UNIVERSITAT ROVIRA i VIRGILI

Increasing of secondary cobalamin deficiencies in newborns in Catalonia in the last eight years

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FINAL DEGREE PROJECT

BIOCHEMESTRY AND MOLECULAR BIOLOGY DEGREE

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I would like to sincerely show my gratitude to Dr. Rodríguez Frías for giving me the opportunity to do an internship at the Vall d'Hebron Hospital.
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"Let food be thy medicine, thy medicine shall be thy food" – Hippocrates 460-370 a.C.

Abstract

Introduction: Cobalamin, also called vitamin B12, is a water-soluble vitamin crucial for protein metabolism. Cobalamin deficiency is one of the most common metabolic disorders in humans, especially in newborns. It can be classified as a primary deficiency -due to the genetic alteration of the enzymes that catalyze the absorption, transport, or metabolization from vitamins to active cofactors-, or as a secondary deficiency -due to dietary deficiencies-. Many of the secondary deficiencies in newborns have their origin in an inadequate maternal diet and are transitory.

Hypothesis: Inadequate maternal nutrition, specifically the lack of aliments of food of animal origin, increases the number of acquired cobalamin deficiencies in newborns in recent years.

Objectives: To determine the incidence of cobalamin disorders and their biochemical alterations over the years in the reported cases in Vall d'Hebron Hospital, Barcelona, Catalonia, Spain, Europe.

Methodology: The heel prick test, a newborn screening test, has been performed on 532,410 newborns, in Hospital Clinic, Barcelona, during the period 2013-2020. Automatized immunoassay methods and indirect methods to analyze the metabolites resulting from cobalamin deficiency were applied only to 606 patients, who tested positive for the primary markers on the heel prick test. Half of these patients with altered biomarkers are referred to Vall d'Hebron Hospital Metabolic Diseases Unit, while the other half are referred to Sant Joan de Déu Hospital, Barcelona.

Results: The secondary cobalamin deficiencies have increased above the years in newborns in Catalonia, but not the primary genetic deficiencies. Only 25-35% of mothers from newborns with secondary deficiencies have a metabolic alteration, originated from inadequate nutrition, related to secondary cobalamin deficiency in newborns.

Conclusion: Secondary cobalamin deficiencies in newborns are steadily increasing in Catalonia from 2013 until the present. Despite the fact that 25-35% of mothers have a biochemical alteration related to cobalamin, 65-75% of cobalamin deficiencies in newborns cannot be explained. For that reason, the hypothesis cannot be confirmed nor rejected.

Discussion: Early detection in neonatal screening programs has substantially increased the efficacy in the clinical management of positive cases, including the secondary vitamin B12 deficiencies, that is consistent with previously published results (1). It is advisable to monitor maternal cobalamin status during pregnancy to prevent newborn cobalamin deficiency.

Key words: Vitamin B12, cobalamin deficiency, methylmalonic acid, homocysteine, newborn screening.

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Abbreviations

AdoCbl: adenosylcobalamin

C2: acetylcarnitine

C3: propionylcarnitine

C16: palmitoylcarnitine

C17: heptadecanoylcarnitine

Cbl: cobalamin

CblA: MMAA-related adenosylcobalamin deficiency

CblB: MMAB-related adenosylcobalamin deficiency

CblC: MMACHC-related adenosylcobalamin and methylcobalamin deficiency

CblD: MMACHD-related adenosylcobalamin and methylcobalamin deficiency

CblE: MTTR-related methylcobalamin deficiency

CblF: LMBRD1-related methylcobalamin deficiency

CblG: MTR-related methylcobalamin deficiency

CBS: cystathionine Beta-synthase

DBS: dried blood spots

FI: intrinsic factor

GC-MS: gas chromatography-mass spectrometry

Hcys: homocysteine

MCA: methylcitric acid

MCM: methylmalonyl CoA mutase

MeCbl: methylcobalamin

Met: methionine

MMA: methylmalonic acid

MMAA: methylmalonic aciduria type A protein gene

MMAB: methylmalonic aciduria type B protein gene

MMACHC: methylmalonic aciduria and homocystinuria type C protein gene

MMACHD: methylmalonic aciduria and homocystinuria type D protein gene

MTRR: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase gene

LMBRD1: Lysosomal cobalamin transport escort LMBD1 protein gene

MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase gene

MMUT: methylmalonyl-CoA mutase protein gene

MTHF: methyltetrahydrofolate

MTHFR: methyltetrahydrofolate reductase

NBS: expanded newborn screening

PA: propionic aciduria.

Ser: serine

SUCLA-2: beta-subunit of the ADP-forming succinyl CoA synthetase deficiency.

TC: transcobalamin II

TCR: transcobalamin receptor

THF: tetrahydrofolate

1. Introduction and background

Cobalamin (Cbl), also named vitamin B12 ($C_{63}H_{88}CoN_{14}O_{14}P$, molecular weight 1,355 kDa) is a coordination complex of cobalt surrounded by 4 pyrrole rings, forming an almost planar macrocyclic group. The cobalt atom has 6 coordination valences, 4 of which establish covalent bonds with the corresponding nitrogen of the pyrrole rings (see **figure 1**) (2). It is a water-soluble vitamin that can be actively synthesized by some intestinal bacteria in the human body. However, its absorption is minimal, because the synthesis site (colon) is located beyond the absorption site, which causes its elimination by feces. Consequently, this vitamin must be taken from the diet, and the only source is animal foods (3,4).

