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Metallothioneins as Dynamic Markers for Brain Disease in Lysosomal Disorders

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Objective: To facilitate development of novel disease-modifying therapies for lysosomal storage disorder (LSDs) characterized by nervous system involvement such as metachromatic leukodystrophy (MLD), molecular markers for monitoring disease progression and therapeutic response are needed. To this end, we sought to identify blood transcripts associated with the progression of MLD.

Methods: Genome-wide expression analysis was performed in primary T lymphocytes of 24 patients with MLD compared to 24 age- and sex-matched healthy controls. Genes associated with MLD were identified, confirmed on a quantitative polymerase chain reaction platform, and replicated in an independent patient cohort. mRNA and protein expression of the prioritized gene family of metallothioneins was evaluated in postmortem patient brains and in mouse models representing 6 other LSDs. Metallothionein expression during disease progression and in response to specific treatment was evaluated in 1 of the tested LSD mouse models. Finally, a set of in vitro studies was planned to dissect the biological functions exerted by this class of molecules.

Results: Metallothionein genes were significantly overexpressed in T lymphocytes and brain of patients with MLD and generally marked nervous tissue damage in the LSDs here evaluated. Overexpression of metallothioneins correlated with measures of disease progression in mice and patients, whereas their levels decreased in mice upon therapeutic treatment. In vitro studies indicated that metallothionein expression is regulated in response to oxidative stress and inflammation, which are biochemical hallmarks of lysosomal storage diseases.

Interpretation: Metallothioneins are potential markers of neurologic disease processes and treatment response in LSDs.

Lysosomal storage disorders (LSDs) comprise a class of inherited diseases characterized by disruption of normal lysosomal function. Incompletely degraded substrates accumulate, accompanied by cellular dysfunction and death. Neuroinflammation occurs as a reaction to substrate accumulation within microglia and astrocytes or as a response to primary neuronal or oligodendrogial damage.1 Neuroinflammation is of particular relevance in mediating the neuropathology associated with LSDs. Metachromatic leukodystrophy (MLD; Online Mendelian Inheritance in Man database #250100), a demyelinating LSD caused by mutations in the arylsulfatase A (ARSA) gene,2 is a prototypical example of LSD with progressive accumulation of undegraded sulfatides in the nervous system as well as...
neuroinflammation and neurodegeneration. MLD is an autosomal recessive disease with an estimated incidence of 1:40,000 to 1:100,000. The disease is classified into late infantile, juvenile, and adult forms according to the age at onset of symptoms. Clinical manifestations, which consist of unrelenting motor and cognitive impairment, progress rapidly and are more severe in the early onset variants, frequently leading to death within the first decade of life. A correlation between MLD phenotype and ARSA mutations has recently been suggested.

Considerable research activity is currently focused on developing strategies to target brain disease in MLD and other LSDs with central nervous system (CNS) involvement. Gene therapy, enzyme replacement therapy, and small molecular weight compounds are advancing from preclinical to early clinical studies and may enable disease-modifying treatments for these thus far incurable, devastating diseases. Clinical phenotypes and disease progression are highly variable, thus complicating the study of new therapies. Tracking aspects of the complex CNS pathology progressively perturbed in patients with MLD, and possibly in other LSDs, could highlight novel markers potentially useful for accelerating therapeutics development.

Materials and Methods

Human Studies

Peripheral blood mononuclear cells (PBMCs) were obtained by drawing blood from MLD patients and age- and sex-matched healthy controls at San Raffaele Scientific Institute upon informed consent collection. A T-lymphocyte culture was established by stimulating PBMCs with 1µg/ml phytohemagglutinin and 300U/ml interleukin-2 in Iscove modified Dulbecco medium 5% human serum for 10 days.

Postmortem snap-frozen or formalin-fixed human brain samples and the cerebrospinal fluid (CSF) from patients and controls were obtained from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at University of Maryland, Baltimore.

Mouse Studies

All the animal procedures described herein (on mouse models of different LSDs at a late symptomatic stage and on age-matched wild-type [WT] C57 siblings) were approved by the institutional animal care and use committee of the San Raffaele Scientific Institute.

