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**Letter to the Editor**

*To the Editor-in-Chief*
*Sir,*

**Rapid diagnosis of medium chain Acyl Co-A dehydrogenase (MCAD) deficiency in a newborn by liquid chromatography/tandem mass spectrometry**

Medium chain Acyl-CoA dehydrogenase (MCAD) deficiency is the most common $\beta$-oxidation defect.[1,2] Its presentation varies from acute neonatal to late-onset and even to adulthood onset,[3,4] and in some cases it is asymptomatic for an entire lifetime.[5] The symptoms are characterised by hypoglycaemia, vomiting, seizures, lethargy, coma, till death. The metabolic disorder is frequently caused by fasting or viral febrile illness. Moreover, MCAD deficiency can be responsible for sudden infant death syndrome (SIDS),[6] for permanent neurological damage due to hypoglycaemic encephalopathy,[7] and for Reye-like syndrome.[8,9]

Molecular analysis of the *MCAD* gene in clinically affected patients has revealed that 80% of patients are homozygous for a common mutation 985A → G, and 18% are heterozygous for this mutation.[7,10] The delayed, or not performed, diagnosis of MCAD deficiency at presentation causes the deaths of about 20–25% of patients.[11,12] Because of its frequency (1:10000–1:17000 newborns)[1,13–15] and high mortality, neonatal screening by electrospray ionisation tandem mass spectrometry (ESI-MS/MS) is highly recommended.[13,16–18] However, in very rapid and acute neonatal onset, frequently within 3 days of life,[3,19] neonatal screening could be too late with reference to the worsening of the gravity of illness. Therefore, clinical symptoms must lead to laboratory tests and to diagnosis as soon as possible.

MCAD deficiency diagnosis can be performed by analysing the acylcarnitine profile from a dried blood spot or plasma spot.[24–33] Acylcarnitine analysis was originally performed by high-performance liquid chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS),[21] or GC of organic acids after hydrolysis of carnitine esters. These processes are time-consuming (typical run times are >30 min) and labour-intensive, thus limiting high-throughput performance.

We describe here a patient with acute severe presentation in his third day of life. In this case a very rapid diagnosis by LC/MS/MS allowed a complete recovery before the decompensation became very important. After diagnosis, LC/MS/MS analyses are also fundamental for follow-up purposes during long-term management therapy.

The patient, a male, was born at term to non-consanguineous parents from Tuscany, with a weight of 3360 g. A brother and a sister are healthy. No clinical abnormalities were found in the first day of life. After 48 h of life, he became hypotonic and hyporeactive. Abnormal laboratory findings included metabolic aciduria (blood gas parameters were pH 7.28; $pCO_2$ 28.3; $HCO_3^-$ 13.1; EB −11.8), high values of transaminases and lactate, hyperammonaemia (291 μM; normal <32 μM), and hypoglycaemia (10 mg/dL; normal 70–120 mg/dL). Because of the presence of these symptoms, a sample from blood spot paper was immediately prepared and immediately analysed by LC/MS/MS. The diagnosis of MCAD deficiency, due to accumulation of medium chain acylcarnitines (see later), was made about 1 h after drawing blood.

Acylcarnitines and labeled standards of amino acids were purchased from Cambridge Isotope Laboratories (Andover, MA, USA); a stock solution was made in methanol. The standard concentrations are in the range 500–2500 μmol/L for amino acids and in the range 7.6–152 μmol/L for acylcarnitines. In order to obtain working solutions, daily dilutions (1:100) were made using methanol. All chemicals and solvents were of the highest purity available from commercial sources, and were used without any further purification.

A dried blood spot was punched into a 1.5-mL tube and 200 μL of methanol containing labeled standards were added. The sample was shaken on a vortex system for 20 min, and was then dried under a nitrogen flow at 50°C. The extracted acylcarnitines and amino acids were derivatised to butyl esters using n-butanol plus HCl (3 M) at 65°C for 25 min. After derivatisation the sample was dried under a nitrogen flow at 55°C and then recovered by 200 μL of water/acetonitrile (1:1) containing 0.1% formic acid. 40 μL of the diluted sample were injected in flow injection analysis (FIA) mode for the MS/MS experiments.

