

# Methylmalonic Acidemia Diagnosis by Laboratory Methods

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## Abstract

Methylmalonic acidemia (MMA) is usually caused by a deficiency of the enzyme methylmalonyl-CoA mutase (MCM), a defect in the transport or synthesis of its cofactor, adenosyl-cobalamin (cblA, cblB, cblC, cblF, cblD, and cblX), or deficiency of the enzyme methylmalonyl-CoA epimerase. A comprehensive diagnostic approach involves investigations of metabolites with tandem mass spectrometry, organic acid analysis with gas chromatography, enzymatic studies with fibroblast cell culture, and finally, mutation analysis. With biochemical techniques and enzymatic assay the reliable characterization of patients with isolated MMA for mutation analysis can be achieved. Reliable classification of these patients is essential for ongoing and prospective studies on treatments, outcomes, and prenatal diagnoses. This article reviews the diagnostic techniques used to characterize patients with MMA.

**Keywords:** Diagnostic techniques, Enzyme assay, Methylmalonic acidemia, Mutation analysis, Organic acid analysis, Tandem mass spectrometry

## Introduction

Methylmalonic acidemia (MMA) is usually caused by a deficiency of the enzyme methylmalonyl-CoA mutase (MCM, EC 5.4.99.2), a defect in the transport or synthesis of its cofactor, adenosyl-cobalamin (cblA, cblB, cblC, cblF, cblD and cblX), or a deficiency of the enzyme methylmalonyl-CoA epimerase. MCM is a mitochondrial enzyme that catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA.

Isolated MMA is found in patients with mutations in MUT, located on chromosome 6p21, causing partial (mut<sup>-</sup>) or complete (mut<sup>0</sup>) enzyme deficiency (1). In general, the mut forms of MMA is unresponsive to vitamin B12 therapy. MCM activity requires 5-prime-deoxyadenosylcobalamin (AdoCbl), a coenzyme form of vitamin B12. Patients with defects in the synthesis of AdoCbl are usually responsive to vitamin B12 therapy and are classified

as 'cbl' type. The cblA type is caused by mutations in the MMAA gene on 4q31. MMAA is involved in the synthesis of adenosylcobalamin (AdoCbl), a coenzyme for MCM. The cblB type is caused by mutations in the MMAB gene on 12q24. MMAB encodes cobalamin adenosyl transferase (ATR), which catalyzes transfer of an adenosyl group from ATP to cobalamin (I) to form AdoCbl (2, 3).

Combined MMA and homocystinuria is a genetically heterogeneous disorder of cobalamin (cbl; vitamin B12) metabolism. The defect causes decreased levels of the coenzymes adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), which result in decreased activity of the respective enzymes MCM and methyltetrahydrofolate homocysteine methyl transferase, also known as methionine synthase (MTR). Different forms of the disorder have been classified according to complementation groups of cells in vitro: cblC, cblD, cblX and cblF. Members of