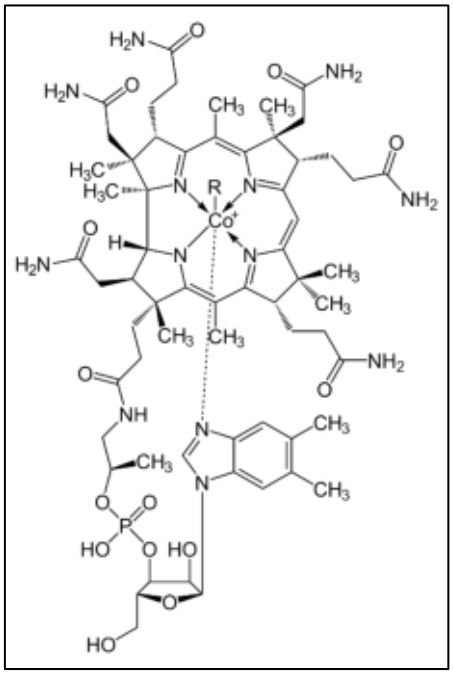

Figure 1. Structure of the cobalamin molecule. Taken from (2).

The main food sources of Cbl are shown in **table 1**. Usually, meat animal foods have higher quantities of Cbl than non-meat animal foods.

Table 1. Quantity of cobalamin in animal foods. Adapted from (5)

Food	Quantity of cobalamin (µg/100 gr)
Beef liver (raw)	81
Canned sardine	29.6
Iberian ham	15.7
Foie gras	15
Chicken (fried)	8.13
Rabbit (cooked)	9.8
Tuna (baked)	8
Tuna (griddle)	5
Cheese	0.66-2.6
Chicken egg (cooked)	1.7

The recommended quantity of Cbl may vary depending on the life stage of the people (see **table 2**).

Table 2. Diary recommended quantity of cobalamin during the human life stages. Adapted from (6).

Life stages	Recommended quantity (µg/day)
Infants from 7 to 11 months	1.5
Children from 1 to 6 years	1.5
Kids from 7 to 10 years	2.5
Kids from 11 to 14 years	3.5
Teenagers from 15 to 17 years	4
Adults	4
Pregnant mothers	4.5
Nursing mothers	5

There are two biologically active forms of vitamin B12 depending on the functional group bonded to the sixth coordination site, that catalyzes two different reactions. Vitamin B12 participates in the first reaction in the form of **methylcobalamin** (MeCbl), which is a cofactor of the cytoplasmatic enzyme methionine synthase. And the second reaction implies the **adenosylcobalamin (AdoCbl)**, which is the cofactor of the mitochondrial enzyme methylmalonyl-coenzyme A mutase (3,7,8).

1.1. Cobalamin metabolism

The transformation of cobalamin (cyanocobalamin or hydroxycobalamin) to the active coenzyme form requires three stages: **intake**, **abortion**, and **metabolization** (8).

The absorption step starts with the linking of diet cobalamin to the protein **haptocorrin,** secreted from the salivary glands, which protects the molecules from the low pH of the stomach. The cobalamin-haptocorrin complex is degraded by the pancreatic enzymes in the duodenum, releasing free cobalamin. Then, the cobalamin links to the **intrinsic factor (FI)** secreted in the stomach. **Cubam** receptor facilitates both intestinal absorption and renal reabsorption of IF-Cbl complex (8).

Once cobalamin reaches the bloodstream, it binds to **transcobalamin II (TC)**, which allows the transport inside of the cell through a specific membrane receptor. The TC-Cbl complex is endocytosed to the lysosome, and free cobalamin is released from it (4).

Cbl enters the cytosol where its central cobalt atom is reduced from 3+ to 2+ oxidation state by simultaneous steps, and then converted in wither MeCbl by methionine synthase reductase in the cytosolic compartment, or AdoCbl by enzymes involved in the synthesis and transfer to methylmalonyl-CoA mutase in the mitochondrial matrix (see **figure 2**) (4,7).