An Applied Biosystems-Sciex (Toronto, Canada) API 2000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray source was employed for this study. The TurboIonSpray source was operated in positive ion mode with a needle potential of +5900 V and with a ''turbo'' gas flow of 10 L/min of air heated at 150°C (nominal heating-gun temperature).

Mass calibration and resolution adjustments on the resolving quadrupoles were performed automatically by using a $10^{-4}$ mol/L solution of PPG introduced via the built-in infusion pump. The peak width was set on both resolving quadrupoles at 0.7 Th (measured at half height) for all MS and MS/MS experiments.

Collision-activated dissociation (CAD) MS/MS was performed in the LINAC Q2 collision cell, operating with 10 mTorr pressure of nitrogen as collision gas. The declustering potential (DP) and collision energy (CE) were automatically optimized for acylcarnitines and amino acids using the Analyst 1.1 software. The resulting DP was +18 V, and optimal CE was found to be 20 eV (laboratory frame) in the case of amino acids. A DP ramp (10–55 V) and a CE ramp (35–50 eV) were needed in the case of acylcarnitines.

MS and MS/MS spectra were collected in continuous flow mode by connecting the infusion pump directly to the TurboIonSpray source. A standard solution of 10 ng/μL of each amino acid and acylcarnitine in water/acetonitrile (1:1) containing 0.1% formic acid was infused at 10 μL/min. The quantitation experiments

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were performed using a series 1100 Agilent Technologies (Waldbronn, Germany) CapPump coupled to an Agilent Micro ALS autosampler, both fully controlled by the API 2000 data system. Mobile phase flow rate was 30 µL/min using water/acetonitrile (1:1) containing 0.1% formic acid. The eluent from the column was directed to the TurboIonSpray probe. The acquired data were processed using the Analyst 1.1 proprietary software including the 'Explore' option (for chromatographic and spectral interpretation) and the ChemoView software (for quantitative information generation).

Genomic DNA was extracted from the patient's and from his parents' peripheral blood lymphocytes using standard methods. The genomic fragments covering all 12 exons and the exon/intron boundaries of the *MCAD*-gene were amplified by a set of primers located in flanking intronic sequences. PCR amplification was performed under the following conditions: initial denaturation at 94°C for 4 min was followed by 30 cycles with denaturation at 94°C for 30 s, annealing temperature of 63°C for 30 s, and extension at 72°C for 2 min. All the amplification reactions were performed in a total volume of 25 µL containing 2.5 U PolyTaq DNA polymerase (Polymed, Sambuca-Firenze, Italy), 25 mM of dNTPs, 200 ng forward primer, 200 ng of the reverse primer and 1XPCR reaction buffer.

PCR fragments were separated on a 2% agarose gel containing ethidium bromide and the bands were visualised by an UV transilluminator. DNA products were purified using a Nucleospin extract kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. The double-stranded purified products were used for direct sequencing with the same PCR amplification primers. The sequencing reactions were performed with Big Dye terminator cycle sequencing ready reaction kit reagents (Applied Biosystems, Foster City, CA, USA). The reactions were run on an ABI PRISM 310 sequencer and were analysed using Sequencing analysis software, version 3.3.

Figure 1 shows a typical MS/MS profile of a healthy newborn. Figure 2 shows the comparison between a normal acylcarnitine profile versus one affected by a MCAD deficiency. The anomalous ions corresponding to very high concentrations of hexanoyl carnitine (C6), octanoyl carnitine (C8), decanoyl carnitine and decenoyl carnitine (C10:1) are clearly observed at *m/z* 316, 344, 372 and 370, respectively, only in the MCADD-affected profile. The amino acid profile was normal. After diagnosis the patient was supplied intravenously with glucose and with insulin as emergency therapy. These administrations gave high cellular energy availability, halting lipolithic pathway activation. In addition, a supplementation of l-carnitine was needed to remove accumulation of toxic intermediates. Figure 3 shows the MCAD deficiency acylcarnitine profile during emergency therapy (8, 18, and 48 h post-diagnosis). The use of an LC/MS/MS system during the first 48 h post-diagnosis permitted the evaluation of the removal of the toxic acylcarnitines (Table 1). After the first 48 h, a