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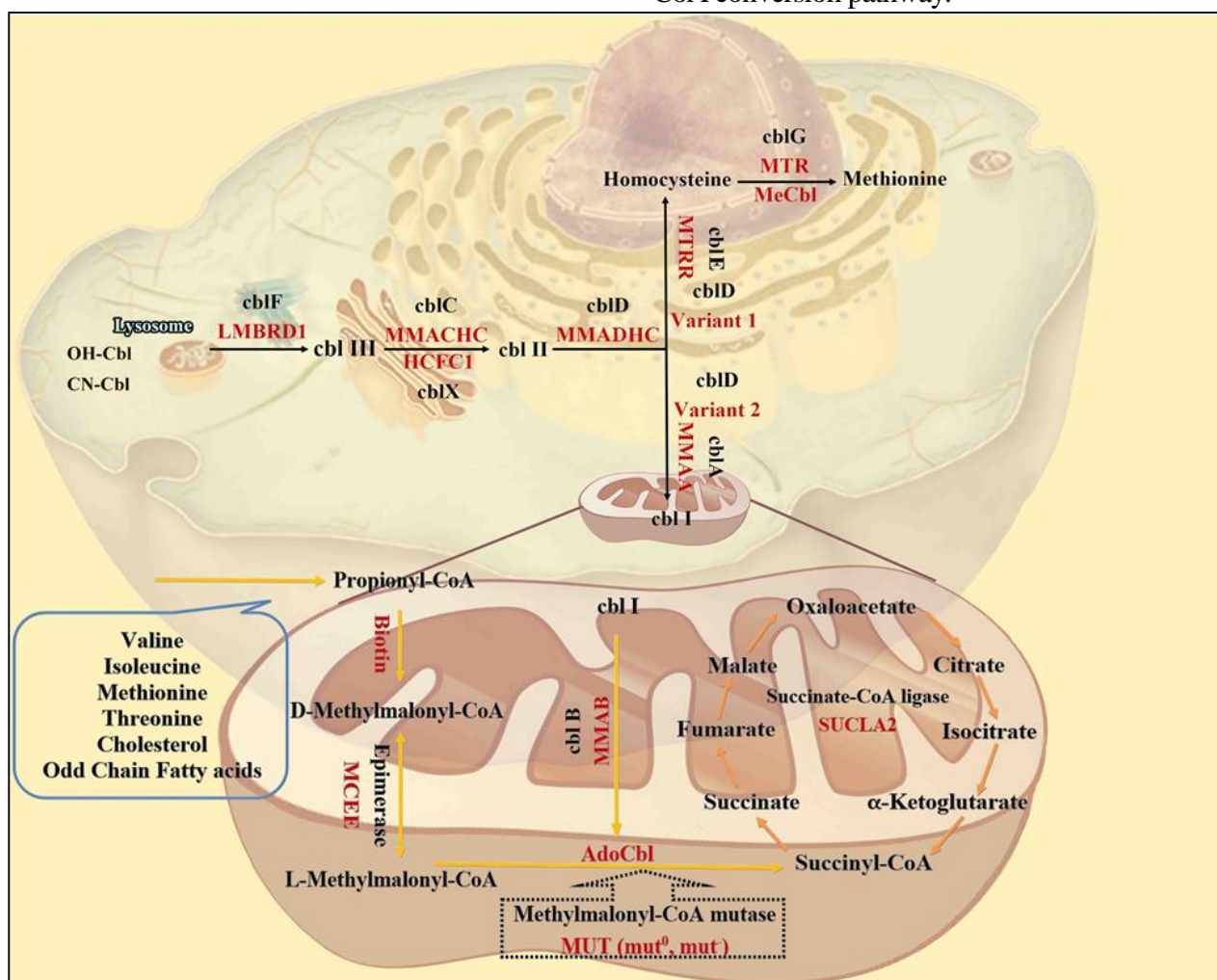
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complementation group cblD all contain homozygous or compound heterozygous mutations in the MMADHC gene, located on chromosome 2q23. The cblC type of combined MMA and homocystinuria is caused by homozygous or compound heterozygous mutations in the MMACHC gene located on chromosome 1p34. MMA and homocystinuria, cblC type, is the most common inborn error of cobalamin metabolism, with about 250 known cases (4). The cblF type is caused by homozygous or compound heterozygous mutations in the LMBRD1 gene on chromosome 6q13 (5). The cblX type is an X-linked, Xq28, recessive metabolic disorder that is caused by

mutations in the HCFC1 gene.

Also deficiency of the enzyme methylmalonyl-CoA epimerase and ADP-forming succinyl-CoA synthetase (SCS-A, EC 6.2.1.5) is found in patients with MMA. Deficiency of the enzyme methylmalonyl-CoA epimerase is caused by mutation in the MCEE gene on chromosome 2p13.3 and deficiency of the SCS is caused by mutation in the SUCLA2 gene on chromosome 13q14.2. SCS is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA. Fig. 1 shows the genes involved in the propionyl-CoA to succinyl-CoA conversion pathway.



**Fig. 1.** The propionyl-CoA to succinyl-CoA conversion pathway

Onset of the manifestations of isolated MMA ranges from the neonatal period to adulthood. All phenotypes demonstrate intermittent periods of relative health and metabolic decompensation, usually associated with intercurrent infections and stress. In the neonatal period the disease can present

with lethargy, vomiting, hypotonia, hypothermia, respiratory distress, severe ketoacidosis, hyperammonemia, neutropenia, and thrombocytopenia, and can result in death. In the infantile/non-B12-responsive phenotype, the most common form, infants are normal at birth but develop

lethargy, vomiting, dehydration, hepatomegaly, hypotonia, and encephalopathy. An intermediate B12-responsive phenotype occasionally presents in neonates, but usually presents in the first months or years of life; affected children exhibit anorexia, failure to thrive, hypotonia, and developmental delay, and sometimes have protein aversion and/or vomiting and lethargy after protein intake.

Atypical and "benign"/adult MMA are associated with increased, albeit mild, urinary excretion of methylmalonate; however, it is uncertain whether individuals with these conditions will develop symptoms. Major secondary complications of MMA include developmental delay (variable), tubulointerstitial nephritis with progressive renal failure, "metabolic stroke" (acute and chronic basal ganglia involvement), disabling movement disorder with choreoathetosis, dystonia and para/quadruparesis, pancreatitis, growth failure, functional immune impairment, and optic nerve atrophy. This review describes the biochemical and molecular genetics methods used to diagnosis isolated MMA.

