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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

Targeted screening, broader detection: A case of D-Bifunctional Protein Deficiency detected through newborn screening for X-linked adrenoleukodystrophy in a pilot study in Eastern Andalusia

Christella Igiraneza

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

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Prof. dr. Jeroen BOGIE

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Expanded detection in XALD newborn screening

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Keywords: X-linked adrenoleukodystrophy, newborn screening, HPLC-MS/MS, C26:0-lysophosphatidylcholine,C24:0-lysophosphatidylcholine, peroxisomal disorders, D-bifunctional protein deficiency, genetic counseling

ABSTRACT

X-linked adrenoleukodystrophy (XALD) is a severe peroxisomal disorder (PD) with a global birth prevalence of 1 in 17,000. It is caused by pathogenic variants in the *ABCD1* gene, resulting in the accumulation of very long-chain fatty acids (VLCFAs), particularly C26:0-lysophosphatidylcholine (C26:0-LPC), in tissues. Early detection through newborn screening (NBS) is crucial, as timely intervention can significantly improve clinical outcomes. This study aimed to validate and implement a sensitive and specific method for detecting XALD in newborns in Eastern Andalusia using C26:0-LPC as the primary biomarker. C24:0-LPC was also measured to improve specificity. In total, 12,593 dried blood spot samples were analyzed using a modified HPLC-MS/MS assay adapted from Haynes et al. (2012). Additionally, this study aimed to assess the specificity and diagnostic scope of XALD NBS, hypothesizing that overlapping biochemical markers may allow incidental detection of other PDs. No cases of XALD were identified during the study period. However, one newborn showed elevated VLCFAs, hypotonia, respiratory distress, and seizures. Family history revealed a sibling who died of D-bifunctional protein (DBP) deficiency. Genetic analysis confirmed homozygosity for the *c.714+1G>A* variant in the *HSD17B4* gene, with both parents as heterozygous carriers. Although no XALD cases were detected, the study highlights the feasibility and value of implementing XALD screening in Spain. The incidental identification of DBP deficiency supports the broader diagnostic utility of VLCFA-based screening and reinforces the importance of early detection and genetic counseling in PDs.

1. INTRODUCTION

1.1 Newborn screening (NBS)

Newborn screening (NBS) aims to achieve early presymptomatic diagnosis of serious but treatable conditions in newborns for which timely intervention is critical to improve the outcome. Early diagnosis can prevent developmental delays, severe health complications or potentially fatal outcomes (1, 2). Many of the conditions included in the NBS programs are inborn errors of metabolism. However, screening for endocrine, hematologic, immunologic, and cardiovascular diseases and hearing loss is also included in many NBS programs. NBS was first implemented in Europe during the 1960s, beginning with small-scale programs to detect phenylketonuria (PKU) in the UK. The detection of the biomarker for the disease, phenylpyruvic acid in urine, was a crucial step toward early screening as early as the 1930s (1). Nonetheless, the major breakthrough was the development of a simple and cheap screening method for PKU using dried blood spots (DBS) on a special collection device of a blood sample

taken from the heel of newborn infants by Guthrie (1, 2). Shortly thereafter, Dussault introduced radioimmunoassay techniques for measuring thyroid hormone levels in DBS to identify congenital hypothyroidism, marking the inclusion of a second disorder in NBS programs. Over time, the number of conditions screened for expanded, particularly in the late 1990s and early 2000s with the advent of tandem mass spectrometry (MS/MS), which enabled the simultaneous screening of 40-50 conditions from a single blood sample (1). Despite advances in molecular technologies expanding NBS programs to include multiple conditions, there is still a need to broaden these programs further to include rare and severe genetic disorders such as X-linked adrenoleukodystrophy (XALD) (1-3).

1.2 X-linked adrenoleukodystrophy (XALD)

XALD is the most common inherited peroxisomal disorder (PD) with an estimated incidence of 1 in 15.000-17.000 (3). The disorder is caused by mutations in the ATP-binding cassette subfamily D member 1 (*ABCD1)* gene located on the X chromosome. The mutations in the *ABCD1* gene exhibit a wide spectrum, including missense, nonsense, frameshift and splice-site variants. Type and location of the variant affect the severity and phenotypic variability of XALD (4). Nonetheless, identical variants can result in highly diverse clinical phenotypes, indicating that there is no phenotype-genotype correlation (4, 5). XALD follows an X-linked inheritance pattern due to the location of the *ABCD1* gene on the X chromosome. Approximately 95% of probands inherit an *ABCD1* pathogenic variant from one parent, and at least 4% of XALD patients have a *de novo* pathogenic variant (6). If the father of the proband is affected, he will transmit the pathogenic variant to all his daughters and none of his sons. However, if the mother of the proband has an *ABCD1* pathogenic variant, there is 50% probability for each pregnancy that the gene is transmitted to her son or daughter (4, 6, 7). Males who inherit the pathogenic variant will be affected, whereas females who inherit the pathogenic variant will typically be asymptomatic in childhood but may manifest symptoms in adulthood (4).

The *ABCD1* gene encodes the adrenoleukodystrophy protein (ALDP), a constitutive part of the peroxisomal membrane. It plays a role in the transportation of saturated very long-chain fatty acids (VLCFA) ≥ C22:0 from the membrane to the matrix where they eventually undergo beta-oxidation. A defect in *ABCD1* gene results in impaired VLCFA beta-oxidation and consequently accumulation of VLCFA in all tissues, primarily affecting the nervous system, adrenal gland, and testis (3, 5). The precise role of the accumulation of VLCFAs in the pathogenesis of XALD still needs to be elucidated (4). Various *in vitro* experiments have demonstrated that VLCFA accumulation is toxic. Excess of VLCFA in cultured cells decreases cortisol release by human adrenocortical cells and causes cell death in astrocytes and oligodendrocytes. Moreover, VLCFA are extremely hydrophobic and their desorption rate from biological membranes is so slow causing disruptive effects on the structure, stability and function of cell membranes (7). In vivo, VLCFA cause oxidative stress and oxidative damage to proteins, microglial activation and apoptosis. Consequently, XALD results in progressive demyelination in the central nervous system, axonopathy in the spinal cord, and adrenal insufficiency (5, 7).

1.3 Spectrum/phenotypes of XALD patients

XALD has diverse clinical presentations, which can be asymptomatic or present in rapid progressive phenotypes. The clinical spectrum of XALD ranges from primary adrenal insufficiency (Addison disease) to slowly progressive adrenomyeloneuropathy (AMN) to rapidly progressive and fatal cerebral ALD (cALD) (3).

1.3.1 Addison's only phenotype

Addison's disease occurs in the first decade of life with a lifetime prevalence of 80-90% and is associated with high morbidity and mortality (3, 7). Rare cases show distinct Addison only or adrenocortical dysfunction without neurological involvement and are characterized by fatigue, hypotension and bronzing of the skin. The patients show elevated plasma concentrations of adrenocorticotropic hormone and lower concentrations of cortisol. In most cases, Addison-only patients also develop cALD or AMN and neurological symptoms (5, 7).

