFR; n=5 for PRAS40; n=6 for ERK1/2 and active β-catenin; n=7 for AKT), phosphorylated proteins were plotted as ratio of phosphorylated versus total protein. GAPDH was employed as loading control. Data were referred to protein expression in RB1818. Statistical significance was determined by one-way ANOVA and Tukey's multiple comparisons test. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

Fig. 5. Cell stress markers in control and KD fibroblasts. Representative western blotting images and densitometric analysis of (A) GRP78, (B) calreticulin, (C) HSP70, (D) Bcl2, (E) total NRF2 and (F) nuclear NRF2 proteins. Data are the mean ± SD of independent experiments (n=4 for GRP78; n=10 for calreticulin and HSP70; n=5 for total NRF2; n=2 for nuclear NRF2). GAPDH was employed as loading control for whole cell and cytoplasmic proteins, histone H3 was used as loading control for nuclear proteins. Data were referred to protein expression in RB1818. Statistical significance was determined by one-way ANOVA and Dunnett's multiple comparisons tests. *: P<0.05; **: P<0.01; ***: P<0.001.

Fig. 6. Treatment of L40 fibroblasts with LacCer. L40 fibroblasts were treated for 0, 5 and 10 min. Representative western blotting images and densitometric analysis of $pAKT^{Ser473}$ and AKT proteins. Data are expressed as mean ± SD of independent experiments (n=2). Statistical significance was determined by one-way ANOVA and Tukey's multiple comparisons tests. *: $P<0.05$ **: $P<0.01$.