### Diagnosis of MMA

Acidosis, ketosis, hyperammonemia, hypoglycemia, hyperglycemia, and neutropenia are symptoms of methylmalonic and propionic acidemia (PA). Laboratory findings suggestive of MMA and PA include low bicarbonate levels less than 22 mmol/l in infants and less than 17 mmol/l in neonates, ketones in the urine, blood ammonia levels greater than 150 µg/dl in neonates, 70 µg/dl in infants, and 35–50 µg/dl in older children and adults, blood glucose levels less than 40 mg/ml in infants and less than 60 mg/ml in children, and absolute neutrophil counts less than 1,500/mm<sup>3</sup> (6). Acylcarnitine levels in patients with any of the listed laboratory findings should be determined by tandem mass spectroscopy (MS/MS). Patients should also be screened for PA/MMA based on C3 and C3:C2 ratios. Patients with suspected disorders of cobalamin or propionate metabolism have C3 values greater than 7 µmol/l and C3:C2 ratios greater than 0.2; however, such screening cannot distinguish between MMA and PA. Table 1 shows the differential diagnoses of MMA and PA.

**Table 1.** Differential diagnosis of MMA and PA.

	Amino Acids pathway affected	Enzyme	Clinical Symptoms	Routine Laboratory Investigation	Plasma Amino Acids (Acylcarnitine profile)	Urine Organic Acids
<b>Methyl malonic Acidemia (MMA)</b>	Isoleucine, valine, methionine, threonine	Methylmalonyl CoA mutase	Acidosis, ketosis, hyperammonemia, hypoglycemia, hyperglycemia, and neutropenia	Low bicarbonate level, positive ketones in the urine, blood ammonia levels, low blood glucose levels	Methylmalonic acid in blood, Acyl carnitines, increased glycine in blood	Methylmalonic acid and methyl citrate in urine
<b>Propionic Acidemia (PA)</b>	Isoleucine, valine, methionine, threonine	Propionyl CoA carboxylase	Acidosis, ketosis, hyperammonemia, hypoglycemia, hyperglycemia, and neutropenia	Low bicarbonate level, positive ketones in the urine, blood ammonia levels, low blood glucose levels	Propionylcarnitine, increased glycine in blood	Propionic acid, 3-OH propionic acid, methyl citrate, propionyl glycine in urine

A definitive diagnosis of the disorder is based on urine organic acid analysis using gas chromatography/mass spectrometry (GC/MS) (6, 7). Organic acids can be measured using any body fluid, but urine is the most efficient for determining the type of disorder. The determination of organic acids and glycine conjugates in urine is key for the diagnosis and follow-up of MMA. Urine collected over 24 h allows for variations in volume excretion during the day. The impracticality of 24 h collection is, however, such that a random specimen, preferably the first morning voiding, an acceptable alternative. Intra-individual variations will occur

with respect to the time of sampling, the patient's clinical status and diet, and whether the sample is collected when the patient is fasted or fed. Sampling during fasting or metabolic decompensation is often considered to be most valuable because, in most cases, metabolites of interest are then excreted selectively or at higher concentrations. Two factors can increase excretion of organic acids; first, an increase in excretion may be nonspecific because some metabolites are reported to be abnormally excreted in conditions not attributable to IEM, such as drug therapy, diet, non-IEM diseases, or physiologic conditions. A second common

misinterpretation may arise from bacterial metabolism. Of possible endogenous origin, such as in intestinal infections, is the abnormal excretion of d-lactate, methylmalonate, p-hydroxyphenylacetate, p-hydroxyphenyllactate, glutarate, benzoate, and hippurate. A urinary organic acid profile is nearly always abnormal during the acute illness phase, but it is commonly barely detectable before or between crises; therefore, it is important to obtain a urine sample for testing during the peak of a crisis. Urine organic acid levels from patients with MMA contain relatively high concentrations of methylmalonic acid and methyl citrate, whereas urine from patients with PA will show relatively high concentrations of metabolites of propionyl CoA, including propionic acid, methyl citric acid, 3-OH propionic acid, and propionyl glycine (6). Table 2 shows methylmalonic acid concentrations in urine for different subtypes of MMA.

**Table 2.** Methylmalonic acid concentrations in urine for different subtypes of MMA.

Subtype	Methylmalonic acid concentration	
	Urine (mmol/mol creatinine)	Blood ( $\mu$ M)
<b>mut, mut<sup>0</sup></b>	1000-10000	100-1000
<b>cbIB, cbIA, cbID</b>	10-100	5-100
<b>MCEE deficiency, SUCLA2</b>	50-1500	7
<b>Normal</b>	<4	<0.27

