

Vitamin K Antagonist Warfarin for Palliative Treatment of Metachromatic Leukodystrophy, A Compassionate Study of Four Subjects

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Abstract: MLD is characterized by accumulation of sulfatides in the brain. Vitamin K regulates two enzymes in sphingolipid biosynthesis and warfarin is known to lower brain sulfatides in rats and mice. We hypothesized that warfarin may mitigate the MLD phenotype by reducing the formation of sulfatides. This compassionate study recruited four advanced patients with clinical, biochemical and genetic confirmation of MLD. The patients were treated with warfarin according to the approved protocol for a total of 45 days. The battery of tests included proton MR spectroscopy (H-MRS) of brain and urinary sulfatide levels recorded at defined intervals. The patients tolerated the medication and there were no bleeding complications.

The urinary sulfatide levels did not decline during the study period. The H-MRS showed decreased N-acetyl aspartate and elevated myoinositol levels in the basal ganglia which remained unchanged after treatment.

Our study did not demonstrate any beneficial effects of warfarin in four advanced cases of MLD. The drug intervention however, was safe and deserves further evaluation through a larger study of longer duration. The metabolite abnormalities reported on H-MRS may be useful in longitudinal follow up of patients with MLD during drug trials.

Keywords: MLD, sulfatide sphingolipids, vitamin K, warfarin

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Introduction

MLD is a severe neuro-metabolic disorder caused by deficient lysosomal enzyme Arylsulfatase A (ARSA). The enzyme catalyses the first step of the degradation of 3-O-sulfogalactosyl-ceramide (sulfatide sphingolipids or simply sulfatides), an essential and abundant component of myelin.¹ In MLD, over-accumulation of sulfatides in the oligodendrocytes and Schwann cells causes progressive demyelination in the central and peripheral nervous systems. The excess sulfatides are also found in the visceral organs and urine. The phenotype is characterized by a relentless neuro-degenerative process; developmental regression, spastic diplegia, ataxia, seizures and peripheral neuropathy.²

ARSA gene consists of 8 exons in a small coding area. More than 100 disease-causing mutations in the ARSA gene have been reported. The enzyme requires a heat stable activator protein, sphingolipid activator B (SAP-B), to hydrolyze the sulfatides. The gene for SAP-B is located on chromosome 10 and consists of 13 exons. Six mutations in the SAP-B gene are described and are responsible for a minority of patients with MLD.³

The modulation of sphingolipid synthesis by vitamin K (VK) has been demonstrated in some micro-organisms, as well as in mice and rats. In 1972, Lev et al observed cessation of sphingolipid biosynthesis in *Bacteroides Levii* secondary to VK depletion.⁴ They showed that VK depletion causes inactivation of serine palmitoyl transferase, the first enzyme in sphingolipid biosynthesis, which catalyses the reaction of serine and palmitoyl-CoA, forming 3-ketodihydrospingosine (3 KDS). The enzyme function was restored with VK supplementation.⁵

The potential involvement of VK in sphingolipid metabolism is also studied in animals by Sundaram et al, who showed a reversible decrease in brain sulfatide concentration in mice. They treated 16 days old mice with warfarin for 2 weeks and documented a 42% reduction in the brain sulfatide level. The other sphingolipids (cerebrosides, gangliosides and sphingomyelins) were only minimally reduced. A 19% decrease in the activity of serine palmitoyl transferase in the brain microsome was demonstrated, which was reversed with VK supplementation. The decrease in the enzyme activity was attributed to a decrease in post-translational carboxylation, which is a VK-dependent step.⁶

Later studies confirmed down regulation of galactosyl-ceramide sulfate transferase in the brain of the warfarin-treated young mice. This is the enzyme responsible for the conversion of cerebrosides to sulfatides. Therefore, warfarin seems to modulate the formation of sulfatides by down regulating 3 KDS synthesis, as well decreasing the conversion of cerebrosides into sulfatides.⁷

Sundaram et al confirmed their findings by creating a dietary deficiency of VK in young rats. This resulted in a 20% reduction in the brain sulfatides, which was corrected with VK supplementation. VK supplementation also enhanced the activity of galactosyl-ceramide sulfate transferase.⁸

The literature cited above indicates that VK controls the rate-limiting step in the production of sphingolipids, and also the conversion of cerebrosides into sulfatides. Therefore, we proposed that warfarin may provide a treatment alternative for MLD by reducing the amount of sulfatide production. We aimed to investigate the safety and efficacy of VK antagonist warfarin in the treatment of MLD and hypothesized that warfarin may reduce the formation of sulfatides and therefore may mitigate the clinical manifestations of MLD.