1.3.2 Adrenomyeloneuropathy (AMN)

Virtually all XALD patients who reach adulthood develop AMN. AMN is characterized as non-inflammatory distal axonopathy mainly affecting males ages 20-30 years and females aged 40-50 years. Initial symptoms are limited to the spinal cord and peripheral nerves (5, 7). AMN can manifest as pure AMN or AMN-cerebral variants whereby pure-AMN patients show gait disturbances and bladder dysfunctions due to pathology in the spinal cord. AMN-cerebral type manifests pure-AMN features as well as cerebral inflammation. However, the mechanisms of the conversion of pure-AMN into AMN-cerebral form are not understood (3, 5).

1.3.3 Cerebral adrenoleukodystrophy (childhood, adolescent and adult)

A newborn male with *ABCD1* mutation is in most cases asymptomatic at birth but has a 31%-35% risk to develop childhood cerebral ALD (cALD) typically between three and ten years (7, 8). cALD is characterized by sudden inflammatory progressive intellectual, psychic, visual and gait disturbances (9). Onset occurs with deficits in cognitive abilities such as visuomotor and visuospatial functions resulting in decline in school performance in boys and adolescents. Further progression occurs with more visible neurologic dysfunctions such as hyperactive behavior, apraxia and seizures (5). Patients show extremely rapid progression and lose the ability to walk and comprehend the language. Within a few weeks, they become blind, unable to speak, bedridden and require full time assistance with feeding within two to four years, they succumb to death or remain in vegetative state for the rest of their lives (5, 7, 9). It is worth emphasizing that cerebral demyelination occurs less frequently in adolescence or adulthood. The symptomatology in these patients strongly resembles cALD but the initial progression of symptoms is usually slower (7).

1.3.4 Women with XALD

As in most X-linked diseases, it was assumed that female carriers remain asymptomatic (3). However, more than 50% of female carriers have some kind of abnormality on neurological examination. Onset of neurologic symptoms mainly occurs between the age of 40 and 50 years and they are similar to those observed in adult males with AMN (5, 7). Sensory ataxia, fecal incontinence and pain in the legs are more prominent in symptomatic women with AMN (7). Ameliorated symptoms in females are due to X-inactivation where enough *ABCD1* functional activity is provided by the normal allele located on the other set of X chromosome in the cells (5). Cerebral involvement and adrenocortical insufficiency are rare, 2% and 1% respectively. It is hypothesized that skewed X-inactivation in neuronal cells may contribute to the manifestation of neurologic symptoms in XALD carriers (7).

1.4 Diagnosis, treatment and genetic counseling of XALD

In most cases, XALD is diagnosed at a late stage whereby irreversible neurological damage has already occurred. While a delayed diagnosis limits treatment options, early detection of XALD enables presymptomatic treatment, and has been proven to significantly alter the disease course (4, 10). Early diagnosis and treatment can be lifesaving, but only if initiated before the onset of neurologic symptoms. To achieve this, newborn screening has emerged as a critical tool allowing for the identification of affected individuals shortly after birth, well before clinical symptoms arise. Early diagnosis allows for timely intervention including hematopoietic stem cell transplantation (HSCT), hormonal replacement therapy (HRT) and dietary interventions. HSCT is currently the most effective treatment for cALD when administered prior to the onset of neurological symptoms. Since cerebral demyelination on MRI typically precedes clinical neurological manifestations, early MRI detection offers a critical window for pre-symptomatic intervention. Initiating HSCT at the earliest signs of MRI abnormality has been shown to halt the progression of cerebral demyelination and significantly improve outcomes (7, 11). MRI severity scores grade the extent and progression of demyelination of white matter and are thus strong indicators of survival outcomes (5). In addition, HRT is another treatment that can significantly improve the prognosis and quality of life for those affected by adrenal dysfunction. On one hand glucocorticoid replacement therapy replaces

cortisol and it should be started with hydrocortisone. On the other hand mineralocorticoid replacement therapy replaces aldosterone and relies on the administration of fludrocortisone (4).

In addition to treatment, XALD can be managed through dietary interventions with three main focuses: the restriction of VLCFAs, the inhibition of fatty acid elongation, and the enhancement of peroxisomal beta-oxidation by the administration of Lorenzo's oil (LO). The adherence to a low-fat diet and LO has shown potential in slowing the progression of XALD. Although, considering the crucial role of dietary lipids for growth and neurodevelopment in the early stages of life, no fat intake restrictions are suggested for patients until the age of two (3). Furthermore, it is recommended to monitor adrenal function, neurological assessments, and brain MRIs for asymptomatic boys from six to thirty months, a period for the highest risk for developing cALD (11). Notably, the early detection of XALD through NBS offers a significant opportunity for genetic counseling. Families of affected children can be informed about the genetic basis of the disease, the inheritance patterns and the availability of reproductive options such as prenatal testing for subsequent pregnancies (7). Implementing NBS programs is crucial for improving clinical outcomes in XALD patients (11).

1.5 Newborn screening for XALD

In the 1980s, elevated plasma VLCFAs, particularly hexacosanoate (C26:0), were recognized as biomarkers for patients with XALD (8, 12). NBS for XALD is now technically feasible and focuses on the measurement of C26:0-lysophosphatidylcholine (C26:0-LPC) in dried blood spot (DBS) samples (12). New York was the first state to introduce the screening of XALD in 2013 and since 2016, XALD has been added to the United States Recommended Uniform Screening Panel (3, 11, 12). To date, 35 states in the USA including California, Georgia and North Carolina have implemented NBS for XALD (12, 13). The inclusion of XALD in NBS programs has been endorsed by these states given the fundamental importance of early diagnosis of the disease. It is significant to highlight that XALD screening in the USA is universal implying that both male and female newborns are screened (11, 12). In New York State, NBS for XALD is conducted using a three-tiered screening approach. The first tier involves a high-throughput flow injection analysis tandem mass spectrometry (FIA-MS/MS) to measure C26:0-LPC. Screen positive samples are then reanalyzed in the second tier which employs a highly specific high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) approach (10-12)). Newborns with elevated levels of C26:0-LPC on the second tier are referred for confirmatory testing. These samples then proceed to the third tier which is sequencing of the *ABCD1* gene (10).

C26:0-LPC, the primary biomarker used in NBS for XALD can also be elevated in other PDs. PDs are a group of inherited metabolic conditions that impair peroxisome function, often affecting the nervous system (14). PDs can be categorized into two main groups: single enzyme deficiencies and peroxisome biogenesis disorders. In the first group, enzyme defects primarily affect metabolic pathways and consist of disorders such as XALD and D-bifunctional protein deficiency (DBP deficiency). The latter gives rise to Zellweger spectrum disorders (ZSD) that result from mutations in different PEX genes (15). Consequently, these PDs can be incidentally detected or cause 'false positive results' during XALD screening since they present similar biochemical profiles. This highlights the broader diagnostic implications of the biomarker (3).