Plasma amino acids in MMA contain carnitines, whereas those in PA contain propionylcarnitine. Both can have high glycine even when well controlled (6). Also, plasma homocysteine can be measured to identify gene types involved in MMA. After urine organic acid analysis and determination of plasma homocysteine concentration, patients are diagnosed based on one of the following criteria:

a) Patients with very high concentrations of methylmalonic acid in urine, but normal homocysteine, have mutations in at least one of the MUT (mut, mut<sup>0</sup>), cbIB, cbIA and cbID (var 2) subtypes. MMA subtypes are diagnosed by enzyme assay analysis and/or molecular studies. Molecular genetics techniques are available for carrier testing of family members to aid in reproductive decision

making and to determine whether prenatal testing is necessary (8).

b) Patients with slightly elevated methylmalonic acid in urine, but normal homocysteine, have mutations in at least one of the MCEE, SUCLA2 and benign MMA subtypes. Subtypes are diagnosed by enzyme assay analysis and/or molecular study.

c) Patients with abnormally high concentrations of methylmalonic acid in urine and homocysteine in plasma have mutations in at least one of the cbIC, cbIF, or cbID (var 1) subtypes. Subtypes are diagnosed by mutation analyses of MMACHC, MMADHC, and LMBRD1. Fig. 2 shows a flowchart for different stages of MMA diagnosis.

### 1. Acylcarnitine profile analysis

In a subset of newborn diseases with severe metabolic disorders, irreversible damage with adverse lifelong consequence may occur. For some of these diseases, a diagnostic method may help to prevent such damages. A critical screening technique used to detect many of these metabolic disorders in newborns is tandem mass spectrometry (MS/MS). MS/MS has the potential to simultaneously detect and quantify many metabolites with similar physicochemical properties. This constitutes a dramatic advance over the classical methods used for newborn screening. MS/MS has improved the detection of inborn errors of metabolism in newborns by making the analysis more sensitive, specific, and reliable than was previously possible. Its inherent ability to detect and quantify multiple metabolites in a single sample permits wide recognition of amino acid, fatty acid, and organic acid disorders.

Many patients have less severe symptoms if diagnosed and treated early. In addition, early diagnosis can decrease medical expenses and allow family planning to be considered before other affected siblings are born. Therefore, MS/MS can provide considerable benefits to patients and their families if integrated into newborn screening programs, provided that adequate funding is made available to cover the costs of the additional medications and foods. One of the disorders that can be diagnosed following MS/MS is MMA. The screening program identifies children that may be considered at risk for these disorders. Newborn screening for PA/MMA based on C3 and C3:C2

was begun in New York State in November 2004. The diagnoses are made at specialty care centers based on test results and evaluation by metabolic specialists. In May 2005, methylmalonyl carnitine

(C4DC) was added to the newborn screening panel as a secondary marker for PA/MMA. In some instances, samples were referred to rule out disorders of propionate metabolism on the basis of persistently elevated C4DC. Until 2008, a protocol was used in New York State that referred patients with suspected disorders of cobalamin or propionate metabolism with C3 concentrations greater than 7  $\mu\text{mol/l}$  and C3:C2 ratios greater than 0.2 (9).