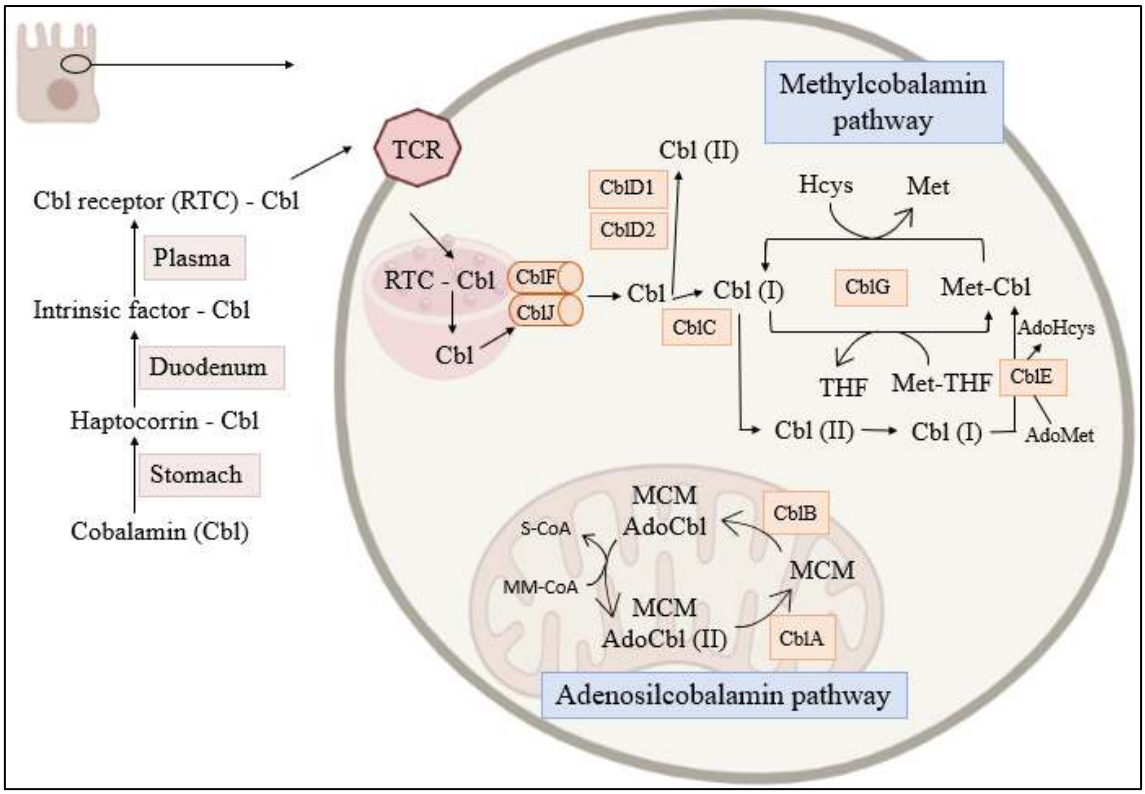

Figure 2. **Cobalamin endocytosis and intracellular metabolism**. AdoMet: adenosylmethionine; AdoHcys: adenosylhomocysteine MMCoA: methylmalonyl CoA; S-CoA: succinyl CoA. See text for details. Adapted from (8).

1.2. Cobalamin related diseases

Cobalamin disorders can be primary or secondary. A primary disorder is that produced in the cell (genetic, for instance, methylmalonic aciduria type A protein gene (*MMAA*)), while the secondary disorder is that which is not produced in the cell but affects it (for instance, poor nutritional intake).

Cobalamin primary disorders include all the inborn diseases in the absorption, transport and intracellular cobalamin metabolism disorders. Cobalamin diseases might also be caused secondarily, without any genetic affectation mostly due to a poor intake of animal foods, and to a lesser extent by decreased intestinal absorption (8).

1.2.1. Secondary cobalamin disorders

The most probable cause of a secondary cobalamin disorder is nutritional vitamin B12 deficiency. This may happen in adults who have a restriction of natural protein intake and do not ingest vitamin supplements, such as vegan diet, or due to cultural-historical influences, like in certain Asian populations (9). Newborns may also develop a secondary deficiency of maternal origin, commonly known as **acquired deficiency** (9,10). Another cause may be alterations in gastric mucosa causing malabsorption of cobalamin (9).

As a result, the clinical manifestations produced by the secondary Cbl deficiency are heterogeneous and include a spectrum that ranges from asymptomatic to clinical manifestations, such as anemia, failure to thrive, muscular atrophy, and even irreversible neurologic damage if the deficiency is prolonged. These diseases can be prevented by intaking more natural proteins or with the ingest of vitamin supplements (10,11).

1.2.2. Primary cobalamin disorders

Primary disorders refer here to congenital or hereditary disorders. The term **congenital** (or inborn) **error of metabolism** was introduced in 1908 by Archibald E. Garrod. This concept has evolved and nowadays is used to encompass those hereditary diseases that are the consequence of biochemical alterations of genetic origin in the structure or function of a protein with metabolic function (11).

Inborn errors of metabolism are a heterogeneous group of rare disorders which involve defects of the metabolic pathways of the break-down or storage of carbohydrates, lipids, proteins and nucleic acids, and its monomeric components (monosaccharides, fatty acids, amino acids, nucleotides) (8,11).

They can be classified into different groups: **(A)** disorders of intermediary metabolism, as organic acidurias and beta-oxidation defects, **(B)** disorders involving complex molecules (macromolecules) and their storage, as glycogenosis or lysosomal storage disorders, and **(C)** disorders involving energy metabolism, either mitochondrial or cytoplasmatic defects (11).

Cobalamin absorption and transport can be caused by a primary disorder (8). The most studied, frequent and severe disorders are intracellular cobalamin metabolism disorders. Based on the biochemical phenotype and genetic analysis, they have been classified as Cbl mutants. Among them, the most known are the **adenosylcobalamin deficiency** –MMAA-related adenosylcobalamin deficiency (CblA), MMAB-related adenosylcobalamin deficiency (CblB)–, **methylcobalamin deficiency** –MTTR-related methylcobalamin deficiency (CblE), MTR-related methylcobalamin deficiency (CblG) and the **combined deficiency** of both cofactors –MMACHC-related adenosylcobalamin and methylcobalamin deficiency (CblC), MMACHD-related adenosylcobalamin and methylcobalamin deficiency (CblD), LMBRD1-related methylcobalamin deficiency (CblF)– (3).

Consanguinity should be considered due to the greater recurrence of some pathogenic variants. Moreover, having descendants from a common ancestor increases the possibility of homozygosity, which means, to have two identical pathogenic variants (4,8).

1.2.2.1. Methylcobalamin deficiency

As mentioned above, MeCbl is the cofactor of the **methionine synthase**, a **cytosolic** enzyme that catalyzes the methylation from homocysteine to methionine (3,8).