Patients and Methods

Study design

This pilot study was designed as a compassionate, open-labeled prospective trial for patients with clinical, laboratory and genetic confirmations of MLD. The exclusion criteria included eligibility for allogeneic bone marrow transplantation (ABMT) or any pre-existing hematological disorder. The study protocol was reviewed and approved by the hospital IRB and the project was funded by the Pro-Roberto Foundation for MLD Research. Written parental consents were obtained and the subjects were recruited into the study for a total of 45 days. The initial warfarin dose was 0.2 mg/kg, which was adjusted to the International Normalized Ratio (INR) values according to the existing guidelines.⁹

Patient safety

For monitoring the INR and the Prothrombin Time (PT), we used the HemoSense INRatio. This is an in-vitro diagnostic system which provides quantitative PT and INR results using fresh capillary whole blood obtained by a finger stick.¹⁰ All suspicious results were



followed by venous puncture and the conventional laboratory method.

Due to warfarin's well-known narrow therapeutic window, we monitored the patients closely during the study period. The patients were evaluated by the physician on a weekly basis and underwent testing for PT/INR. When necessary, the parents received instructions to adjust the warfarin dosage and the INR was repeated in 3–4 days. The parents were educated on the use of warfarin, side effects, and avoidance of potential factors that may result in bleeding. The physician was to be informed of any significant changes in diet or medications, abnormal bleeding, sudden neurological deterioration, as well as concurrent infections. A detailed protocol, in accordance to the existing guidelines, was implemented to treat potential bleeding complications.

Battery of tests

We hypothesized that the drug intervention would ameliorate the inborn error by reducing the production of sulfatides in the brain. Therefore, we predicted a decrease in the overall sulfatide levels and a subsequent reduction in the urinary excretion of sulfatides. To monitor the impact of treatment, urine samples were collected at baseline and at two week intervals, and the sulfatide levels were quantified using thin layer chromatography as described in the literature.^{11,12}

Dali et al have studied a cohort of 13 children with MLD and established a correlation between the levels of N-acetyl aspartate (NAA) and cognitive and motor function. They have proposed that Proton MR Spectroscopy (H-MRS) may provide a valuable tool for measuring the effects of treatment interventions in MLD.¹³ Modeling this approach, we implemented H-MRS of the brain before and after treatment. Single voxel H-MRS was implemented to measure concentrations of NAA, creatine (Cr), myoinositol (ml) and choline (cho) at the level of the right basal ganglia (BG) according to the parameters described before.¹⁴ Metabolite concentration

ratios to Cr were calculated before and after treatment and compared by statistical analysis.

Results

Four patients with biochemical and genetic confirmation of MLD were recruited into the study and were treated with warfarin for 45 days according to the IRB approved protocol. Table 1 briefly summarizes the phenotypic and genotypic features in the study cohort. All patients were advanced in their disease and had profound developmental delay and spastic diplegia, none were candidates for ABMT.

Patient safety

The patients tolerated the drug without any complications. None of the patients experienced any adverse events. The INR's were carefully monitored during the study period and were maintained in the therapeutic range of 2–2.5. There were no bleeding complications necessitating the reversal of anti-coagulation.

Test results

The MRI studies demonstrated confluent areas of white matter degeneration in the brain (Fig. 1) consistent with advanced leukodystrophy.

The urine sulfatide levels were recorded at baseline and at 2, 4 and 6 weeks after treatment. The mean for the urinary sulfatide level at baseline was 5.624 micrograms of sulfatide per milligrams of creatinine, which is twenty times greater than the normal range. The values showed marked fluctuations without a discernible pattern. A paired *t*-test did not show any significant changes during the study period ($P < 0.9$). Figure 2 illustrates the urinary sulfatide levels at baseline and during the follow-up period.

The H-MRS recorded at baseline at the level of the right BG demonstrated elevated ml/Cr and decreased NAA/Cr ratios compared to the control values¹⁴ (Fig. 3). For normal values please see Table 2. The H-MRS

Table 1. Genotypic and phenotypic manifestations in the four subjects.