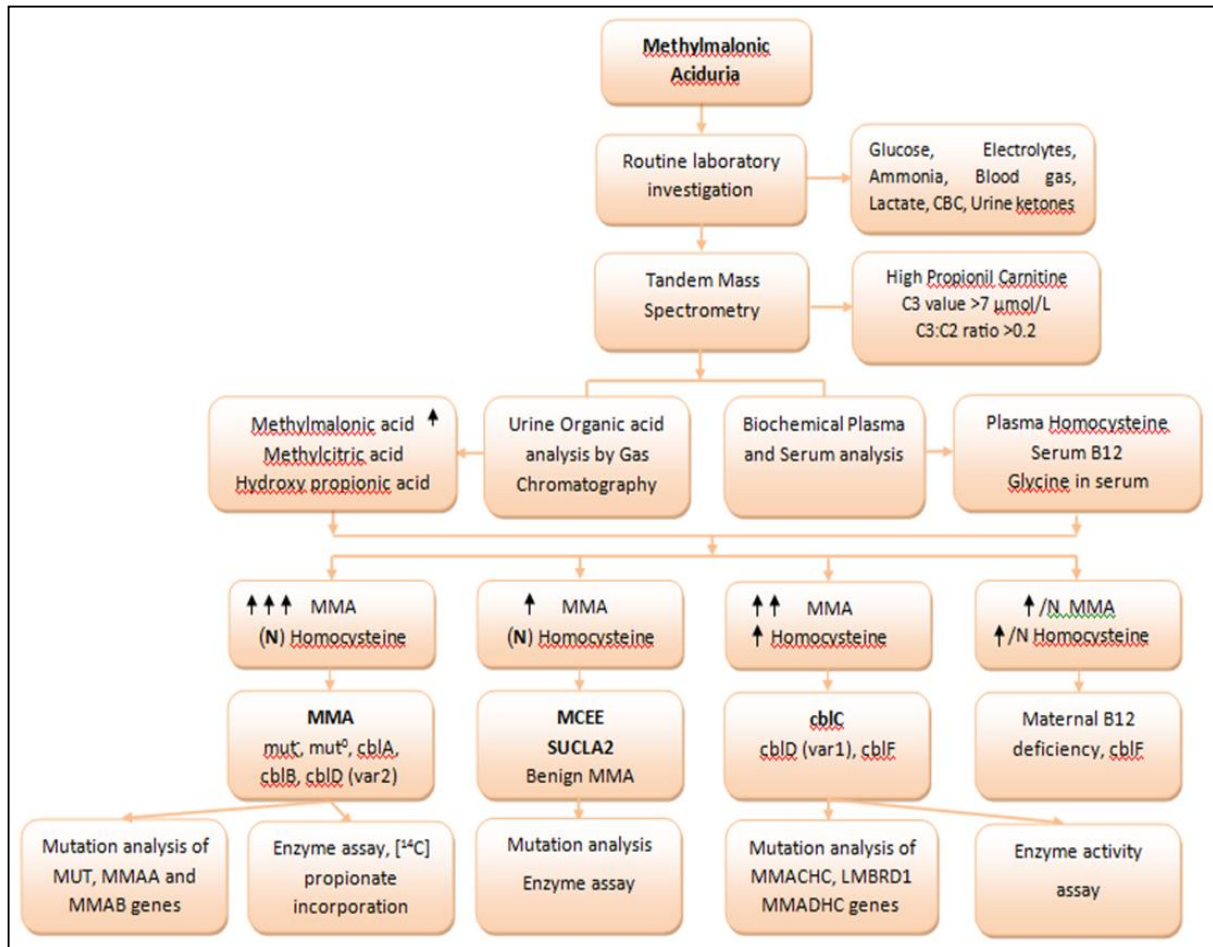


Fig. 2. A flowchart for different stages of MMA diagnosis

## 2. Organic acid analysis

In the hereditary diseases known as organic acidemia, an enzyme or co-factor defect in a metabolic pathway leads to the accumulation and increased excretion of one or more of these acidic metabolites in urine. Therefore, human urine contains numerous organic acids and other chemical compounds at a variety of concentrations (10) and urinary organic acid analysis has become an important tool for laboratories involved in the diagnosis of these inherited metabolic disorders. Gas chromatography (GC) is the technique of

choice to separate and identify more than 250 normal and pathological acidic metabolites detected in these diseases (11-13).

In 1963, Cox and White demonstrated an increase in urinary excretion of methylmalonic acid in patients with vitamin B12 deficiencies (14). Since then numerous methods have been described for the determination of urinary methylmalonic acid using colorimetry or GC (15, 16). Some of these methods utilize pre-purification of methylmalonic acid by thin-layer chromatography or ion-exchange resins in conjunction with calorimetric procedures or GC.



They were sensitive enough to measure a greatly increased amount of methylmalonic acid, but accurate determination of small amounts of methylmalonic acid in biological specimens has been difficult (17).

Millar *et al.* (1974) described an improved method based on gas-liquid chromatography (GLC) of the butyl ester of methylmalonic acid, which was produced by reacting a diethyl ether extract of urine or freeze-dried urine with a mixture of boron trifluoride and butanol. Therefore, methylmalonic acid was directly extracted from urine and measured as its butyl ester (18). Tanaka *et al.* (1980) described a practical gas-chromatographic method of urinary organic acid analysis that was designed to be used in organic acidemia screening programs. This method involves extraction of urine with ethyl acetate, dehydration of the extracted residues, trimethylsilylation, and use of the list of retention indices to identify the organic acids (19).

In 1981 Maties *et al.* modified Millar's method and adapted it for use with urine specimens absorbed into filter paper. The advantage of this method was that methylmalonic acid was quantitated with acceptable confidence from the small amount of urine present on filter paper specimens that were easily collected and mailed to a central testing laboratory. This technique was also applicable to the detection of other types of organic acidemias (20).

In 1983, Hyman *et al.* described a rapid method for MMA detection that utilizes DEAE-cellulose paper for sample collection and diazotized *p*-nitroaniline for color development. Using anion-exchange filter paper, urine organic acids are selectively adsorbed to the paper. The color reaction with diazotized *p*-nitroaniline, which takes place *in situ*, is more sensitive than a reaction in solution, and substances producing interfering colors with the reagent can be rinsed from the disc prior to the assay, making the assay more accurate (21).

Nakamura *et al.* (1987) described a method for microanalysis of short chain dicarboxylic acids including methylmalonic, succinic, and methylsuccinic acids, which consists of pre-fractionation of the dicarboxylic acid fraction by ion-exchange chromatography, extraction of the eluate with ethyl acetate, and analyses of dicarboxylic acids as dimethyl esters by GC. They

suggested that small amounts of these dicarboxylic acids in normal human urine, amniotic fluid, and serum can be accurately measured with this method (17).