The MeCbl deficiency leads to a functional deficiency of **folate**. This affects the rapidly dividing cells of the bone marrow, which require increased thymidylate for DNA synthesis, eventually leading to macrocytic anemia. For that reason, patients suffer

neurological diseases, apart from poor feeding, vomiting, failure to thrive, developmental delay, nystagmus, hypotonia or hypertonia, ataxia, seizures, and blindness. Most newborns are symptomatic in the 1st year of life, and many of them have a poor outcome (8).
When the formation of methylcobalamin is disturbed, the concentration of homocysteine increases to pathological levels (3). Isolated homocystinuria is always found in the absence of methylmalonic acidaemia (7).
Homocysteine is a non-essential surfed amino acid resulting from the essential process of methylation. The main donor of methyl groups for these processes is methionine. After this donation, methionine finally results in homocysteine. That amino acid is found in the transulfurization and remethylation ways (7):

(i) Homocysteine can be **remethylated to methionine** by two different pathways. The most important one is catalyzed by the **methionine synthase**, which uses the **methyltetrahydrofolate (MTHF)** as substrate, and is activated by the **methionine synthase reductase**. That reaction requires the **MeCbl** as a coenzyme (2).
The homocystinuria can be caused by a functional deficit of **methionine synthase** (as in CblE disease), caused by the 5-methyltetrahydrofolate-homocysteine methyltransferase reductase gene (***MTRR***). Moreover, homocystinuria can be caused by the CblG disease, which disturbs the formation of **methylcobalamin** (8).
In **CblG** disease the methionine synthase deficiency is due to a mutation that affects the apoenzyme, while in **CblE** disease the mutation affects the gene that codifies for the methionine synthase reductase (8).

(ii) In the transulfurization pathway (see **figure 3**) homocysteine is transformed to cystathionine by the **cystathionine beta-synthase (CBS)** and the cofactor **pyridoxal-phosphate**, the activator of the S-adenosylmethionine in many tissues. Cystathionine is subsequently transformed into cysteine, which may be a precursor of glutathione or taurine. Finally, the sulfide group from cysteine, as well as cysteine itself, is oxidized and excreted by urine as sulfate form (9).

Classic homocystinuria is caused by **CBS deficiency** and it leads to toxic concentrations of **homocysteine (Hcys)** (7,8).
The eye, skeleton, central nervous and vascular system are all involved in the homocystinuria typical presentation. The patient is usually normal at birth, but progressively develops to dislocation of the ocular lens, myopia and glaucoma, osteoporosis, developmental delay and mental retardation, and thromboembolic complications (7).

When the CBS enzyme is disturbed, it leads to **tissue accumulation of methionine and homocysteine and their S-adenosyl derivatives**. In addition, the -SH group of two homocysteine reacts between them forming the **homocystine** (two homocysteines linked

by a sulfur bridge) or with the -SH group of other molecules, leading to the formation of disulfide compounds, another reason to find low levels of cysteine (8).

Homocistinuria is a group of congenital errors of homocysteine metabolism. It is characterized by high concentrations of homocysteine in plasma and urine, and it is a consequence of an enzymatic deficiency in homocysteine metabolism (7).

The aim of the treatment for homocystinuria is to reduce plasma total homocysteine to normal or high-normal concentration. About half of all patients with CBS deficiency respond partially to large oral doses of **pyridoxine**. In addition, folic acid, and vitamin B12 might be added to the treatment. Nevertheless, nowadays the best treatment for homocystinuria is the intake of **betaine** to promote a secondary pathway that transforms the homocysteine in methionine (7,8).

Thromboembolic complications are due to high levels of Hcys alters the balance of clotting factors, damaging the walls of blood vessels, and the defects in the coagulation system are a risk factor to cause vascular accidents. That symptom constitutes the major cause of morbidity and mortality and is highly frequent in all severe forms of homocystinuria (plasma total homocysteine > 100 µmol/L) (7).