Primary cultures of cortical astrocytes and primary cocultures of astrocytes and microglia were stimulated 10 to 14 days after plating as follows: 6 hours of incubation with 10ng/ml lipopolysaccharide (LPS), 100µM H2O2—1mM L-buthionine-sulfoximine (BSO) or 1mM Trolox; or 24 hours of incubation with 10µm dexamethasone or 10% serum from WT and globoid cell leukodystrophy (GLD) mice.

Microarray Analysis

Biotinilated cRNA (Ambion, Austin, TX) from MLD patients and controls was hybridized on 6-sample Illumina (San Diego, CA) WG_v3 Beadchips. Microarray analysis was performed according to the Minimum Information about a Microarray Experiment guidelines. Microarray data have been deposited in National Center for Biotechnology Information (NCBI) Gene Expression Omnibus with accession number GSE23350 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23350).

Single gene analysis, significance analysis of microarrays (SAM), and ingenuity pathway analysis were performed on normalized data only on probes with a detection p < 0.0001 on at least 1 array.

Metallothionein Detection

Quantitative polymerase chain reaction (qPCR) was performed using Taqman Gene Expression Assay primers and probes for human MT1A, MT1E, MT2A, MTE, mouse Mt1, Mt2, Ltp2 and Cxcl11, and human and mouse GAPDH as reference genes (Life Technologies, Carlsbad, CA).

Mouse α-metallothionein (z-MT) antibody (DAKO, Carpinteria, CA; clone E9) was used for MT detection in immunohistochemical stainings on formalin-fixed human brains (1:50) together with routine hematoxylin & eosin and Klwer–Barrera methods, and in Western blot analyses (1:1,000) on brain samples and CSF.

Please also refer to the Supplementary Methods.

Results

MTs Are Overexpressed in the T Lymphocytes of MLD Patients

To identify biomarkers for MLD, we performed a genome-wide expression analysis on primary T lymphocytes from 24 MLD patients and 24 age- and sex-matched healthy controls. T cells were chosen because a well-established culture method allows obtaining a valuable quantity of good-quality RNA even starting from the limited amount of blood that could be drawn from pediatric patients. Moreover, aethylsulfatase A is known to be biologically active in this subset of circulating cells. We found 23 transcripts (targeted by 26 probes) that were significantly differentially expressed in patients compared to controls, with a false discovery rate of <0.01 by SAM with a cutoff fold change of 1.5. Twenty transcripts were overexpressed and 3 were underexpressed in patients (Fig 1A and Table). Four members of the metallothionein (MT) gene family (MT1A, MT1E, MT2A, and MTE-MT1IP; NCBI RefSeq: NM_005946.2, NM_175617.3, NM_005953.3, and
NR_003669.1, respectively) and the MT pseudogene MT1P3-C20ORF127 were overexpressed in primary T lymphocytes of patients with MLD. Overexpression of MTs in patients was confirmed by qPCR (see Fig 1B). To confirm these associations in an independent population, we evaluated MT expression in cultured primary T lymphocytes derived from a new cohort of 7 early onset MLD patients and 9 controls. Satisfyingly, marked MT overexpression was confirmed in this independent population (see Fig 1C).

To gain clues on the pathways altered in MLD and the possible correlation between their perturbation and MTs, we analyzed the expression levels and ontology of the 11,883 genes from our microarray study through Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) software. Interestingly, the majority of the top 10 most significantly perturbed pathways \( (p < 0.05, \text{Fisher exact test}) \) in patients were related to cellular oxidative stress and/or inflammatory responses. Most of the molecules belonging to these pathways were significantly upregulated in MLD patients as compared to controls (Supplementary Fig S1).

**MTs Are Overexpressed in the Brain of MLD Patients**

To investigate whether MTs are overexpressed in patients’ brains in addition to patient-derived T lymphocytes, we analyzed postmortem brain tissue of 4 MLD patients who died at advanced disease stages, compared to 4 age-, sex-, post-mortem interval–, and RNA quality–matched controls without neurologic disease. MTs were robustly overexpressed in the frontal cortex of patients with MLD as compared to controls by qPCR (Fig 2A). MT overexpression was particularly striking in the white matter, consistent with the prevalent involvement of myelin-rich regions in this disease. Of note, MT overexpression in the brain of MLD patients greatly exceeded the levels observed in primary T lymphocytes (average MT fold change of 25.5 in brain tissue, compared to average fold change of 1.6 in T lymphocytes).