Verhaeghe *et al.* (1988) developed a method that combines the specificity, reproducibility, and high extraction yield of anion-exchange chromatography with the speed and simplicity of solvent extraction using a gas chromatography-flame ionization detector (GC-FID) to measure urine organic acids. They suggested that this convenient procedure is selective, reproducible, and a suitable alternative to the more cumbersome diethylaminoethyl-Sephadex extraction method (10).

Hoffman *et al.* (1989) described a procedure for analysis for organic acids in various biological samples that incorporates the *O*-(2,3,4,5,6-pentafluorobenzyl) oxime-trimethylsilyl (*O*-PFBOxime-TMS) esters of oxoacids, aldehydes, and ketones. The gas-chromatographic properties of the *O*-PFEOxime-TMS esters have distinct advantages over the commonly used *O*-ethoxime-TMS esters; each is processed in a manner identical to that for aqueous standards, and requires no deproteinization. They suggested that there are no limitations on sample volumes and it is likely that cerebrospinal fluid and homogenized tissue samples can also be analyzed without further modifications. Proteins and peptides, as well as basic and polar low-molecular mass compounds, such as amino acids, inorganic acids, creatinine, purines, amines, sugars, and urea are retained on the silicic acid column. An additional advantage is that orotate and uracil, compounds valuable in distinguishing some urea-cycle disorders, are efficiently extracted (22).

One of the most critical points in a metabolic-profiling scheme is the isolation of the compounds of interest from the biological matrix. These should be extracted in high, uniform, and reproducible yields, accompanied by as few compounds as possible from other product classes. Because organic acids cover such a wide range of polarity and have different chemical properties related to the various functional groups present, this requirement has been difficult to meet. Moreover, for a procedure to be useful as a routine diagnostic method, one should be able to process several samples simultaneously with reasonable accuracy and speed.

Methods currently in use consist of isolating acidic constituents from urine before derivatization and GC. These include anion-exchange chromatography based on organic polymers or cellulose and solvent extraction with ethyl acetate or diethyl ether. The principal advantage of diethylaminoethyl-Sephadex column chromatography is its specificity and high and reproducible extraction recovery of polar and nonpolar acids. This method is strongly recommended for quantitative monitoring and for recognizing subtle changes in excretion profiles. Disadvantages of this method are that it is a laborious and complex procedure and the profile may be obscured by dominant peaks of some polar acids and inorganic sulfate and phosphate, which can mask some important organic acids in the gas chromatogram. These shortcomings impede diagnoses of metabolic diseases, especially in instances in which the increases in organic acid metabolites are small, as in vitamin-responsive organic acidopathies. Because of these practical difficulties, many groups have found solvent extraction to be an attractive approach for routine diagnoses of organic acidemias. However, solvent extraction is more widely used because it is fast and simple and yields an adequate recovery of aromatic and less-polar aliphatic acids with minimal co-extraction of sulfate and phosphate (10).

Solvent extraction has serious limitations imposed by the low and unreproducible extractability of polar acids such as 3-hydroxyisovalerate, 3-hydroxypropionate, methyl citrate, and citrate (19).

In addition, an organic acid profile enables one to evaluate metabolic disorders pathobiochemically on the basis of their relations to one another as precursors or products. Therefore, careful consideration of small changes in organic acid ratios is essential for accurate diagnosis and optimal management for prognosis after treatment in addition to quantitative determination of organic acids. GC-MS is more specific, in that quantification is based on the relative intensities of characteristic fragment ions in a reconstructed ion chromatogram. Mass chromatography usually yields lower precision and sensitivity than GC-FID detection, and single- or multiple-ion monitoring can be used to quantify only a few target

compounds. With GC-FID detection, on the other hand, compounds that are 100- to 1000-fold less concentrated than the major components of the sample can still be quantified (10). GC/MS requires expensive instrumentation, and maintenance, operation, and data interpretation require highly-specialized training and technical expertise. In addition, a computer is almost indispensable for data processing. Thus, organic acidemia has been screened in only a few major medical centers, where such instruments and expertise are available.

In conclusion, at present, solvent extraction with ethyl acetate, diethyl ether, or both, is widely used. This type of liquid extraction yields poor analytical recoveries of the more-polar compounds and is inconvenient for use with large numbers of samples. Also, GC-MS has become a well-established, easily automated, and reliable technology in the research field of metabolomics (23). Human urine contains many metabolites, and GC-MS analysis of urinary organic acids is an important technique for the diagnosis of inborn errors of lipid, amino acid, and carbohydrate metabolism (24). By means of urease pretreatment of urine samples and other methodological improvements, GC-MS has been applied to simultaneously analyze the numerous metabolic intermediates of multiple categories in urine, providing diagnostic evidence for more than 130 inborn errors of metabolism (IEM) (25- 27).