Europe has yet to standardize NBS for XALD. Recent advancements in genome-wide screening and newborn metabolic screening have reinforced the need to include XALD as shown by Kemper et al. who argue that the benefits of early detection far outweigh the risks (16). The screening for XALD in the Netherlands (SCAN) project was launched to evaluate the feasibility and outcomes of adding XALD to the national NBS program. The Netherlands is the only European country that implemented screening for XALD up to date. However the screening is selective implying that only male newborns are screened because only male patients develop treatable complications in childhood (10). This assertion

is not entirely accurate as current clinical trials such as "the observational study on disease progression in women with XALD" are actively exploring treatment options for female XALD patients. An Italian group also launched a pilot study for XALD implementation in Italy (3).

The Spanish NBS program includes screening between eight and 29 diseases. The specific number of diseases covered varies slightly depending on the region, as some regions have slightly different screening protocols or have implemented additional tests (17). The East Andalusian NBS program currently includes 38 diseases and is conducted between 48 and 72 hours after birth (18). In 2022, this pilot study was launched for the implementation of XALD in the East Andalusian NBS program. To our knowledge, this is the first project in Spain to screen for XALD and aims to elaborate more on the clinical benefits of early diagnosis and intervention. The study aims to develop a model for a regional NBS program that includes XALD for all newborns in Eastern Andalusia as a part of the general NBS program. Implementing XALD screening has the potential to improve early diagnosis, enable timely therapeutic intervention and ensure subsequent follow-up for patients.

The results presented in this thesis are derived from a specific phase of a broader three-year study. Data and outcomes generated before this phase are not considered within the scope of this report. This study presents a case in which a newborn presented with elevated VLCFA levels during XALD screening but was ultimately diagnosed with DBP deficiency. This finding highlights the diagnostic overlap in PDs and underscores the importance of robust follow-up testing and genetic counseling (15). Primarily, this study aimed to evaluate the effectiveness of a sensitive and specific XALD screening framework using the HPLC-MS/MS method adapted and modified from the protocol described by Haynes et al (19). The novelty of this study is the simultaneous quantification of C26:0-LPC and C24:0-LPC, both VLCFA associated with XALD. Previous studies have shown that the combined measurement of C26:0-LPC and C24:0-LPC yields the highest diagnostic accuracy in XALD, outperforming the other VLCFA (5, 20, 21). The integration of both analytes is expected to enhance the sensitivity of the screening method. Additionally, this study aimed to assess the specificity and scope of NBS for XALD, with a focus on the incidental detection of other PDs. We hypothesize that due to overlapping biochemical markers, targeted NBS for XALD can lead to the incidental detection of other PDs, highlighting the strengths of this screening.

2. EXPERIMENTAL PROCEDURES

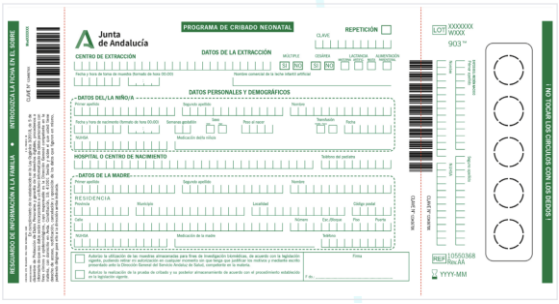
2.1 Recruitment of newborns and ethical approval

Neonatal Care Units from all public and private health centres in the East Andalusian region of Spain joined the screening program led by a multidisciplinary NBS team at the Malaga Regional University Hospital (MRUH) in Malaga. The program integrated the expertise of two laboratories: the laboratory of metabolic disorders and newborn screening center in Eastern Andalusia at MRUH which is the reference lab for NBS in Eastern Andalusia and the Molecular Diseases Diagnostic Centre (CEDEM) in Madrid which is one of the reference centres for the genetic diagnosis. The screening for XALD was included into the existing NBS program for endocrine-metabolic diseases at the MRUH. This study was centrally approved by the Comité Provincial de Ética e Investigación de Málaga. The screening of XALD was proposed and explained to the parents of newborns born in the provinces: Almería, Jaén, Granada and Málaga, in Eastern Andalusia. The screening was universal implying that both male and female newborns were included in this study. Adherence to XALD screening was voluntary and participation in this study was based on informed consent signed by both parents (Fig.1). In cases of positive screening results, a separate written informed consent specific to genetic analysis was requested to ensure ethical compliance and parental understanding of further diagnostic procedures.

2.2 Preparation of the internal standard and calibrators

The internal standard (IS) stock solutions of both isotope-labelled tetradeuterated analog of

A



B

Figure 1. Newborn screening form: A. The front side of the form shows four sections: family information receipt (left margin); identification details of the newborn, mother and healthcare center; blood sample collection circles (right margin) and a consent section where parents indicate their decision to participate in the newborn screening program. **B.** The back side contains, in the central area, the space designated for the newborn's identification label.

C24:0 (d4-C24:0-LPC, 156,25µmol/L) and C26:0 (d4-C26:0-LPC, 156,25µmol/L) from Fisher Scientific were prepared by dissolving powders in high performance liquid chromatography (HPLC) grade methanol (Romil-pure chemistry). The IS working solutions were prepared by firstly allowing both IS stock solutions to reach room temperature (RT) for 30 minutes, then ultrasonicated in an ultrasonic bath (Fisher Bioblock Scientific) for 30 minutes and again let to stand at RT for 30 minutes. An equal volume of both IS stock solutions was added to HPLC grade methanol to attain a working IS concentration of 0,398µmol/L for both d4-C24:0-LPC and d4-C26:0-LPC. Standard stock solutions of C24:0-LPC and C26:0-LPC were prepared by dissolving the purified reference compounds in HPLC grade methanol. Calibrators were generated by spiking various concentrations of the reference compounds into a matrix consisting of steroid-free human serum mixed with washed peripheral blood cells from 20 healthy donors (55:45, v/v). Calibrators were serially diluted at concentrations 0.02, 0.25, 0.5, and 2µmol/L for the calibration curve. The resulting mixtures were spotted on Whatman 903 filter paper, left to air dry for four hours and stored at -20°C until analysis. The four calibrators were added to each 96-well plate with the DBS samples and served as controls to make the regression.