Immunoreactivity against MTs was elevated in both the white and gray matter of postmortem brains from the same MLD patients, particularly in astrocytes (see Fig 2B). Consistent with the disease, a diffuse disruption of tissue architecture was also observed, particularly in the white matter, with presence of engulfed tissue macrophages and disruption of the myelin reticulum.

**MTs Are General Markers of Nervous Tissue Damage in LSDs**

More than 40 different LSDs exist, the majority of which involve the nervous system.\(^{18}\) We assessed whether MTs are overexpressed in other LSDs with nervous system involvement in addition to MLD. We analyzed MT expression in postmortem brain samples from a total of...
FIGURE 2: Metallothioneins (MTs) are overexpressed in the brains of metachromatic leukodystrophy (MLD) patients. (A) Relative mRNA abundance of MT1A, MT1E, MT2A, and MTE in the white and gray matter of the frontal cortex of 4 MLD patients compared to 4 controls (CTR; mean ± standard error of the mean; *p < 0.05). (B) Immunohistochemistry images of the hematoxylin & eosin (H&E), Kluver–Barrera, and α-MT stainings on white and gray matter of a representative MLD brain and a related CTR brain (original magnification, ×20). Strong immunoreactivity for MTs can be observed in MLD in subcortical white matter areas and in cortical regions, particularly restricted to cells morphologically identifiable as astrocytes (asterisks). MLD brain samples show diffuse disruption of the normal cytoarchitecture, with medium-to-large tissue macrophages engulfing the perivascular zones of the white matter (arrows). Kluver–Barrera staining for myelin highlights diffuse dysmyelination in subcortical areas, with granular overload of phospholipidic components of myelin in the cytoplasm of the tissue macrophages (arrowheads).
20 patients affected by different LSDs with CNS involvement, such as mucopolysaccharidosis type I (MPSI), mucopolysaccharidosis type-III (MPSIII), Niemann–Pick disease (NPC), neuronal ceroidlipofuscinosis (NCL), and Sandhoff disease (SD), comparing them with the expression values in 17 age-, sex-, postmortem interval–, and RNA quality–matched controls without neurologic disease. MTs (MT1A, MT1E, MT2A, and MTE) were significantly overexpressed in frontal cortex homogenates from LSD patients versus controls (Fig 3A, B and Supplementary Fig S2). Expression changes for each of 4 MTs in the overall group of LSDs with CNS involvement versus controls are shown in Figure 3A. Expression changes for MT2A in each of the 6 LSDs with CNS involvement are shown separately in Figure 3B. Expression changes for MT1A, MT1E, and MTE in the individual LSDs are shown in Supplementary Figure S2.

The increase in MT protein expression was confirmed by immunoblot (see Fig 3C). Relevant for future clinical biomarker assay development, MT protein levels appeared elevated also in the CSF of an LSD patient for whom postmortem CSF was available (see Fig 3D).

To further confirm that brain involvement in LSDs is associated with MT overexpression, we analyzed animal models of different LSDs. Mice modeling MLD, globoid cell leukodystrophy (GLD), MPSI, MPSIII, and SD were analyzed at late symptomatic stages that are characterized by demyelination and/or neurodegeneration of various severity. Murine Mt1 and Mt2, orthologs of the human MT isoforms, were significantly overexpressed in brain in these model mice (Supplementary Fig S3A). As observed in humans, the magnitude of overexpression varied across different LSDs, with the mouse model for GLD showing the highest transcript levels (fold change: 5.5 for Mt1 and 8.0 for Mt2). Immunohistochemistry confirmed MT overexpression at the protein level in brain samples from GLD mice and suggested that MTs are expressed predominantly by reactive astrocytes (see Supplementary Fig S3B, C).

We then began to estimate the relative specificity of MT overexpression as a marker for an MLD-relevant molecular process. We analyzed 5 large, publicly available blood expression data sets from patients with another neuroinflammatory disease (multiple sclerosis), as well as from 2 neurodegenerative diseases with a neuroinflammatory contribution (Parkinson disease) or without a major neuroinflammatory component (Huntington disease). Satisfyingly, in these in silico analyses the MTs MT1A, MT1E, MT2A, and MTE were not overexpressed in blood cells from a total of 181 disease controls with other inflammatory or degenerative neurologic disease and 101 controls free of neurologic diseases (Supplementary Fig S4A, B). Similarly, no significant change in Mt1 and Mt2 expression levels could be appreciated in the blood or in the brain of experimental autoimmune encephalomyelitis (EAE) mice, modeling multiple sclerosis, throughout the
progression of the clinical phenotype (see Supplementary Fig S4C). Thus, blood MTs likely mark a process that is pathobiologically linked to MLD and other LSDs.