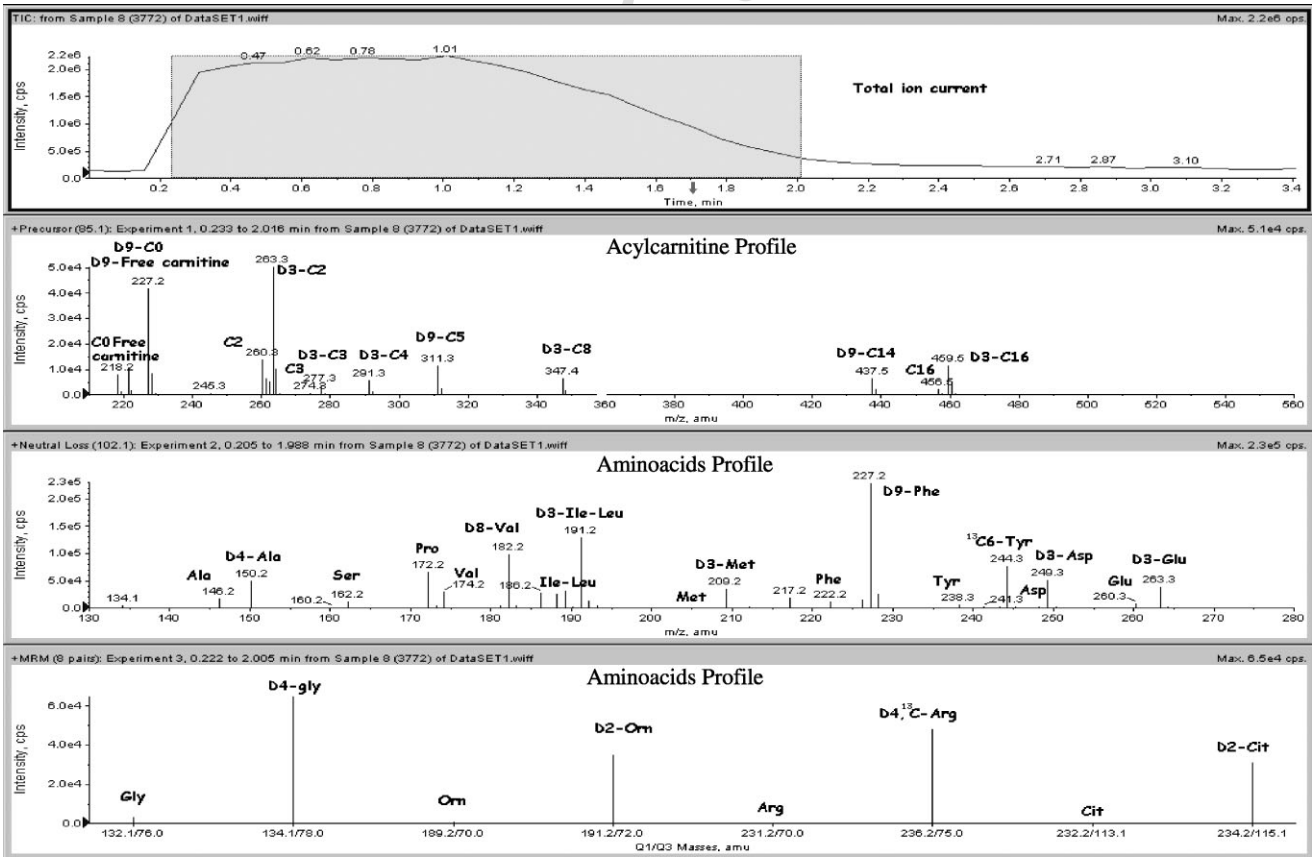


**Figure 1.** ESI-[Q1]MS/MS profiles of acylcarnitines and amino acids of a healthy newborn.

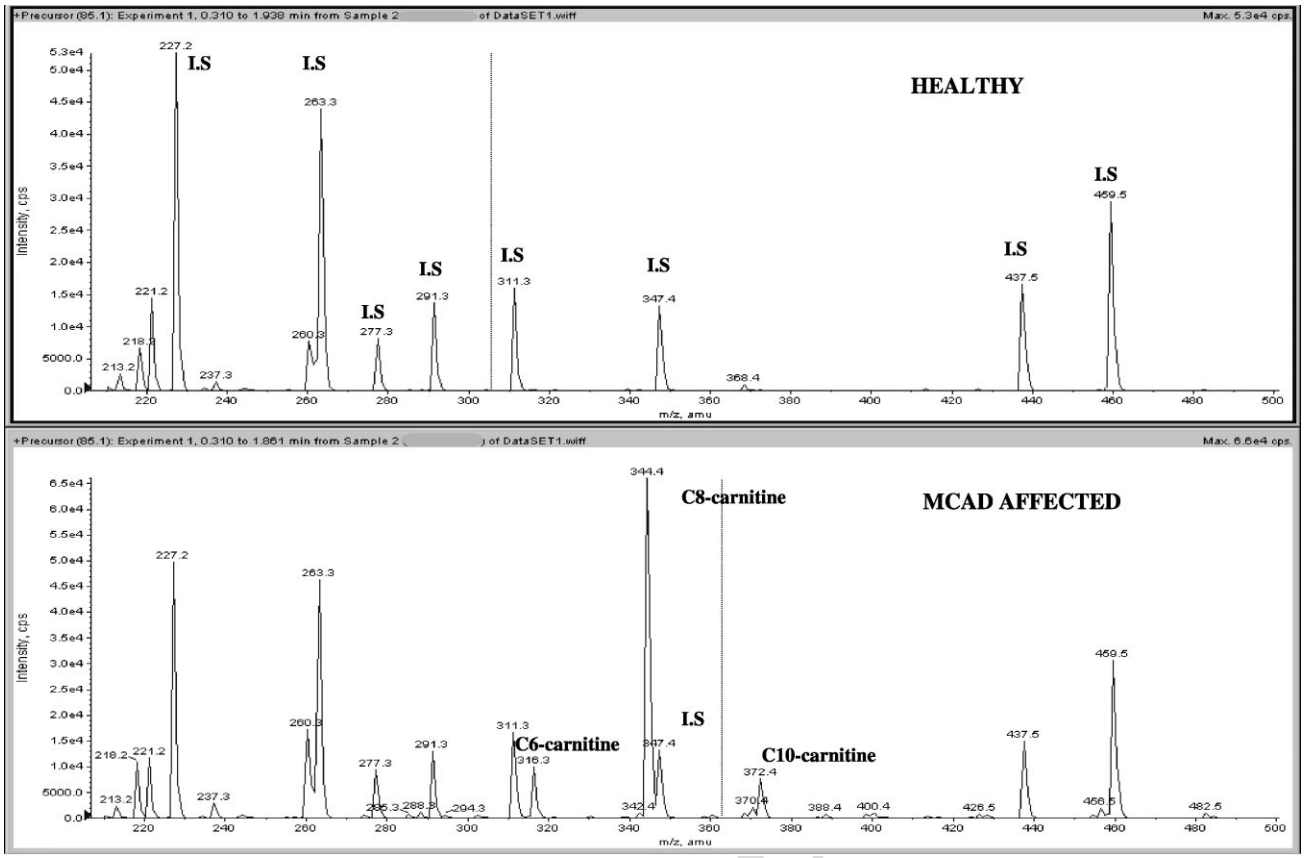


**Figure 2[Q1].** Normal acylcarnitine profile compared with one from a patient with MCAD deficiency.

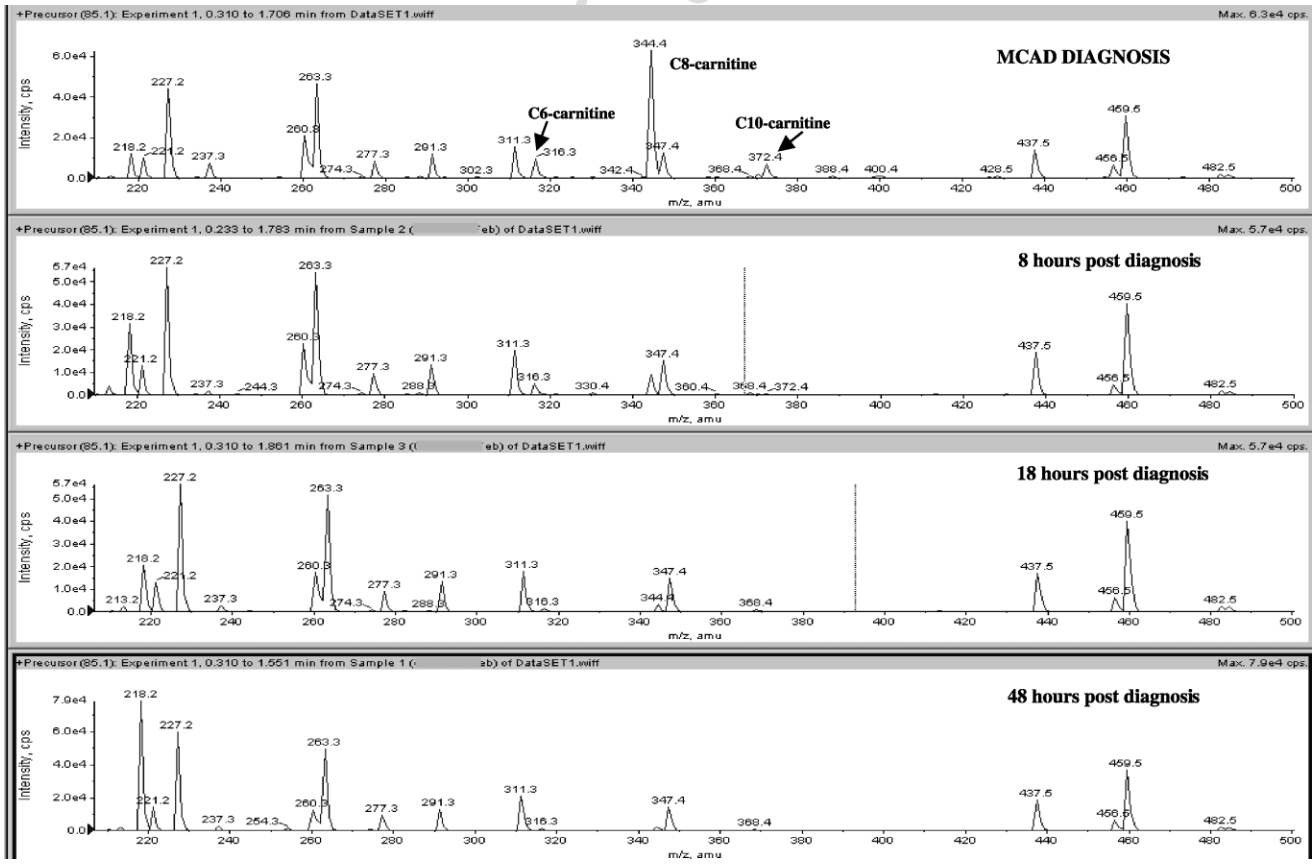


**Figure 3[Q1].** Acylcarnitine profile of a patient with MCAD deficiency during emergency therapy.

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**Table 1.** Decrease in levels of medium chain acylcarnitines during emergency therapy

| | C6 | C8 | C10 | C10:1 |
|---|---|---|---|---|