2.3 Sample collection and preparation

DBS samples were collected from newborns using the standard heel-prick procedures. DBS cards (Fig.1) were then labeled and securely transported to the laboratory of metabolic disorders and newborn screening center at MRUH for analysis. Unique study IDs were assigned to each sample to ensure confidentiality during the testing. DBS cards were stored at 4ºC before use. Single 3.2-mm disks of DBS samples and calibrator samples were punched with a DBS puncher (PerkinElmer) into 96-well microplates (Chromsystems). 100µl IS was added to each well. Plates were covered with protective sheets for 96 well plates (Chromsystems) and incubated at 31°C for 30 minutes and shaken at 650 rpm on the microplate titramax/incubator (Heidolph 1000). The protective sheet was removed and the extracting solution was transferred to new 96-well microplate pureGrade (Thermofisher) and heat-sealed with aluminium seals (Thermofisher) before HPLC-MS/MS analysis.

2.4 HPLC-MS/MS analysis

The analysis was performed using a Shimadzu HPLC system with a LC-20AD pump at a flow rate of 1.5ml/min. Analytes were separated by an isocratic mobile phase composed of 50:50 (v:v) HPLC grade methanol : HPLC grade acetonitrile containing 5mM of ammonium acetate all purchased from Romil-pure chemistry. Analysis started with the injection of a 20µl sample using a SIL-30ACMP thermostatted multi-plate autosampler (Shimadzu). Total run time was 3.5 minutes per sample with elution of C24:0-LPC and C26:0-LPC at 1.5 minutes and backpressure of approximately 300 bars. Analytes were separated using a Mediterranea C8 column (5 X 0.21cm, 3µm particle diameter from

Teknokroma). The column temperature was kept constant at 40°C and the compartment of the autosampler was kept at 4°C. An API4000 triple quadrupole mass spectrometer with a Turbo V electrospray ion source (Applied Biosystems, Sciex) in negative ionization mode was used with multiple reaction monitoring (MRM) with parameters as shown in table 1. Analytes were detected by selected reaction monitoring using the transitions as shown in table 1. Quantitation of C24:0-LPC and C26:0-LPC levels is based on the integral of the peak areas from MRM analyses and was performed using the MultiQuant Software system.

Table 1. Parameters for the API4000 *HPLC-MS/MS* during analysis of C24:0-LPC and C26:0-LPC.

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
C24:0-LPC	592,6	367,3	100	-150	-11	-46	-8
D4-24:0-LPC	596,7	371,5	100	-150	-11	-46	-8
C26:0-LPC	620,7	395,4	100	-150	-11	-43	-11
D4-26:0-LPC	624,7	399,4	100	-150	-11	-45	-8

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential

2.5 Screening algorithm

The screening of XALD is based on the quantification of the biomarker: C26:0-LPC. However, in this study, we also quantified for another VLCFA namely, C24:0-LPC. DBS samples were analyzed using HPLC-MS/MS assay. In the first two months of the study, reference intervals and cut-off values were analyzed in 3000 anonymous DBS samples. Cut-off values of 0.18μmol/L for C24:0-LPC and 0.14μmol/L for C26:0-LPC were used to retest samples in duplicate using the same DBS sample. For newborns who exceeded the cut-off values in the repeat test, the results were explained to parents and collection of a second DBS sample was requested. The second DBS sample was reanalyzed in triplicate by HPLC-MS/MS assay. The values were examined and if the mean concentration exceeded cut-off values, the sample was classified as screen-positive and the parents of the infant would be referred for genetic testing.

2.6 Genetic testing and counseling

Results that exceeded cut-off values were classified as screen-positive. The screen-positive newborns were referred to CEDEM for genetic testing. A second written informed consent for specific genetic analyses was collected after providing families with specific information regarding the results from VLCFA analysis. Targeted clinical exome sequencing was performed on screen positive samples with suspicion of a PD. Genetic counseling was offered to the families of newborns who were screened with mutations in genes related to PD.

2.7 Statistical analysis

Statistical analysis including descriptive statistics, such as means, SDs and SEDs were performed using GraphPad Prism 10 software. As the dataset lacked confirmed positive cases for XALD or other PDs, construction of a reliable receiver operating characteristic (ROC) curve to determine cut-off values for C24:0-LPC and C26:0-LPC was not feasible. Therefore, the 99th percentile of the C24:0-LPC and C26:0-LPC concentration distributions among the 3000 newborns screened in the first two months was calculated and used as the cut-off value. This method ensures that the cut-offs reflect the upper end of the normal distribution, reducing the risk of false positives while maintaining sensitivity.

3. RESULTS

3.1 Chromatographic analysis of VLCFAs in newborn samples and calibrators

To evaluate VLCFA (C26:0-LPC) levels in DBS samples, chromatographic analysis was performed using HPLC-MS/MS following derivatization. IS was included in all samples to ensure accurate quantification and to normalize instrument variability. To establish a framework for interpreting the analyte signals, four calibrators with increasing known concentrations of C24:0-LPC and C26:0-LPC were analyzed. Supplementary figure 1 (A-D) demonstrates that the peak intensities for both VLCFAs increased proportionally with calibrator concentration, demonstrating a strong linear response and validating the quantitative accuracy of the assay. The linear regression (Supplementary figure 1E) of calibrators

provided a reference to estimate the concentrat ion of VLCFAs in the newborn samples using the MultiQuant Software system. Fig. 2 demon strates the extracted ion chromatogram of a healthy newborn (Fig. 2A) and a case newborn DBS sample (Fig. 2B). The IS (control) peaks in red (d4-C24:0-LPC) and black (d4-C26:0-LPC) are more intense than the analyte peaks. The analyte peak areas of C24:0-LPC (blue) and C26:0-LPC (green) in Fig. 2A are relatively low in intensity, suggesting normal peroxisomal beta-oxidation activity. In contrast, both C24:0-LPC and C26:0-LPC peak areas in Fig. 2B exhibit substantially elevated intensity compared to those in the healthy newborn, indicating accumulation of these VLCFAs. This elevated profile is characteristic of VLCFA accumulation observed in PDs such as X-ALD or DBP deficiency.

screen-positive. The analysis detected one positive case with elevated VLCFA results for both C24:0-LPC and C26:0-LPC (Fig. 3).