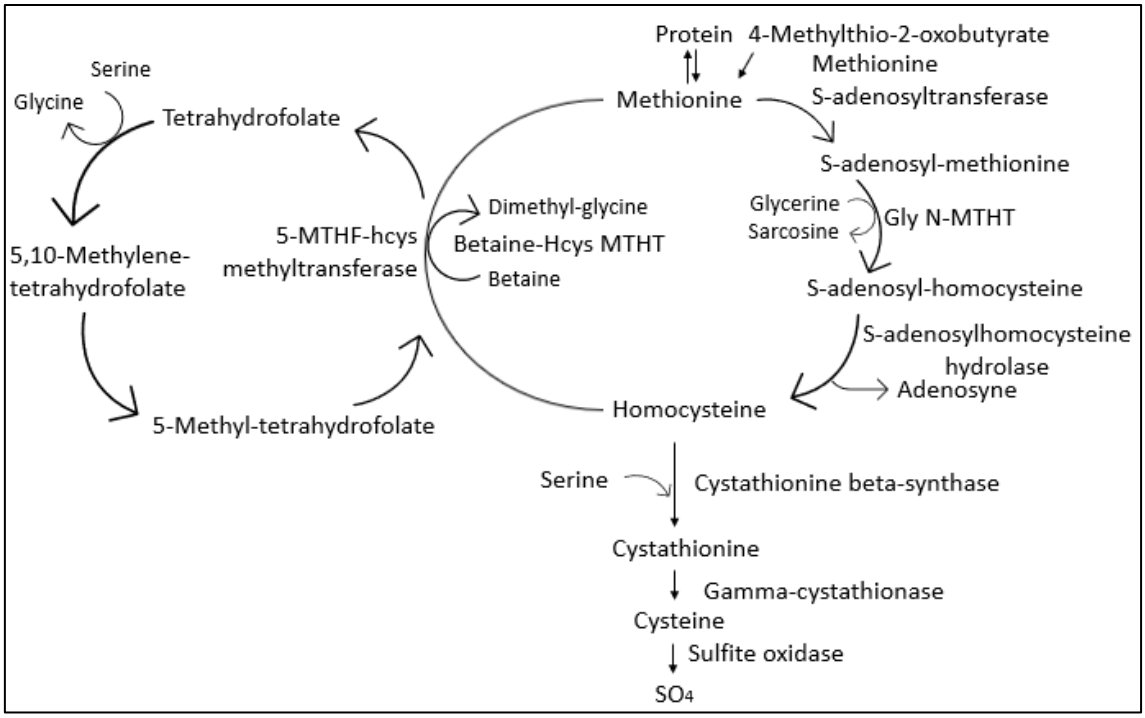

Figure 3. **Metabolism of the sulfur-containing amino acids.** MTHT: methyltransferase. See text for details. Adapted from (7).

1.2.2.2. Adenosylcobalamin deficiency.

The deficiency of AdoCbl comprises **CblA** and **CblB** disorders, characterized by methylmalonic aciduria, vomiting, dehydration, tachypnoea, lethargy, failure to thrive, developmental retardation, hypotonia and encephalopathy.

Usually, total serum Cbl is normal, and urinary methylmalonic acid levels are elevated, but there is no increase of plasma total homocysteine (3).

The mutation in the **methylmalonic aciduria type A protein gene** (***MMAB)*** causes the CblB disease. This disorder is characterized by isolated methylmalonic aciduria (MMA) which is caused by an adenosylcobalamin (AdoCbl) deficiency (3).
The *MMAB* gene encodes for the cobalamin adenosyl-transferase, which catalyzes the final step in intramitochondrial synthesis of the cofactor of **methylmalonyl CoA mutase**, the adenosylcobalamin (9).

On the other hand, the defect in **CblA** is caused by a mutation in the ***MMAA*** gene. It encodes a polypeptide that belongs to the G3E family of GTP-binding proteins (3) and is involved in the transfer of AdoCbl from adenosyltransferase to methylmalonyl-CoA mutase and maintaining mutase-bound AdoCbl in its active form (8).
To distinguish between them is necessary a **genetic or fibroblast study**, because in both cases the activity of methylmalonyl-CoA mutase is normal in presence of added AdoCbl (3).
Most of the patients respond to protein restriction and hydroxocobalamin treatment. However, some patients appear to become resistant to Cbl supplements (4,8).

In this type of disease, hydrocarbon chains with an odd number of carbons, such as: **(A)** branched-chain amino acids valine, leucine, and isoleucine, **(B)** pyrimidine, such as uracil and thymine, **(C)** cholesterol, and **(D)** odd fatty acids, results finally in **Propionyl CoA** (3C) as the end degradation product, which is very toxic in high levels because can mimic acetyl-CoA in some essential enzymes (7).

Propionyl CoA (3C) is naturally metabolized by carboxylation with **propionyl CoA carboxylase** and **biotin** resulting in **methylmalonyl CoA**, which is a ramified molecule of 4 carbons. Through an additional isomerization process by the **methylmalonyl CoA mutase (MCM)** and **B12 cofactor adenosylcobalamin (AdoCbl)**, it becomes **succinyl CoA**, which is a linear molecule of 4 carbons that can enter directly in the Krebs cycle pathway (9).
If the enzyme propionyl CoA carboxylase or the biotin does not work correctly, propionyl CoA accumulates in the cytoplasm and condenses with the **oxalacetate** from the Krebs cycle to form **methylcitric acid**, of 7 carbons, instead of citric acid, of 6 carbons. It would create an obstruction in the Krebs cycle and the production of by-products (see **figure 4**). On the other hand, its detection allows the diagnostic and follow-up studies.
If the enzyme methylmalonyl CoA mutase has any alteration, methylmalonyl CoA levels will be incremented, which results in **methylmalonic acidemia** (8).

Methylmalonic acidemia can also be caused due to MCM deficiency. It involves another possible mutation in the **methylmalonyl-CoA mutase protein gene *(MMUT*)**. The symptoms and treatment are the same. Nonetheless, the patients used to be resistant to hydroxocobalamin (3,7).

The **methylmalonyl CoA mutase** is a mitochondrial homodimeric enzyme that catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA, and its deficiency is caused by a pathogenic alteration in the ***MMUT*** gene (7)(9).

The *MMUT* gene alterations are classified into two subclasses: ***mut-*** lines have a MCM enzyme with detectable activity but exhibit a greatly reduced affinity for the AdoCbl cofactor. These patients might respond to the treatment and have a lover occurrence of mortality, morbidity, and long-term complications. However, ***mut^0*** lines do not show detectable MCM activity even if AdoCbl is provided in excess (8).

A variety of pathogenic variations in *MMUT* have been discovered and firstly, it was associated with forms of the disease produced abnormally low levels of MCM mRNA, indicative of a primary defect on mRNA transcription or processing. But also, changes in the amino-terminal half of the protein which eliminates the enzyme activity have been detected, between others (7).