**MTs Are Dynamic Markers of Disease Progression and Therapeutic Response**

GLD, also known as Krabbe disease, is an LSD that causes rapidly progressive neurologic deterioration and death in early childhood. Transplantation of hematopoietic stem cells from healthy donors in newborns with infantile Krabbe disease can favorably alter the natural history of the disease upon trafficking of transplant-derived cells to the brain, where they become an in situ source of replacement enzyme.\(^{19,20}\) GLD mice model this rapidly progressive CNS involvement as well as the response to treatment.\(^{21,22}\) We thus used this model to investigate MT expression at progressive disease stages and in response to hematopoietic stem cell transplantation.

MT expression in GLD mice dramatically increased from the presymptomatic stage (at 9–12 days postnatal) to the late symptomatic stage (at 35 days postnatal) in both blood cells and brain homogenates (Fig 4A). GLD animals underwent hematopoietic stem cell transplantation from WT donors at postnatal day 8. At 40 days of age, transplanted mice showed only mild symptoms consistent with a response to treatment, as previously reported.\(^{21,22}\) Remarkably, MT expression levels in treated animals were significantly lower than the levels measured in untreated age-matched GLD mice, which were moribund at this disease stage (see Fig 4A). These data indicate that MT expression may serve as a marker of disease progression and response to treatment.

MT overexpression could exert its effects through Lrp2/megalin, a member of the low-density lipoprotein receptor gene family with both endocytic and signal transduction functions.\(^{23–25}\) Lrp2 mRNA levels in brain extracts significantly increased along the course of the disease in GLD mice, but not EAE mice (used as controls of specificity; see Fig 4B).

We further explored the relationship between MT expression and disease progression in patients with the

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**FIGURE 4:** Metallothioneins (MTs) are a dynamic marker of disease progression and response to treatment. (A) Relative abundance of MT1 and MT2 mRNA in blood cells of presymptomatic (9–12 days, n=9) and late symptomatic (25–35 days, n=18) globoid cell leukodystrophy (GLD) mice and in the brain tissue of presymptomatic (9–12 days, n=8), late symptomatic (35 days, n=6), and transplanted (40 days, n=3) GLD mice compared to age-matched controls (mean ± standard error of the mean [SEM]; **p < 0.01). PBMC = peripheral blood mononuclear cell. (B) Relative abundance of Lrp2 mRNA in brain of presymptomatic and late symptomatic GLD and experimental autoimmune encephalomyelitis (EAE) mice, compared to age-matched wild-type animals and complete Freund adjuvant–treated animals, respectively (mean ± SEM; n = 5–9; **p < 0.01). (C) Comparison of the correlation between relative mRNA abundance of the average of MT1A, MT1E, MT2A, and MT2E and age (expressed in months) for early onset metachromatic leukodystrophy (MLD) patients (dark gray squares) and for age-matched healthy controls (CTR; light gray dots). Pearson correlation coefficient = 0.617 (p = 0.0432) for patients and 0.474 (p = 0.119) for controls.
late infantile form of MLD. In this variant, the disease invariably presents itself between 12 and 24 months of age and progresses more homogeneously with time than in other subtypes. We used patient age at the time of blood drawing as an approximate indicator of disease progression and correlated MT expression to age at blood drawing. MT expression in blood increased in correlation with the patients’ age at drawing (see Fig 4C; Pearson correlation coefficient = 0.617; p = 0.0432). In contrast, MTs did not correlate with age in healthy controls (Pearson correlation coefficient = 0.474; p = 0.119).

**MTs Play a Role in Oxidative Stress and Neuroinflammation In Vitro**

Microglia activation and neuroinflammation play a pivotal role in the pathogenesis of LSDs affecting the CNS. Microglia activation is a constant finding in LSD brains and triggers focal inflammation that precedes demyelination and/or neurodegeneration. MTs, however, appear to be chiefly expressed in astrocytes of diseased brain, as suggested by our data (see Fig 2B) and those of others.