3.3 Case presentation

The female newborn was born through ceasarean section delivery at 38+4 weeks of gestational age with birth weight if 3250g (83rd percentile), height51cm (84th percentile) and head circumference 37.5cm (98th percentile) and Apgar score of 5, 7, and 7 at 1, 5 and 10 minutes respectively. The patient was born to consanguineous parents of Moroccan origin (first cousins) both of whom are heterozygous carriers of the *c.714+1G>A* variant in the Hydroxysteroid (17-beta) dehydrogenase 4 (*HSD17B4*) gene. She is the third out of three pregnancies. One brother died from DBP deficiency and the other brother is a healthy five

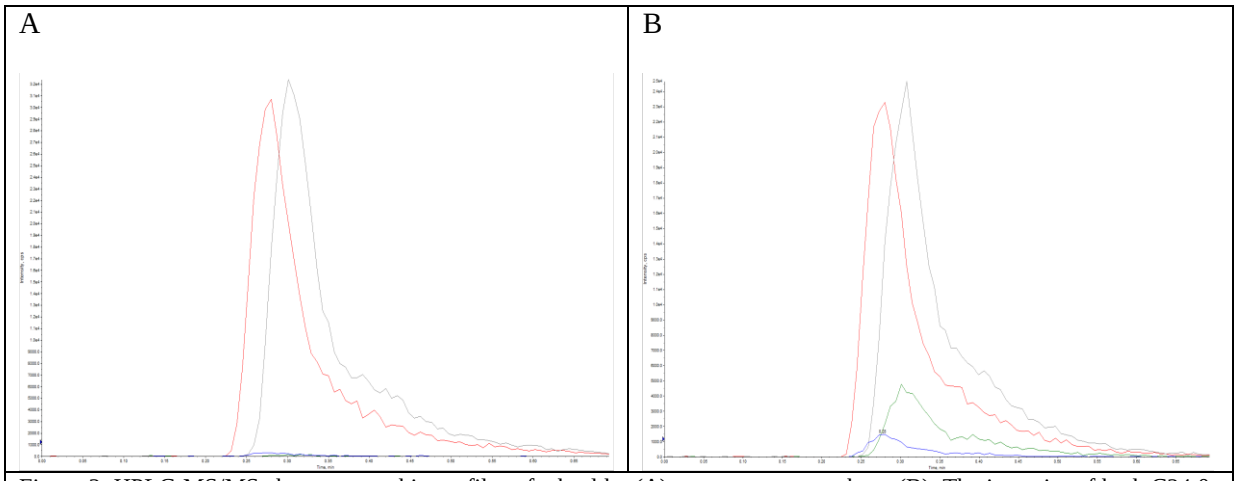


Figure 2: HPLC-MS/MS chromatographic profiles of a healthy (A) versus a case newborn (B). The intensity of both C24:0-LPC and C26:0-LPC levels is more elevated in the case newborn (B).

3.2 VLCFA analysis

A total of 12,593 newborns were screened for elevated VLCFA (C24:0-LPC and C26:0-LPC) in the laboratory of metabolic disorders and newborn screening center at MRUH. The mean VLCFA concentration was 0.04179 ± 0.002013 (SEM) for C24:0-LPC and 0.05790 ± 0.002294 (SEM) for C26:0-LPC. 126 newborns with C24:0-LPC and C26:0-LPC levels above the cut-off value of 0.18µmol/L and 0.14µmol/L respectively had to be retested. in duplicate. Although, 69 of these newborns exhibited only elevated levels of C24:0-LPC and were not retested. Based on the initial and retest results of the original DBS sample, a second DBS sample was requested. If VLCFA levels remained above the established cut-off values in the second DBS, the case was classified as a

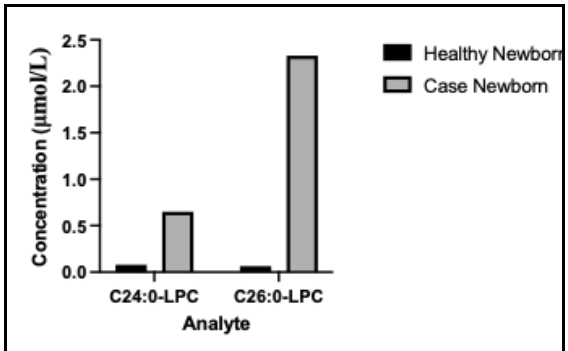


Figure 3. C24:0-LPC and C26:0-LPC plasma concentrations in dried blood spot samples from a healthy and a case newborn. Concentrations were determined using calibration curves generated by the MultiQuant software in the HPLC-MS/MS. The suspected case shows elevated C24:0-LPC and C26:0-LPC levels, consistent with a peroxisomal disorder. Data represents a single measurement for each case.

year old. Furthermore, a detailed family history revealed that paternal grandparents were also first cousins. The father has epilepsy and was previously treated with valproate for an unspecified period of time.

The patient reported poor vitality, hypotonia and respiratory distress at birth at the hospital in Melilla, requiring intubation and mechanical ventilation. At four hours of life, she presented a generalized tonic-clonic seizure with decreased responsiveness and fixed gaze, self-limited without medication, lasting 20 seconds. 30 minutes later, a similar seizure occurred and was treated with IV phenobarbital 20mg/kg, with no further seizures afterward. Given the family history of DBP deficiency, the patient was transferred to the neonatal intensive care unit (NICU) of MRUH for further evaluation.

Prenatal ultrasounds were normal. Non-invasive prenatal screening did not detect any aneuploidies in chromosomes 13, 18, 21 and X. The mother reported reduced fetal movements during pregnancy. At the first physical examination after birth, the patient presented with clear syndromic features with relative macrocephaly, slightly prominent anterior fontanelle, microretrognathia and severe hypotonia. Additionally, she presented with an "open book" posture, few spontaneous movements with slow response to stimuli, weak cry, absent palmar and plantar reflexes and weak sucking.

Additional examinations were performed: cranial ultrasound detected mild periventricular white matter hyperechogenicity with small pseudo-cystic lesions in the temporal cortex, poor differentiation between white and gray matter in the posterior parietal lobes with loss of sulci. MRI imaging (Fig. 4A) demonstrates notable hyperintensity on T2-weighted sequences in the periventricular white matter, particularly within the posterior cerebral region and involving the cerebellar white matter (green box), suggestive of early myelination abnormalities. A spectroscopy study demonstrated markedly pathological elevation of the choline peak (I=13,7) and reduction of N-acetylaspartate (NAA) (I=7,61) (Fig. 4B), indicating intracranial metabolite abnormality.

Given the increased clinical suspicion of DBP deficiency, biochemical testing for VLCFA was required. NBS revealed markedly elevated levels of C24:0-LPC (0,657μmol/L) and C26:0-LPC (2,297μmol/L) compared to a healthy newborn (Fig. 3). The increase was more pronounced for C26:0-LPC which is the key biomarker in PDs. These findings were consistent with impaired peroxisomal beta-oxidation supporting the biochemical diagnosis of a PD. This prompted further genetic analysis at CEDEM with suspicion of a PD, particularly DBP deficiency because of the family history.

Written informed consent was obtained from the parents of the newborn for genetic studies. In order to confirm the suspicion of DBP deficiency, targeted clinical exome sequencing was requested at CEDEM. The results showed that the patient had a homozygous mutation: *c.714+1G>A* in the *HSD17B4* gene, inherited from both parents who are unaffected carriers. This variant affects the canonical donor splice site at intron 6 and is predicted to severely disrupt normal mRNA splicing, leading to a loss of function of the DBP. The variant has not yet been reported in the OMIM allelic variant section, but a similar substitution at the same nucleotide position (*c.714+1G>C)* has been classified as likely pathogenic in ClinVar (22,23). The patient was transferred back to the NICU at the hospital of Melilla and remains medically complex and alive with the age of two months and is being followed by multiple subspecialists. Genetic counseling was offered to the parents to address recurrence risk, inheritance patterns, availability of reproductive options such as prenatal testing for subsequent pregnancies and support family planning.