| Diagnosis | 3.35 | 26.7 | 3.03 | 0.897 |
| 8 hours PD | 2.6 | 19 | 1.79 | 0.65 |
| 18 hours PD | 1.43 | 3.28 | 0.269 | 0.225 |
| 48 hours PD | 0.457 | 1.22 | 0.104 | 0.12 |
| Normal values* | 0.06–0.24 | 0.02–0.23 | 0.03–0.2 | 0.05–0.24 |

C6, Hexanoyl carnitine; C8, octanoyl carnitine; C10, decanoyl carnitine; C10:1, decenoyl carnitine; PD, post-diagnosis.
* 5th–95th percentile.

new management regime was started, consisting of an administration of a milk specific for $\beta$-oxidation defects, containing no fats but only carbohydrates and proteins, and in addition an aliquot of human milk containing fats, that provided 15–16% of the total daily intake of kilocalories. Figure 4 shows the acylcarnitine MS/MS profiles during the 48 h after the start of this management regime. The values of the increase in medium chain acylcarnitines allowed the evaluation that, in this patient, the MCAD residue activity was probably very low. Therefore, the management regime was corrected with respect to the human milk administration, changing to 8% the contribution of fats to the total daily intake of kilocalories.

Figure 5 shows the MCADD-affected acylcarnitine profile following the definitive long-term management regime, in comparison with a normal profile.