We started to investigate how crosstalk between microglia and astrocytes relates to MT expression. MT transcripts increased in pure mouse astrocyte cultures upon contact with a microglia-conditioned medium. MT expression in astrocytes increased even more significantly in culture with a medium conditioned by LPS-activated microglia (Fig 5A). These initial results implied a relationship between microglia resting/activated status and MT expression by surrounding astrocytes. This prompted us to further explore the microglia–astrocyte crosstalk in a coculture constituted of approximately 5% microglial cells and 95% astrocytes.

When GLD cells were put in in vitro culture, MT expression returned to normal levels over time, suggesting that the MT overexpression observed in vivo may be a response to the extracellular milieu more than to intracellular stimuli (see Fig 5B; see also Supplementary Fig 5 for the effects of serum on MT expression). We thus limited our analysis to WT cells.

To dissect the relation of MTs to inflammation and oxidative stress, we stimulated astrocyte–microglia cocultures with proinflammatory (LPS) and pro-oxidative (H$_2$O$_2$ with glutathione depletion) stimuli, alone or pre-treated with dexamethasone, an anti-inflammatory, or Trolox, an antioxidative drug. As expected, MT expression increased in response to proinflammatory and pro-oxidative stimuli (see Fig 5C). Surprisingly, pretreatment with dexamethasone did not block LPS- or oxidative stress–induced MT expression. Instead, pretreatment with dexamethasone generally increased MT expression. Pretreatment with an antioxidant blocked MT expression in response to oxidative stress, but had no effect on MT expression per se nor in response to LPS (see Fig 5C).

Interestingly, the addition of 10% serum from late-symptomatic GLD mice to WT astrocyte–microglia cocultures was sufficient to significantly increase MT expression compared to WT serum, independently from astrocyte activation (as monitored by the expression of chemokine Cxcl11; see Fig 5C). Serum of affected mice thus may contain unidentified soluble factors that induce a cellular response leading to an increase in MT expression.

**Discussion**

LSDs cause immeasurable burden for affected children and their families, as well as public health. As a group, these are common childhood genetic disorders, with an estimated combined frequency of 1 in 7,700 live births. In these diseases, autosomal or X-linked recessive mutations reduce or eliminate the activity of a variety of lysosomal enzymes. Delivery of replacement enzymes is particularly challenging in LSDs that affect the central nervous system. Some early phase clinical trials are beginning to test the efficacy of enzyme delivery by means of either direct brain gene transfer or lentivirus-modified patient-derived hematopoietic stem cells. Simple circulating biomarkers that inform about CNS pathology in LSDs would be beneficial for accelerating these efforts.

Here we began to delineate a potential biomarker for MLD and possibly other LSDs: overexpression of the MT gene family in T cells derived from patient blood. MTs were identified based on an mRNA-wide search and replicated in independent patient samples. MT expression was elevated in both blood cells and brain of patients. The relative abundance of the transcripts in brain from MLD patients was substantially higher than that observed in T lymphocytes, suggesting that increased expression of MTs could be directly linked to disease pathogenesis in the most affected tissue.

This marker could be more generally useful in LSDs beyond its association with MLD. In human and mouse brains representing other LSDs characterized by nervous tissue involvement, MTs were generally overexpressed. Important for developing a molecular marker of treatment response, our study suggests that MT expression correlates with disease progression and disease modification, as shown in MLD patients and in a mouse model of globoid cell leukodystrophy. Excitingly, when the model mice were transplanted with normal hematopoietic stem cells (in which transplant-derived cells traffic to the brain and become an in situ source of replacement enzymes), enzyme delivery was elevated in both blood cells and brain of patients.
enzyme), their brains showed a remarkable correction of MT expression.

Disease specificity is valuable, but not essential, for markers of a progressive molecular disease process and therapeutic response. Satisfyingly, MT overexpression in peripheral blood cells appears to be preferentially associated with LSDs and was not observed in blood of patients with multiple sclerosis, Parkinson disease, or Huntington disease, nor in blood and brain of mice modeling multiple sclerosis throughout the progression of the disease. Although MT overexpression marks a molecular process that may be relevant to neurodegenerative disorders as well,²⁹–³¹ to our knowledge, this is the first report of increased MT levels in peripheral cells of patients with a neurologic disease.