4. DISCUSSION

4.1 Study aims and key findings

Given the fundamental importance of early diagnosis of XALD, over the past years, several countries have implemented their NBS program with the assessment of C26:0-LPC values in DBS for screening XALD (12). This is the first pilot study of NBS for XALD in Spain and was designed to assess the utility of NBS for XALD through quantification of VLCFAs, particularly C24:0-LPC and C26:0-LPC, in DBS using a modified and adopted HPLC-MS/MS method from Haynes et al. (15). The rationale for screening is based on the potential for early

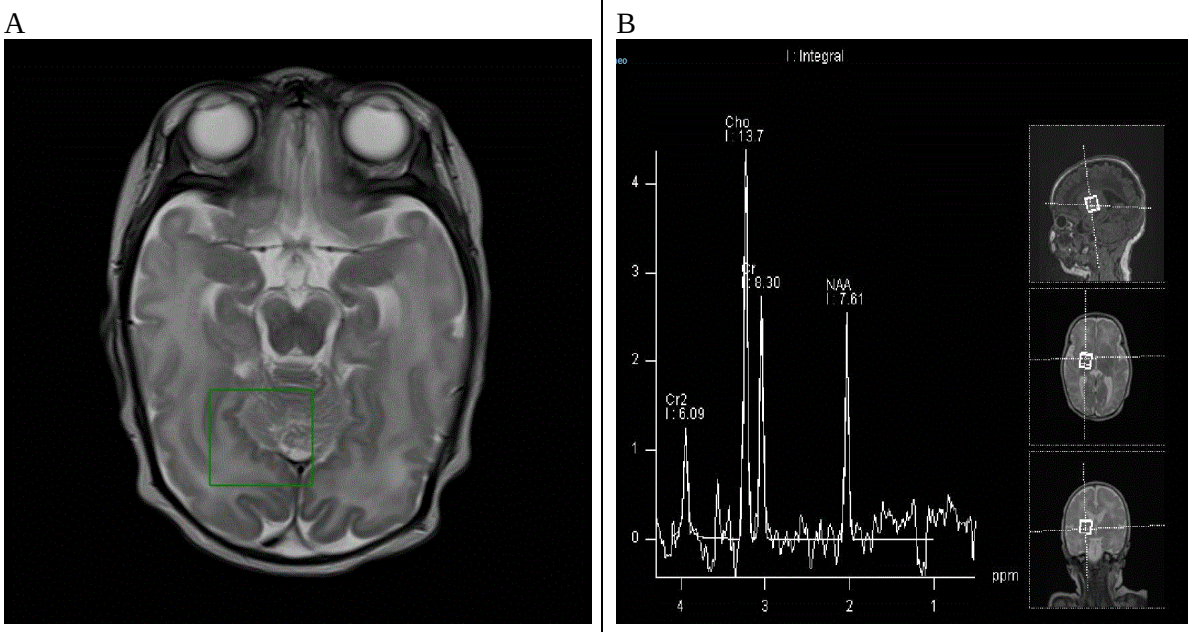


Fig. 4 Brain MR images of a newborn with suspected D-bifunctional protein deficiency– **A**. Axial T2-weighted MRI of the brain: Diffuse hyperintensity of the periventricular and deep white matter, with loss of normal white-gray matter differentiation. The green box highlights the posterior periventricular region with abnormal white matter signal. **B.** MR spectroscopy of the basal ganglia: the voxel (white square) is positioned in the deep gray matter. The spectrum shows a markedly elevated choline (Cho) peak, elevated creatine (Cre) peak and a reduced N-acetylasparatase (NAA) peak, suggesting neuronal loss and demyelination.

identification of individuals at risk of developing cerebral demyelination, where timely intervention of HSCT can significantly improve neurological outcomes if performed pre-symptomatically (7). The primary objective of this project was to evaluate the effectiveness of VLCFA-based screening in identifying newborns with XALD and to assess its potential integration into the general NBS program in Eastern Andalusia. Early diagnosis is critical as boys with XALD remain asymptomatic during infancy but may present with adrenal insufficiency or rapidly progressive neurological deterioration later in childhood (3). The window for therapeutic intervention is narrow and often missed without systematic early detection. Thus, expanding NBS to include X-ALD can transform the clinical course of this otherwise devastating disease, providing affected families with the opportunity for proactive surveillance and management (7, 11).

While the screening protocol was specifically optimized for detecting XALD, an unexpected case of DBP deficiency was identified in one of the newborns. A female newborn presented with markedly elevated C24:0-LPC (0,657μmol/L) and C26:0-LPC (2,297μmol/L) levels during NBS for XALD. The positive-screen newborn was referred to genetic testing and was diagnosed with DBP deficiency, a rare autosomal recessive PD with an estimated prevalence of 1/100.000. DBP deficiency is a single-enzyme deficiency causing an inborn error of peroxisomal metabolism caused by biallelic pathogenic variants in the *HSD17B4* gene (20). The *HSD17B4* gene located on chromosome 5 is responsible for oxidizing numerous peroxisomal substrates and encodes the DBP. Symptoms typically manifest during the neonatal period including hypotonia, seizures and facial dysmorphism. The condition progresses to severe psychomotor retardation and death within the first two years of life in most cases. The DBP consists of both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase enzyme domains that catalyze multiple steps during beta-oxidation of VLCFA ≥ C22:0 among other things (15, 20). Based on which enzyme domain is affected, DBP deficiency was classified into type I where both 3-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase are deficient, type II where only hydratase is deficient and type III where only dehydrogenase is deficient (15).

4.2 DBP deficiency newborn case

The patient described here exhibited a severe phenotype at birth with seizures, and hypotonia as well as elevated abnormal VLCFA newborn screening results. The neuroimaging findings supported a leukodystrophy process, consistent with peroxisomal dysfunction, and correlated with the clinical and biochemical features observed in DBP deficiency (22). A detailed family history revealed that both parents were heterozygous carriers of the *c.714+1G>A* variant of the *HSD17B4* gene and that an elder brother had died from DBP deficiency. Targeted clinical exome screening confirmed the suspected DBP deficiency diagnosis. The identification of a homozygous *c.714+1G>A* splice-site mutation in the *HSD17B4* gene provides a clear molecular diagnosis of DBP deficiency. The variant is located at the canonical +1 position of the splice donor site in intron six and is predicted to result in aberrant splicing. Though not previously described in OMIM or ClinVar, this mutation is strongly presumed to be pathogenic, based on its nature, position, and similarity to the nearby reported variant *c.714+1G>C*, which has been classified as likely pathogenic (20, 23). The severe biochemical abnormalities, *c.714+1G>A* variant in the *HSD17B4* gene and early-onset neurological manifestations observed in this case are consistent with Type I DBP deficiency, in which both the hydratase and dehydrogenase domains of the protein are impaired (15,20). The early detection of this disorder, although incidental, allowed for rapid diagnostic clarification, initiation of palliative care measures and targeted genetic counseling.