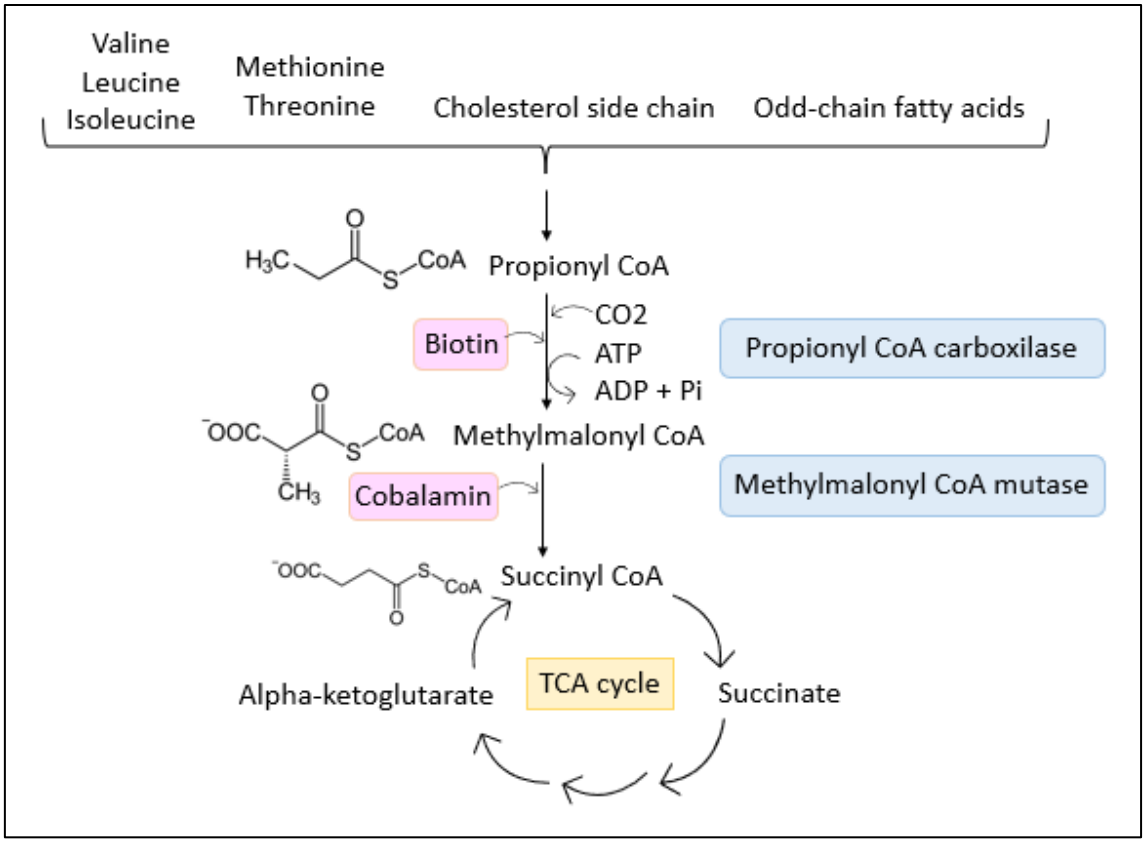

Figure 4. Methylmalonyl CoA pathway. TCA cycle: Tricarboxylic acid cycle. See text for details. Adapted from (7,12).

1.2.2.3. Combined deficiencies of adenosylcobalamin and methylcobalamin.

Among the combined cobalamin defects, three distinct disorders lead to combined deficiency of both **methylmalonyl-coenzyme A mutase** and **methionine synthase**.

CblC is the most frequent inborn error of Cbl metabolism. It is caused by a mutation in the **methylmalonic aciduria and homocystinuria type C protein gene *(MMACHC)***. It encodes a protein that plays a role in the early steps of cellular cobalamin metabolism (8). There are more than 50 known identified pathogenic variants in this gene, and it results in the altered intracellular synthesis of adenosylcobalamin and methylcobalamin. Phenotype-genotype correlation may predict disease severity depending on mRNA stability and protein residual function in some cases (13).

MMACHC protein may interact with the lysosomal efflux transporter of cobalamin, LMBD1, which accepts different derivates of cobalamin for passage into the cytoplasm. Additional roles in cobalamin transport and metabolism are suggested because MMACHC protein can be found in the mitochondria (13).

This disease shows feeding difficulties, lethargy, and progressive neurological deterioration, which includes hypotonia, hypertonia, abnormal movements or seizures, and coma (13). Patients might develop multisystem pathologies, such as renal and hepatic failure, cardiomyopathy, pneumonia, or hemolytic uremic syndrome (7).

Increased homocysteine in plasma, low to normal methionine, and methylmalonic aciduria are the hallmarks of this disease. The treatment with hydroxocobalamin decreases the elevated metabolite levels (8).

Kidney manifestations may appear in this disease, although they are rare, for example, hemolytic uremic syndrome (14).

The hemolytic uremic syndrome is an important affection caused by the inflammation of blood vessels. This illness is characterized by renal insufficiency, thrombocytopenia, and hemolytic anemia. The relation between the symptoms and the CblC deficiency is still not well known. The usual symptoms are nephropathy, growth retard, arterial hypertension, hypotonia, and lethargy. Usually, is accompanied by gastrointestinal symptoms and convulsions (14,15).

On the other hand, **CblD** defect is caused by a mutation in the **methylmalonic aciduria and homocystinuria type D protein gene *(MMADHC***). It encodes a multifunctional protein or encodes for two different products related to intracellular Cbl trafficking. In the diagnostic test, methylmalonic aciduria with or without increased plasma total homocysteine, or isolated homocystinuria may be found. Moreover, the clinical symptomatology is highly similar to CblC (3).