Cases	Age	Mutations	Clinical type	Seizures	Urine sulfatides	Developmental delay	Neuropathy
1	4 years	ARSA gene	Late infantile	+	>20 times normal	Severe	+
2	5 years	SAP B gene	Late infantile	–	>20 times normal	Severe	+
3	4 years	SAP B gene	Late infantile	–	>20 times normal	Severe	+
4	3 years	ARSA gene	Late infantile	–	>20 times normal	Severe	+

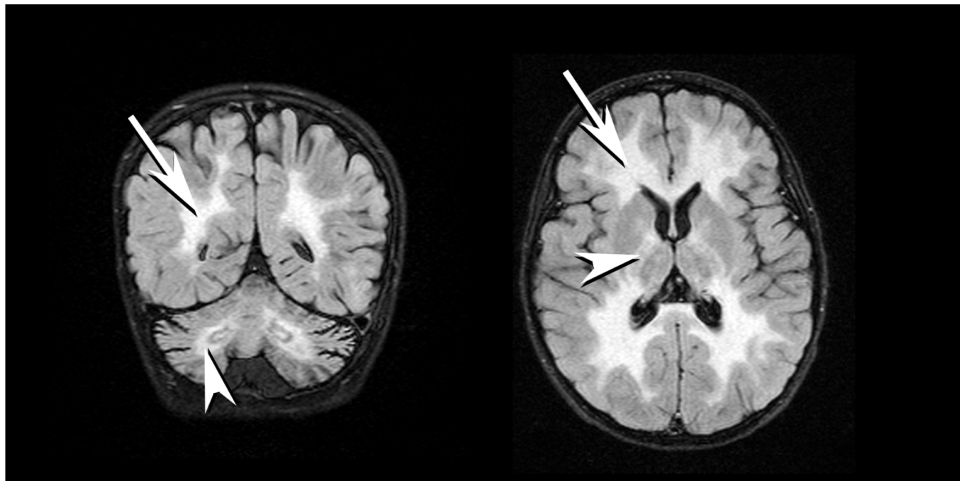


Figure 1. Sample MR images from our study cohort showing advanced leukodystrophy.

Notes: **Left:** case #1, coronal FLAIR images obtained at the level of posterior fossa demonstrating confluent areas of white matter degeneration in the periventricular region (long arrow) as well as the cerebellum (arrow head). **Right:** case #2, axial FLAIR images obtained at the level of the basal ganglia showing confluent areas of signal abnormality in the sub-cortical regions (long arrow) as well as the internal capsule (arrow head).

studies post-treatment failed to show a significant change in the metabolite ratios as summarized in Figure 4 (paired student *t*-test, *P* values ranged from 0.38 to 0.46).

Discussion

VK has been implicated in brain sphingolipid metabolism; the in-vivo and in-vitro studies indicate that VK regulates at least two enzymes involved in sphingolipid synthesis. The exact mechanism of action, however, is not fully understood. VK is a known

cofactor for the gamma carboxylase enzyme, which is involved in the post-translational modification of approximately 12 VK dependent proteins. Modification of these proteins is accomplished by attaching Gamma Carboxyglutamic acid (Gla) residues to the molecule. In the absence of adequate amounts of VK, these proteins lack some of the Gla residues which are critical for their function. The degree of under-carboxylation necessary for protein dysfunction is not precisely determined for each VK-dependent protein.