4.3 Overlap in biochemical profiles among PDs

The unexpected finding of a DBP deficiency case highlights the overlap in biochemical profiles among PDs detectable by HPLC-MS/MS particularly the accumulation of VLCFAs. Both XALD and DBP deficiency are characterized by impaired peroxisomal beta-oxidation leading to elevated levels of VLCFAs such as C26:0-LPC which is typically used as a biomarker in NBS for XALD (3, 20, 24). Therefore, it is plausible that a newborn with DBP deficiency may yield a positive result in an XALD screening due to the shared metabolic profiles (20,24). Subsequent confirmatory testing, including molecular genetic analysis is crucial in differentiating between PDs, in this case between XALD and DBP deficiency (25, 26). The identification of the likely pathogenic variant in the *HSD17B4* gene, rather than in the *ABCD1* gene, confirmed the diagnosis of DBP deficiency. Moreover, the detailed family history specifically of an older brother who died from the same condition and both parents being heterozygous carriers of the pathogenic variant *c.714+1G>A* significantly expedited in confirming the diagnosis. The screening of this case illustrates a strength in expanded NBS: the ability to detect multiple conditions with overlapping biomarkers can facilitate early diagnosis of rare disorders.

4.4 Cultural and familial context

Of note, the parents of the affected infant were first cousins of Moroccan origin. They had already received genetic counseling following the death of their son, who was diagnosed with the same disorder. Despite being informed of the autosomal recessive inheritance and recurrence risks during genetic counseling, the family chose to conceive again. While this reflects their personal reproductive autonomy, it also illustrates the complex interplay between cultural beliefs, religious values, and genetic information. In certain communities, consanguineous unions are traditional and may be seen as culturally normative (27, 28). Additionally, in some religious contexts, including among Muslims, there can be a tendency to place greater emphasis on divine will or fate, which may influence how genetic information is perceived or acted upon (29, 30). These factors underscore the importance of culturally competent genetic counseling that not only provides accurate scientific information but also addresses the family's beliefs, values, and decision-making framework (30, 31). Respectful communication is key in empowering families to make informed choices without imposing value judgments. In regions or populations with high rates of consanguinity, community-level education and proactive screening initiatives may be particularly impactful in reducing the incidence of autosomal recessive disorders like DBP deficiency (27, 32). Although genetic counseling was offered following the diagnosis of their son, the birth of a second affected child highlights the need for ongoing counseling to reinforce understanding of inheritance, provide updated genetic information, and explore reproductive options such as carrier testing for family members, prenatal diagnosis, and

preimplantation genetic testing (31). Continued counseling also plays a crucial role in addressing the emotional, religonal, cultural and psychosocial challenges faced by families managing multiple affected children.

4.5 Broader implications

The detection of DBP deficiency during XALD screening carries important implications for newborn screening policy and practice. It raises the question of whether screening programs that target X-ALD should be designed or interpreted in a way that anticipates detection of other PDs, such as ZSD and DBP deficiency. While this broad diagnostic scope is beneficial in some cases, it also introduces challenges, including ethical considerations around the detection of untreatable or severe conditions not originally intended for screening. Families may be confronted with life-altering diagnoses unexpectedly and the implications of identifying disorders with no current treatment options. Additionally, the burden of follow-up testing and counseling increases significantly for healthcare systems (33, 34). However, from a clinical genetics perspective, the early detection of DBP deficiency through NBS is valuable, despite the poor prognosis, as it avoids prolonged diagnostic odysseys, allows for better-informed reproductive decisions, and ensures that families receive appropriate psychosocial support (35). Furthermore, this case contributes to the expanding mutation spectrum of *HSD17B4* and contributes to the limited body of literature on newborn presentation of DBP deficiency (36). Additionally, it highlights the importance of integrating biochemical, neuroimaging, and genetic data to arrive at a definitive diagnosis. Lastly, it reinforces the clinical utility of NBS programs in identifying PDs early, even in the absence of prior family history or confirmed variant pathogenicity in OMIM or ClinVar (25, 35).

Importantly, the value of NBS for X-ALD extends beyond the detection of affected male infants. While the SCAN project in the Netherlands, has limited screening to boys due to the X-linked inheritance pattern and the higher likelihood of severe phenotypes, this sex-specific approach may inadvertently delay diagnosis in female carriers (10). Excluding female newborns from X-ALD screening not only limits the detection of asymptomatic carriers but also reduces the opportunity to identify other PDs that may present with similar biochemical profiles. X-ALD screening in Eastern Andalusia, being universal, is of critical importance due to the broader inclusion of both sexes. By implementing screening for both boys and girls, Eastern Andalusia ensures the identification of potentially asymptomatic female carriers and those at risk of developing other PDs like in this study.

4.6 XALD screening remains crucial

Although no X-ALD cases were identified in the current cohort, this finding should be interpreted with caution and in light of the known epidemiology of the disease. X-ALD has a relatively low incidence, estimated at approximately 1 in 17,000 births (3), and the limited sample size of 12,593 in this study may have reduced the likelihood of detecting a true positive case. Therefore, the absence of identified cases does not necessarily reflect a limitation in the screening protocol's sensitivity. Previous large-scale NBS programs have demonstrated high analytical sensitivity and specificity for C26:0 and C24:0 quantification in DBS using HPLC-MS/MS, especially when combined with *ABCD1* gene sequencing to improve diagnostic accuracy and reduce false positives (5, 21)). These results reinforce the robustness of VLCFA-based screening as a first-line tool for identifying peroxisomal dysfunction. The incidental identification of a case with D-bifunctional protein (DBP) deficiency further underscores the method's high sensitivity for detecting peroxisomal β-oxidation defects, though it also highlights the limited specificity of VLCFA elevation in distinguishing among PDs. This supports the integration of molecular confirmation in NBS algorithms to enhance disease-specific specificity and diagnostic clarity (25, 26).

Importantly, the incidental detection of DBP deficiency does not diminish the value of X-ALD screening. On the contrary, this finding underscores the robustness and potential of VLCFA-based screening to identify multiple peroxisomal disorders, some of which, like X-ALD, may be amenable to early treatment (25). Although DBP deficiency currently lacks curative therapy, early diagnosis enables the implementation of supportive care and prevents a protracted and distressing diagnostic process

for families (25). For X-ALD, the clinical benefit of early detection remains well established, particularly in male infants who may be monitored and referred for HSCT before irreversible neurological damage occurs.