### 3. Enzyme activity assay

MMA may be diagnosed by measuring MCM activity, with or without the addition of AdoCbl. This can be used to distinguish between two MMA variants (Cbl-responsive and Cbl-unresponsive) and distinguish between two MUT subtypes (mut<sup>-</sup> and mut<sup>0</sup>). Thus, the *in vitro* measurement of MCM activity, with and without AdoCbl, is useful to investigate the Cbl pathway, diagnose MMA, identify MUT and cbl mutations, and gain insight into the biochemical changes accompanying vitamin B12 deficiencies. Methods described and employed to measure MCM activity include radiometric methods, in which [14C] succinyl-CoA is produced and separated from the substrate DL[CH<sub>3</sub>-14C]methylmalonyl-CoA by paper chromatography (28, 29), thin layer

chromatography (30, 31), electrophoresis (32), potassium permanganate oxidation (33, 34), microwell filtration (35), extraction into ethyl acetate (36), high performance liquid chromatography (HPLC) (37) and GC (38). There are also nonradioactive assays based on the separation of methylmalonyl-CoA and succinyl-CoA by reverse-phase HPLC (39, 40), or on the direct spectrophotometric assay of succinyl-CoA (41- 43). The first six methods are reputed to be laborious because they require many manipulations, and time-consuming because of the numerous incubations, and they have been criticized for their lack of sensitivity. The permanganate oxidation method is also criticized because the optimal conditions for oxidation vary depending on the permanganate concentration and heating time. The gas chromatographic radiometric assay method appears to be sensitive, but is also time-consuming. The lack of sensitivity and reproducibility, and the inconvenience of the radiometric assay for MCM activity make HPLC the method of choice. Therefore, the nonradioactive HPLC assay seems to be satisfactory for measuring the conversion of small fractions of methylmalonyl-CoA to succinyl-CoA, and is said to be simple, rapid, reliable, and highly reproducible. This method is sufficiently sensitive to measure low MCM activity, such as the holo-MCM activity in tissue extracts, or the total MCM activity of cells. We therefore believe this method is suitable for detecting abnormal MCM apoenzyme, whether to diagnose MMA or detect errors of cobalamin metabolism (44).

#### 4. Mutation analysis

##### *MUT*

MMA is an inborn error of metabolism due to the impaired isomerization of L-methylmalonyl-CoA to succinyl-CoA. This reaction is catalyzed by the mitochondrial protein MCM, an adenosylcobalamin-dependent enzyme (45). The human MUT gene, located on chromosome 6, is comprised of 13 exons spanning over 35 kb. The open reading frame consists of 2.7 kb, encoding 750 amino acids (46). Two classes of mutations in MUT are classically distinguished by studies of [<sup>14</sup>C]-propionate metabolism in primary fibroblasts from patients with MMA (47). Mut<sup>0</sup>

mutations result in no detectable MCM activity. Mut<sup>-</sup> mutations result in low residual enzyme activity. The human MUT gene was identified by Ledley *et al.*, who screened an expression library with mutase antibodies to isolate the first human cDNA (48). Over the last 27 years a number of studies have described the spectrum of mutations observed at the MUT locus in human patients (45, 49- 57). To date, 272 different mutations have been identified, including 187 missense/nonsense mutations, 24 splice-site mutations, 37 small deletions, 20 small insertions, three small indels, and one gross deletion.

##### *cbIC*

MMA, cobalamin deficiency type C (*cbIC*) with homocystinuria (MMACHC gene) is the most common genetic defect in cobalamin metabolism (4, 58). The MMACHC gene responsible for *cbIC* disorder is located on chromosome 1p34.1 and encodes a polypeptide of 282 amino acids. Exons 1-4 are coding and exon 5 is non-coding. The MMACHC protein may act as an intracellular cobalamin-trafficking chaperone and has been shown to act, in part, catalyzing the reductive decyanation of cyanocobalamin, generating cob(II)alamin, which is the substrate for assimilation into the active cofactor forms methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) (59). To date, mutation analyses of MMACHC (60, 61) have shown 81 different mutations, which include 43 missense/nonsense mutations, five splice site mutations, 19 small deletions, eight small insertions, one small indel, four gross deletions, and one gross insertion.

##### *cbIA*

The *cbIA* type is caused by mutations in the MMAA gene on 4q31. MMAA is involved in the synthesis of adenosylcobalamin (AdoCbl) (2). Two different enzymatic functions have been identified for the MMAA gene product: a role in vitamin B12 transport into the mitochondria, reduction of cobalamin II to cobalamin I, and the conservation or re-activation of MCM. Multiple mutations in various regions of the gene have been identified, which will help guide future structure and function studies (62, 63). The mutations includ



30 missense/nonsense mutations, four splice site mutations, five small deletions, five small insertions, and one gross deletion.