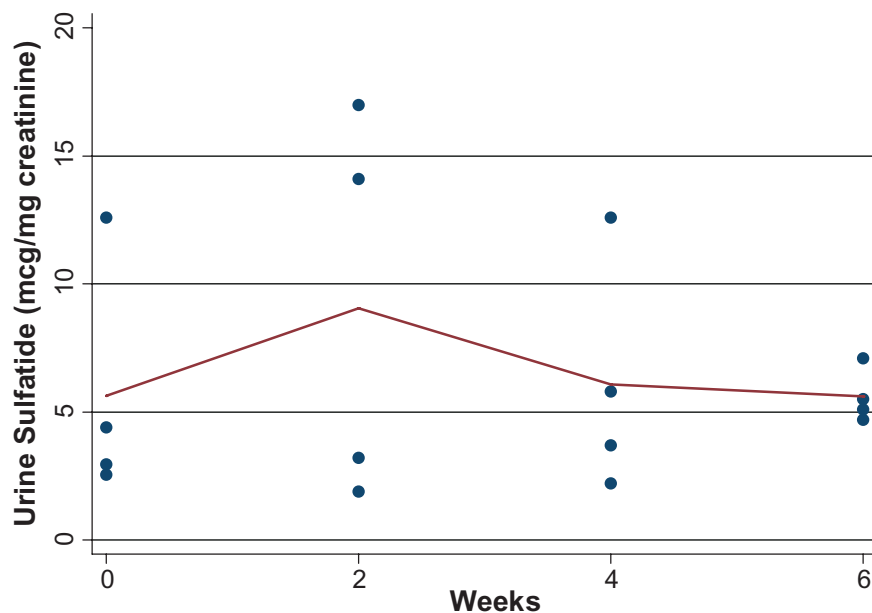


Figure 2. Scatter plot demonstrating the urine sulfatide levels in the four subjects obtained at two week intervals.

Note: The red line represents the mean of the sulfatide levels among the four subjects.

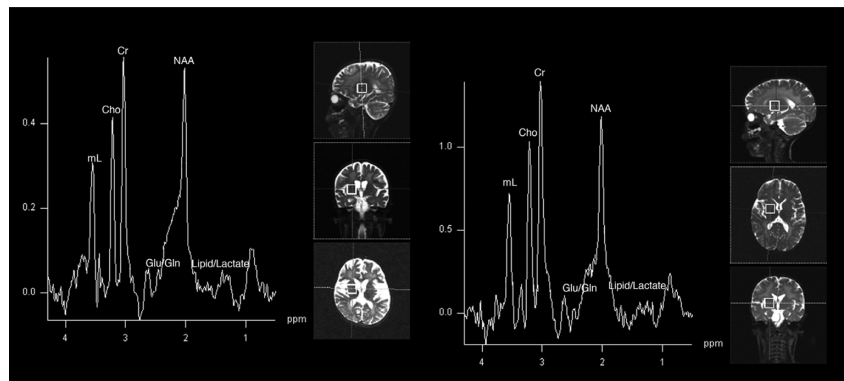


Figure 3. MR spectra obtained on case 1 (left) and case 4 (right) illustrating decreased NAA and increased mL at the level of the basal ganglia.

Some of the VK-dependent proteins, such as prothrombin and serine palmitoyl transferase, are more susceptible to decreased levels of VK.⁶ It has also been suggested that VK is involved in the post-translational protein phosphorylation of the enzyme galactosyl-ceramide sulfate transferase, resulting in a decline in formation of sulfatides.¹⁵

Alteration in sphingolipid metabolism has also been implicated in aging. Loss of myelin, frequently noted in the brain imaging studies of the elderly, is thought to be related to the reduction in cerebroside and sulfatides in the brain.¹⁶ In rats, VK supplementation may have a protective role against the aging of the retinae manifested as retinal thinning,¹⁷ which is yet to be confirmed in humans.

This work, which was designed on the premise of substrate reduction strategy, is an attempt to offer a treatment alternative to children with MLD who are not eligible for ABMT. We acknowledge the shortcomings of the study, including the very small sample size and also the short duration of the trial. These limitations were dictated by the potential safety concerns related to the use of warfarin. Additionally, the fact that our subjects had advanced disease made it very difficult to demonstrate any beneficial changes. Further investigations of this topic will be necessary to examine the prospects of this approach and will require testing the mouse model of MLD.

Presently, the only available treatment for MLD is ABMT.¹⁸ The treatment is invasive, costly, and not readily available in most centers. ABMT is not beneficial in late-infantile or juvenile cases of MLD who have already developed clinical symptoms. Stem cell transplantation has been employed in a few patients and

has resulted in the improvement of some of the laboratory findings in these patients, although amelioration in the clinical course has not been achieved.¹⁹ Overall, the treatment does not seem to be effective in infantile cases of MLD due to the rapid progression of the disease process. Enzyme replacement trial for MLD is in clinical phase I/II and gene therapy is likely to be attempted in a small number of subjects in the future.²

The dose of warfarin administered to the mice by Sundaram was much larger than the FDA recommended dose in humans. This may reflect the differences in drug metabolism among species. In our study, we had to adhere to the common wisdom of maintaining the INR in the therapeutic range of 2–2.5. It is conceivable that higher doses of the drug may provide better efficacy in reducing the sulfatides. Needless to say, this strategy was refuted due to potential safety concerns.