CONCLUSION

In conclusion, although the primary aim of this study was the detection of X-ALD, the incidental identification of a newborn with DBP deficiency underscores the broader utility and complexity of VLCFA-based screening. The integration of VLCFA-based NBS for XALD represents a transformative advance in PDs diagnostics. This approach not only facilitates early intervention for treatable conditions such as XALD but also enables the incidental discovery of other severe metabolic disorders such as DBP deficiency. While such incidental findings raise important ethical questions, the overall benefit to affected families through early support and genetic counseling emphasizes the value of broad-spectrum biochemical screening methods in neonatal care. Future screening algorithms may benefit from incorporating targeted gene panels to enhance diagnostic accuracy.

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Author contributions – RY, MIG and IC conceived and designed the research. MIG and IC performed experiments. IC performed data analysis.MIG provided assistance and guidance to work with the HPLC:MS/MS machine. IC wrote the thesis. RY helped in editing the first draft of the manuscript.

Supplementary figures

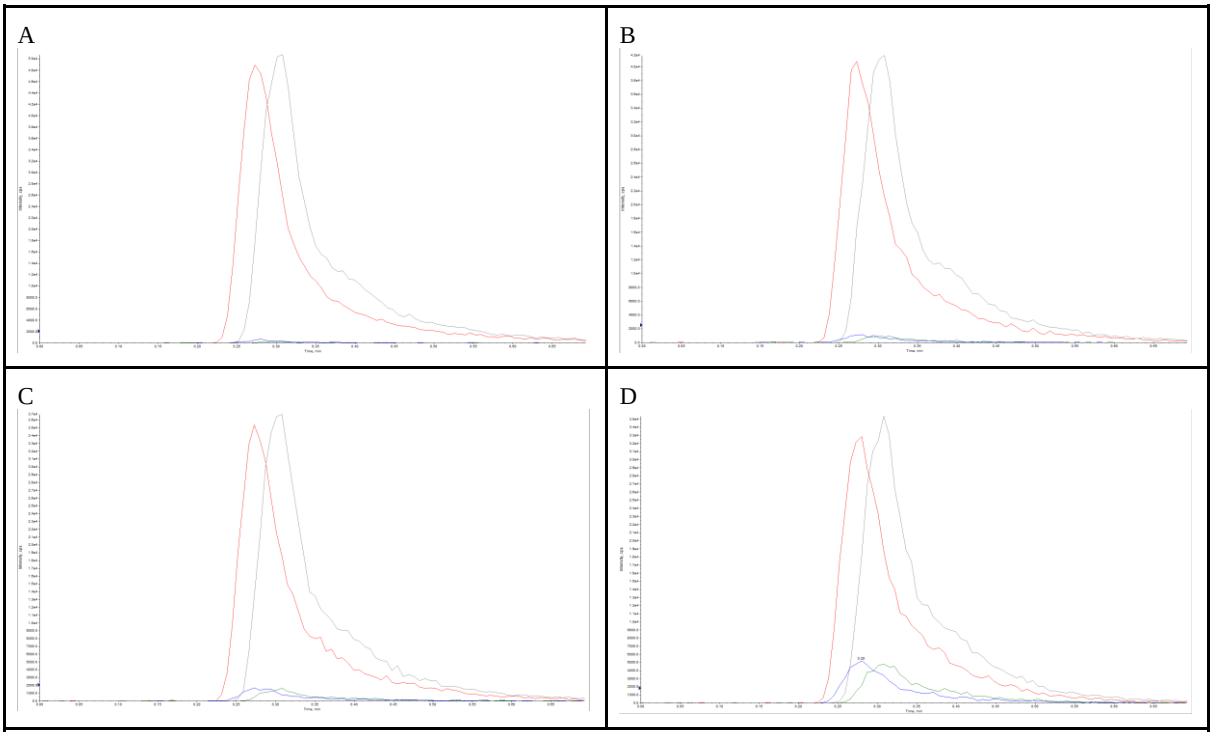
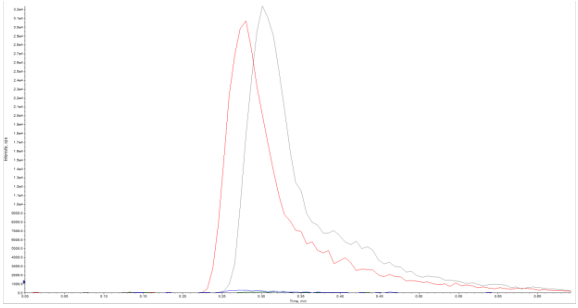


Figure A: Representative HPLC-MS/MS chromatographic profiles of calibrators with increasing concentration of C24:0-LPC and C26:0-LPC; **A**:0,02; **B**: 0,25; **C**: 0,5; **D**: 2μmol/L. The intensity in counts per second increases with the concentration of the calibrators.

A



B

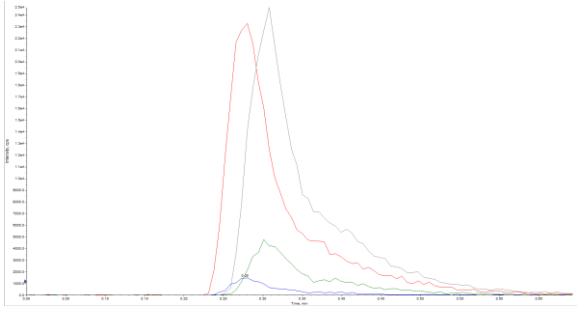
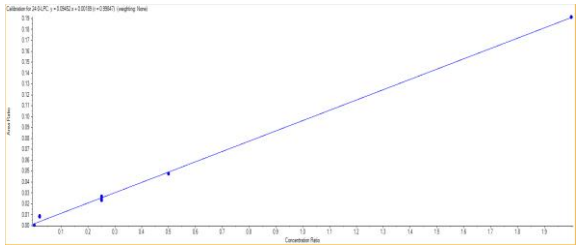


Figure 2: HPLC-MS/MS chromatographic profiles of a healthy (A) versus a case newborn (B). The intensity of both C24:0-LPC and C26:0-LPC levels is more elevated in the case newborn (B).

A



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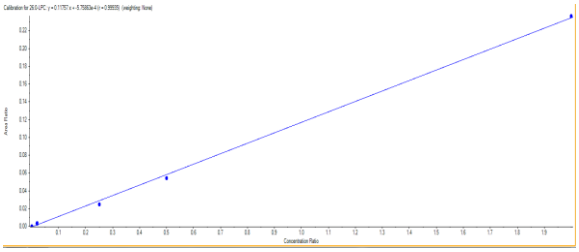

Figure : Linear calibration curves for C24:0-LPC (A) and C26:0-LPC (B) concentrations in µmol/L versus the peak area ratio in calibrator samples (0; 0,02; 0,25; 0,5; 2µmol/L)