Molecular analysis indicated that the patient was homozygous for the known K329E mutation. This mutation is due to the transition 985A→G, which maps on exon 11 of the *MCAD* gene. His parents were heterozygous for this mutation. The K329E amino acid change is the most common mutation in the Caucasoid group of humans. Some authors have estimated its frequency to be about 90% of all MCAD mutations.[22,23]

In conclusion, the newborn screening method is characterised by a simple sample preparation and the instrumental analysis time is less than 3.3 min. In this case we performed the diagnosis about 1 h after receiving the blood spot paper, allowing the start of an emergency therapy immediately. The limitation of Italian newborn screening is that the drawing blood procedure must be performed after 48 h post-birth, which does not allow diagnosis of some acute neonatal onset pathologies. In these cases the driving force is clinical symptoms which indicate a potential metabolic disease. After this manifestation of clinical symptoms a fundamental role is played by an LC/



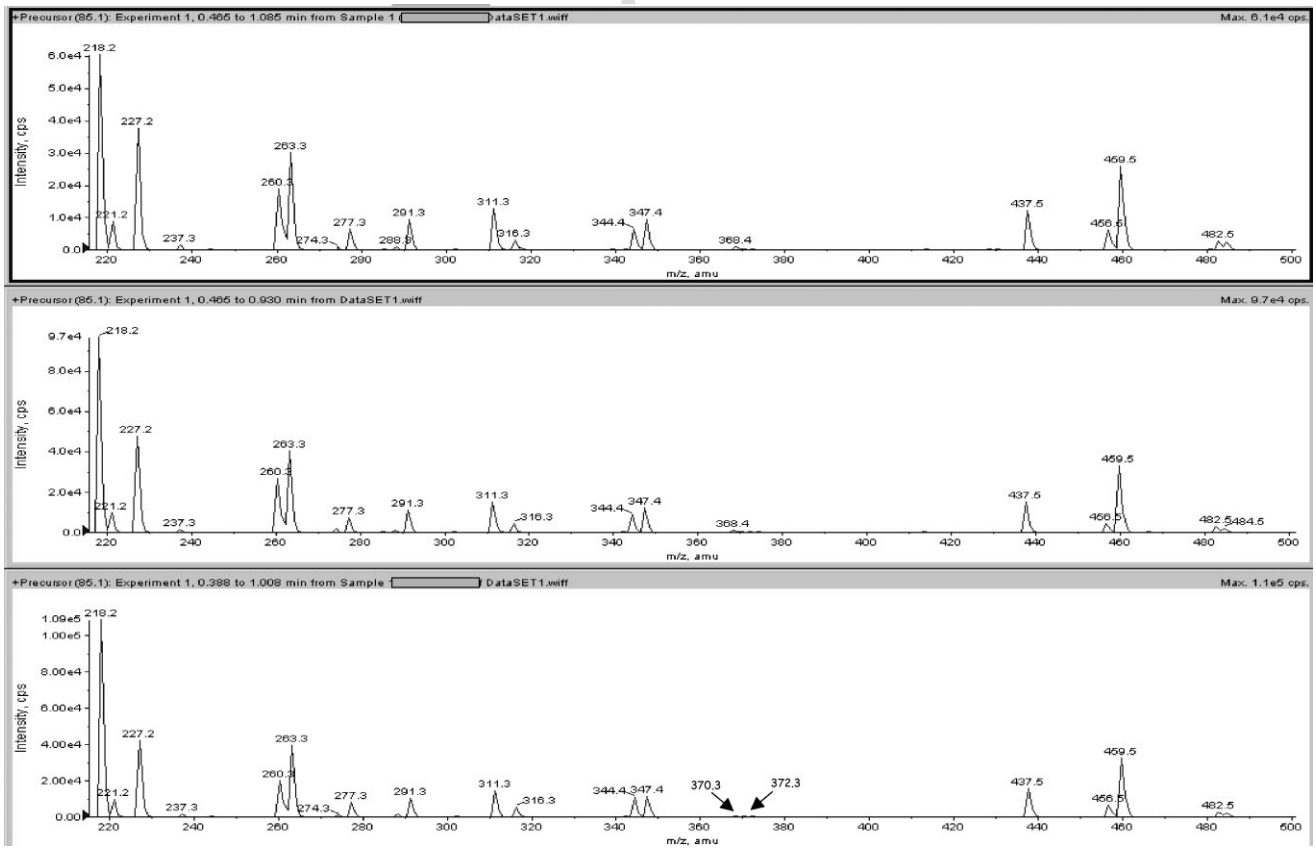


**Figure 4**[Q1]. Restoration of medium chain acylcarnitines during the 48 h after the start of the management regime.

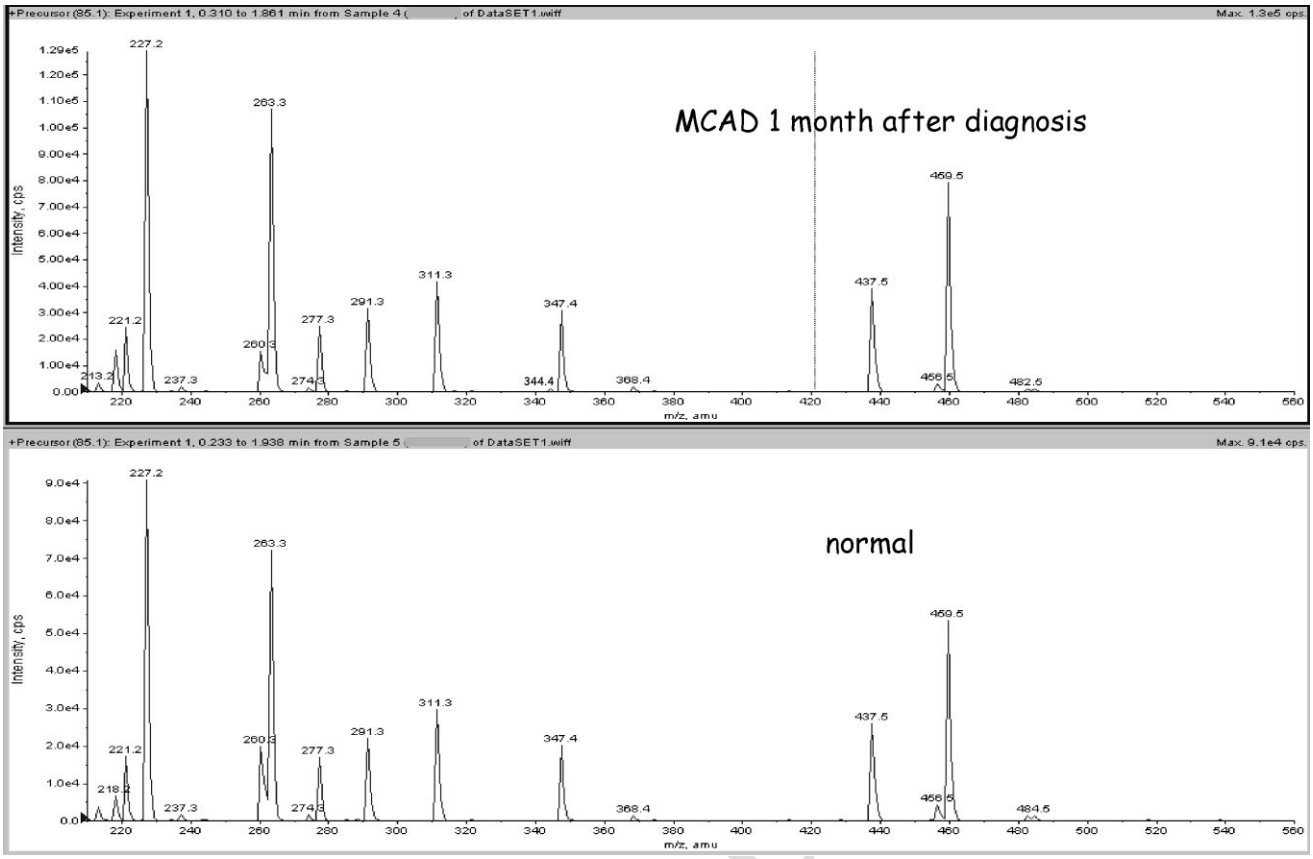


**Figure 5**[Q1]**.** The MCAD deficiency acylcarnitine profile following the definitive long-term management compared with a normal one. Q1

MS/MS system that permits a very fast identification of defects in comparison with other analytical techniques.

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