And last, the **CblF** disease consists of a pathological variant in the **lysosomal cobalamin transport escort LMBD1 protein gene (*LMBRD1*)**. It causes a failure of Cbl transport across the lysosomal membrane following the degradation of TC in the lysosome. For that reason, Cbl cannot be converted to adenosylcobalamin or MeCbl. It means the IF-Cbl must pass through a lysosomal stage in the enterocyte before Cbl is released into the

portal circulation. The patients reported a mutation in the *LMBRD1* gene and its serum Cbl level is low, but increased plasma homocysteine, low to normal plasma methionine and methylmalonic aciduria are found (3).
The clinical symptomatology is different than in other combined deficiencies. The patients show developmental delay, megaloblastic anemia, hypotonia, stomatitis, and skin rashes (16).

In addition to the isolated deficiencies, there are also certain diseases that affect the mitochondria and share the same primary and secondary tests as MMA (17).
Beta-subunit of the ADP-forming succinyl CoA synthetase deficiency (SUCLA)-related mitochondrial DNA depletion syndrome is an encephalomyopathic form with methylmalonic aciduria. The *SUCLA* gene encodes the beta-subunit of the ADP-forming succinyl-CoA synthetase, which is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA (17,18).
It has mitochondrial inheritance with an incidence of less than 1/1,000,000 that appears in childhood and the syndrome is an autosomal recessive manner (19). This syndrome is characterized by neonatal onset of global development delay, hypotonia and muscle weakness, failure to thrive, progressive neurologic decline, sensorineural deafness, and abnormal movement disorder (18). But also, cardiomyopathies, tubular dysfunction, and polyneuropathy have been reported (19).
In urine organic acid analysis, moderate MMA elevations are seen. Also, several other metabolites may be elevated in urine, such as methylcitrate, 3-methylglutaconic acid, 3-hydroxyisovaleric acid, and Krebs cycle intermediates, such as succinate, fumarate, and 2-ketoglutarate. Moreover, profile high levels of propionylcarnitine (C3) must be found in the newborn screening at acylcarnitine (17).

1.2.2.4. Treatment for primary cobalamin deficiency.

These diseases can be prevented or at least minimized by early diagnosis and emergency treatment, currently through neonatal screening programs. Actual specific treatments consist in **(i)** regularly intramuscular doses of hydroxocobalamin, **(ii)** a long-term special diet low in propionicogenic amino acids and whose aim is to reduce the accumulation of toxic metabolites, **(iii)** to prevent catabolic episodes and ensure enough caloric intake through carbohydrates during these episodes (7).
Hydroxocobalamin is the form of vitamin B12 that lacks the cyanide moiety and has a hydroxyl group instead. It is rapidly absorbed from intramuscular deposits, stored in the liver, and eliminated by glomerular filtration (20).

When supplementation and preventive dietary is not effective enough to provide reasonable disease management, liver transplantation is necessary (5,17). The transplant is helpful to decrease the dietary restriction of branched-chain amino acids, but it is not able of reversing some neurological or renal damage already caused before transplantation. For that reason, sometimes a kidney transplant is additionally necessary (21).

Nevertheless, long-term effects should be studied, due to the genetic deficiency is present in all the body cells, not only in the liver. Moreover, the metabolites are still being produced (although at lower levels) by the rest of the cells. For that reason, research on novel treatments (i.e. gene therapy) is highly necessary (21).
Despite the efficacy of successful liver transplantation in the correction of the metabolic instability that characterizes **methylmalonic aciduria (MMA)**, some patients have experienced metabolic strokes in the basal ganglia after transplantation (22).

Gene therapy has been postulated as a potential treatment for monogenic disorders (22). Early proof of experiments at the beginning delivered murine M*mut* (methylmalonyl-CoA mutase) and human *MMUT* activity by chemical transfection or viral infection to human and murine cells lines deficient in methylmalonyl-CoA mutase activity. In aggregate, the cellular experiments clearly show that in the cellular system restoring methylmalonyl-CoA mutase expression can restore enzymatic activity *in vitro* (23).
The first successful *in vivo* gene therapy experiments were performed using an early generation adenovirus type 5 (E1, E3 deleted). These studies showed an adenovirus, configured to express the murine M*mut* cDNA under the control of the cytomegalovirus promoter. Nonetheless, the treated MMA mice exhibited a temporal loss of transgene expression and eventually died (23). More recent adeno-associated viruses' experiments, such as AAV9, have demonstrated can penetrate the central nervous system, although persistent expression remains a challenge for this type of virus gene therapy (22).

In other experiments, the expression of the MCM in extrahepatic tissues can also mitigate the metabolic phenotype of MMA has been proven. This is proof that the expression of the MCM enzyme in only the skeletal and cardiac muscle can fully rescue the lethal effects that accompany whole body MCM deficiency (23).

Thus, it appears that a suite of new gene-based therapies and novel small molecule treatments are emerging for the treatment of MMA that will provide, for the first time, definitive therapies for this severe inborn error of metabolism (22–24).
Recently, a small molecule approach for the treatment of methylmalonic acidemia has been discovered. That novel molecule could be important to treat the multi-system affectation (affects renal, gastrointestinal, immune, central nervous system, hepatic, hematologic, and cardiovascular system). The pharmacodynamic activity of the compound has been studied *in vitro* for propionic and methylmalonic acidemia. The result is a substantial reduction of C3, propionylcarnitine/acetylcarnitine (C3/C2) ratio, methylmalonyl CoA, and propionyl CoA (24).