Mechanistically, we hypothesize that MT expression in LSDs is a response to oxidative and inflammatory processes that are associated with the block of autophagy, which has been shown to be a direct consequence of lysosomal dysfunction.³²,³³ Our in vitro experiments on purified astrocyte–microglia cocultures suggest a complex crosstalk between microglia activation and MT expression in astrocytes and indicate that an as yet unidentified soluble factor present in the serum of GLD mice is

![Figure 5: Metallothioneins (MTs) play a role in oxidative stress and neuroinflammation in vitro.](image-url)

**FIGURE 5:** Metallothioneins (MTs) play a role in oxidative stress and neuroinflammation in vitro. (A) Relative MT1 (left-hand panel) and MT2 (right-hand panel) mRNA expression in pure mouse astrocyte cultures after 6 (dark gray bars) and 24 (light gray bars) hours of contact with conditioned medium from resting or lipopolysaccharide (LPS)-activated microglia, respectively, compared to LPS-treated control astrocytes (*p < 0.05, **p < 0.01). (B) Relative MT1 and MT2 mRNA expression in cells freshly dissected from the brain (0 days in culture) and astrocyte–microglia cocultures (3, 7, 10, and 14 days in culture) isolated from 30-day old globoid cell leukodystrophy (GLD) mice, as compared to matched wild-type (WT) samples (n = 5-4; mean ± standard error of the mean [SEM]). (C) Relative MT1 and MT2 mRNA expression in WT astrocyte–microglia cocultures exposed to proinflammatory (LPS) and pro-oxidative (H₂O₂, l-buthionine-sulfoximine (BSO)) stimuli, alone or combined with 1 of the following 4 elements: dexamethasone, an anti-inflammatory; Trolox, an antioxidative drug; 10% serum from WT mice; or 10% serum from late symptomatic GLD mice (n = 5-16; mean ± SEM; **p < 0.01, n.s. = p > 0.05 compared to control condition [first column]; *p < 0.05, **p < 0.01, n.s. = p > 0.05 compared to dexamethasone-, Trolox-, or WT serum–treated controls). Astrocyte activation was assessed by CXCL11 expression (− = absence of transcript; + = moderate presence of transcript; ++ = strong presence of transcript).
sufficient to stimulate MT expression in cultured glial cells. Upregulation of MTs may play a role in mitigating the abovementioned LSD-associated inflammation and oxidative stress.\(^{34,35}\) Moreover, the specific increase in Lrp2 receptor in the LSD setting suggests, for the first time to our knowledge, a positive correlation between MT and Lrp2 levels in the central nervous system. These observations may help in further elucidating the role of MTs in the pathobiology of LSDs.

This study has the merit of evaluating MT levels on multiple gene and protein expression platforms and in multiple human tissues, including T cells, brain, and CSF, as well as in multiple mouse models. However, limitations should also be kept in mind when interpreting these observations. Biomarker development is a difficult process that proceeds through multiple clinical phases.\(^{36}\) Many markers fail at various stages of this process. Replication of the candidate markers identified here in much larger, independent, and longitudinal patient populations will be needed to develop these leads into a mature clinical marker. Moreover, transitioning the biomarker to a simple clinically feasible platform that uses routinely

### TABLE. Genes Most Differentially Expressed in Metachromatic Leukodystrophy Patients Compared to Age- and Sex-Matched Controls

<table>
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<th>Symbol</th>
<th>Definition</th>
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Genes are ranked by probability value.
\(^{a}\)Genes belonging to the metallothionein family.
\(^{b}\)Underexpressed genes.
available biospecimens such as whole blood or CSF is a goal of further investigation.

In conclusion, direct analysis of LSD brain tissues and gene expression analysis performed on the blood of MLD patients revealed MTs as a novel, potential biomarker of disease progression for this class of diseases. The analysis performed on various LSDs suggests that MT expression changes could reflect a core molecular pathway and potentially an early marker of treatment response. Moreover, these findings provide a novel clue into the molecular mechanisms of tissue damage in LSD brains.

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Potential Conflicts of Interest

References