### ***cb1B***

In *cb1B* type of MMA, the defective gene is *MMAB*. The *MMAB* gene, on chromosome 12q24.1, encodes the mitochondrial enzyme ATP: cobalamin adenosyl transferase (ATR), which catalyzes transfer of an adenosyl group from ATP to cobalamin (I) to form AdoCbl (3). Mutation analysis of the *MMAB* gene identified 20 missense/nonsense mutations, seven splice site mutations, two regulatory mutation, five small deletions, three small insertions, and one small indel (63, 64).

### ***MCEE***

D-methylmalonyl-CoA (D-MMCoA) is formed as a product of the propionyl-CoA carboxylase reaction. D-MMCoA requires racemization prior to becoming a substrate for the MCM reaction, and a deficiency of D-methylmalonyl-CoA racemase (MCR, EC 5.1.99.1) has long been postulated as a potential etiology of hereditary MMA (65). The epimerase gene (*MCEE*) on chromosome 2p13.3 was the first cobalamin-related gene to be identified on the basis of prokaryotic gene arrangements (66). To date, three missense/nonsense mutations have been identified.

### **Other subtypes**

At least four other genetic entities pathways can be associated with isolated MMA. *cb1D* types are caused by homozygous or compound heterozygous mutations in the *MMADHC* gene, found on chromosome 2q23; these include eight missense/nonsense mutations, two small deletions, and three small insertions (67). Function of the product of this gene remains unknown; it shows homology to the putative ATPase component of a bacterial ABC transporter. Mutations in the C-terminal region were identified in patients with *cb1D* variant 2, mutations in the N-terminal region were identified in patients with *cb1D* variant 1, and

truncating mutations were associated with the classic *cb1D* phenotype. This supports suggestions that the *MMADHC* gene product plays a role in directing cobalamin to the 2 cobalamin-dependent enzymes of mammalian cells, Methylmalonyl CoA mutase and Methionine synthase (68).

*cb1F* type is caused by homozygous or compound heterozygous mutations in the *LMBRD1* gene, found on chromosome 6q13. This gene produces a lysosomal cobalamin transporter protein that facilitates lysosomal cobalamin export (69). To date, no mutations have been reported for this gene. The *SUCLA2* gene encodes the beta-subunit of the ADP-forming succinyl-CoA synthetase (SCS-A; EC 6.2.1.5). SCS is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA. The reverse reaction occurs in the Krebs cycle, while the forward reaction may produce succinyl-CoA for activation of ketone bodies and heme synthesis. To date, ten missense/nonsense mutations, one splice site mutation, one small insertion, one gross deletion and one small indel have been identified (70). The *cb1X* type is an X-linked (Xq28) recessive metabolic disorder characterized by severely delayed psychomotor development apparent in infancy and is caused by mutations in the *HCFC1* gene. Mutation in *HCFC1* gene inhibits its function in the transcriptional activation of *MMACHC* gene and showed that disorder of transcription can cause an inborn error of metabolism (71). To date, no mutations have been reported for this gene. Gene subtypes and mutations involved in MMA are shown in Table 3.

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**Table 3.** Subtypes and mutations in genes involved in MMA (HGMD data base)

Subtype	Gene	Mutation	Number of mutation	Percentage
<b>Unresponsive to vitamin B12</b>	MUT	Missense/nonsense	187	<b>58.37</b>
		Splice site mutation	24	
		Small deletion	37	
		Small insertion	20	
		Small indel	3	
		Gross deletion	1	
<b>cbIA</b>	MMAA	Missense/nonsense	30	<b>9.65</b>
		Splice site mutation	4	
		Small deletion	5	
		Small insertion	5	
		Gross deletion	1	
<b>cbIB</b>	MMAB	Missense/nonsense	20	<b>8.15</b>
		Splice site mutation	7	
		Regulatory	2	
		Small deletion	5	
		Small insertion	3	
		Small indel	1	
<b>cbIC</b>	MMACHC	Missense/nonsense	43	<b>17.39</b>
		Splice site mutation	5	
		Small deletion	19	
		Small insertion	8	
		Small indel	1	
		Gross deletion	4	
		Gross insertion	1	
<b>cbID</b>	MMADHC	Missense/nonsense	8	<b>2.79</b>
		Small deletion	2	
		Small insertion	3	
<b>SUCLA2</b>	SUCLA2	Missense/nonsense	10	<b>3.01</b>
		Splice site mutation	1	
		Gross deletion	1	
		Small indel	1	
		Small insertion	1	
<b>cbIF</b>	LMBRD1	Not reported	-	<b>0</b>
<b>cbIX</b>	HCFC1	Not reported	-	<b>0</b>
<b>MCEE</b>	MCEE	Missense/nonsense	3	<b>0.64</b>

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