Quantifying the urinary sulfatide levels has long been considered an important diagnostic feature

Table 2. H-MRS control values obtained at the level of the right basal ganglia in six normal children in our lab.

Age		mL	Cr	NAA
11 months	Metabolite	1.9	4.4	4.0
	Ratio/Cr	0.43	1.00	0.88
29 months	Metabolite	1.8	4.4	4.3
	Ratio/Cr	0.41	1.00	0.96
27 months	Metabolite	1.8	4.0	4.8
	Ratio/Cr	0.45	1.00	1.2
10 months	Metabolite	1.2	4.0	4.1
	Ratio/Cr	0.3	1.00	1.02
29 months	Metabolite	2.2	3.8	4.3
	Ratio/Cr	0.57	1.00	1.13
50 months	Metabolite	2.7	4.2	4.5
	Ratio/Cr	0.52	1.00	1.07

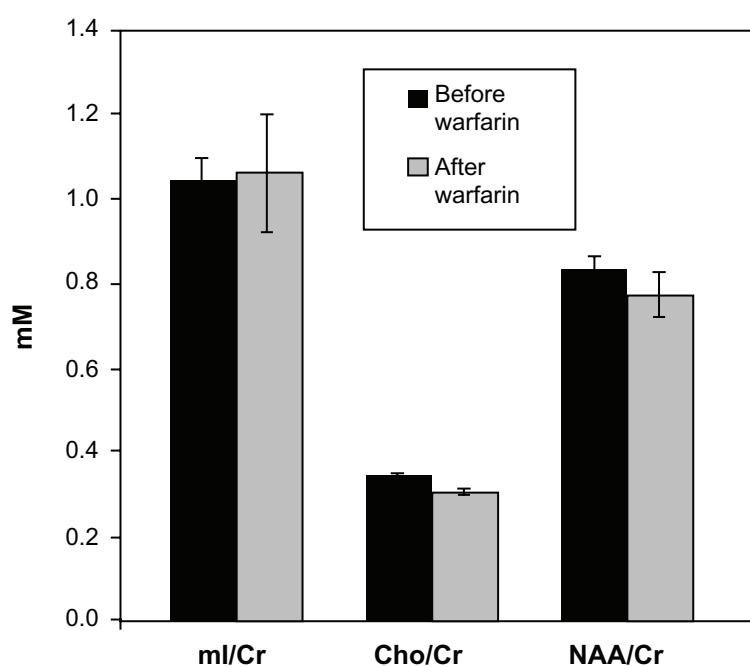


Figure 4. Metabolite ratios to creatine before and after treatment.

Notes: Mean of the metabolite ratios obtained from the four subjects are shown. The treatment did not produce any significant changes in the values.

of MLD. The diagnosis of MLD is often established by demonstrating decreased function of ARSA in-vitro and markedly elevated urinary sulfatide levels. In our study, the urinary sulfatide levels recorded at two week intervals showed significant variability, which may be attributed to dietary factors. It is also conceivable that the urinary sulfatides were sustained by the stored sulfatides in the visceral organs. The future studies should take this dilemma as well as the dietary factors into consideration. Longer trials may be necessary to demonstrate any potential changes in the urinary sulfatides.

H-MRS allows for the quantification of selected brain metabolites in-vivo. Our study demonstrated decreased NAA levels which is secondary to neuronal loss. We also documented elevated mI levels, similar to a recent study performed on a few patients with MLD.²⁰ This metabolite is generated by the glial cells, and therefore the elevated levels of mI possibly reflect the histopathological finding of reactive gliosis characteristic of MLD. Longer follow up periods may be necessary to demonstrate significant changes in the brain metabolite levels on H-MRS.

Conclusions

This compassionate study has established the safety of warfarin along with the HemoSense INRatio

monitoring system in a small group of young children with advanced MLD. The small sample size and also the advanced clinical stage of the participants precluded us from demonstrating any potential efficacy. We concur with Dali et al that H-MRS may be a useful tool for monitoring MLD patient during treatment trials.

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Author Contributions

Designed the experiment: PL. Prepared the IRB protocol and contributed to patient care, obtained parental consent: SB. Performed and analyzed the imaging studies: DW, LB. Evaluated all subjects, analyzed the results, prepared and revised the draft: MA. Assisted with the experiment and manuscript preparation: MC, KA. All authors have reviewed and agreed to the manuscript.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance



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