1.3. Other related pathways

Since the primary deficiencies of methylmalonyl-CoA mutase and propionyl-CoA carboxylase share alteration of corresponding markers, it is important to know how they interact with the cobalamin and the enzymes of its metabolic pathway (7).

1.3.1. Folate pathway

The **folate** is a molecule whose metabolism involves the reduction to dihydrofolate (DHF) and tetrahydrofolate (THF), and the addition of a single carbon unit provided by serine or histidine in various redox states (3).

The principal cause of folic acid deficiency is a poor diet, typical of elder people poorly fed, homeless and have chronic alcoholism. But also, people who have had an augment of its necessities due to physiological causes, like pregnancy, lactation, infancy or adolescence, and also pathological causes, such as acquired immune deficiency syndrome (AIDS) patients or medication for some diseases (chemotherapy) can be affected (9).

During the **pregnancy**, the increment of the erythropoiesis or erythrocytes formation requires between 5 and 10 times more folate. Consequently, the low folate diet leads to a cobalamin decompensation. (10).

The folate is the **methyl donor** for **methionine synthase** (see **figure 5**). For that reason, a low concentration of folate in plasma results in the conversion impairment of homocysteine to methionine. Consequently, the mother's **homocysteine** increases (3).

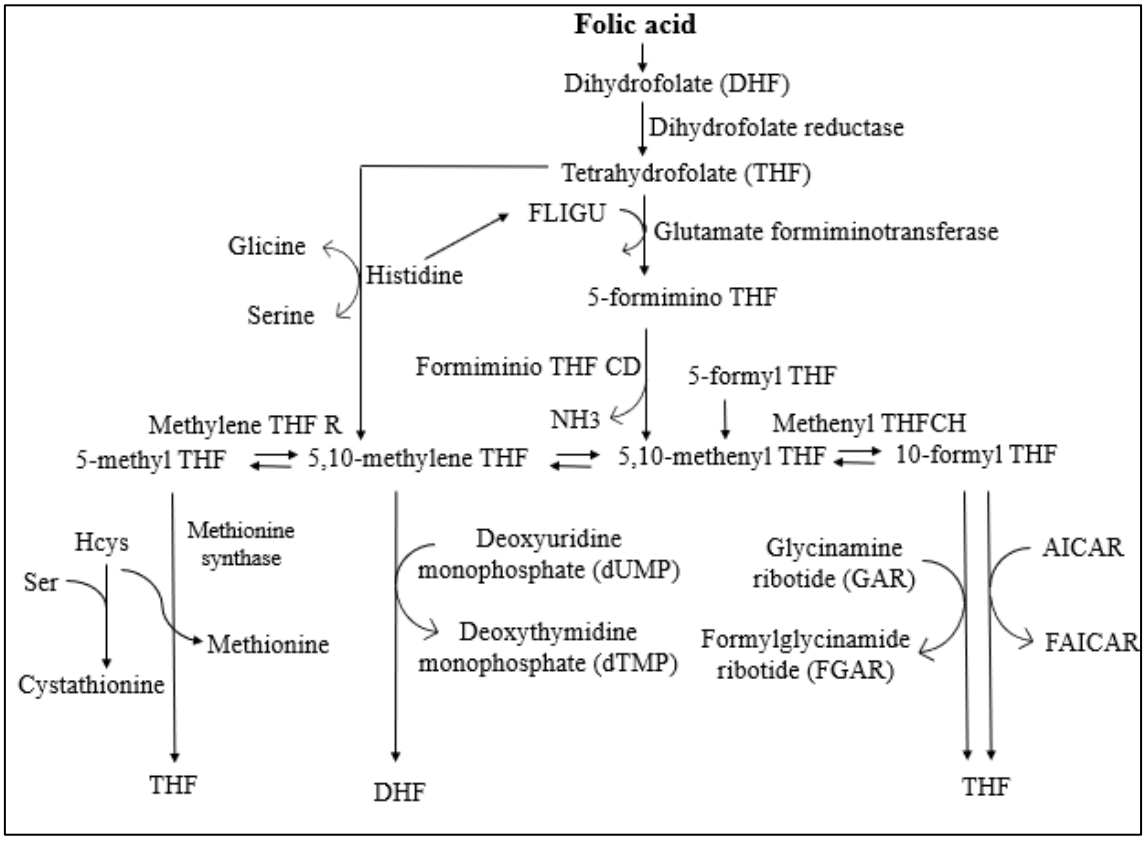

Figure 5. **Folic acid metabolism.** THFCD: tetrahydrofolate cyclodeaminase; FIGLU: formiminoglutamate; THFCH: tetrahydrofolate cyclohydrolase; AICAR: Aminoimidazole carboxamide ribotide; FAICAR: formylaminoimidazole carboxamide ribotide. See text for details. Adapted from (3).

1.3.2. Propionic aciduria

The primary deficiencies methylmalonyl-CoA mutase and propionyl-CoA carboxylase have the same primary and secondary altered biomarkers as the cobalamin deficiency. Consequently, it is important to understand these pathologies to perform a differential diagnosis (25).

Propionic aciduria (PA) is caused by a deficiency of the mitochondrial enzyme propionyl-CoA carboxylase, which is a multimeric protein. So far, all patients with isolated PA are biotin resistant (which is the cofactor of this enzyme) (8).

Both have high concentrations of C3 and methylcitric acid (MCA) in the screening tests (25). Nonetheless, PA is characterized by greatly increased concentrations of free propionic acid and multiple organic acid products in blood and urine. Between them, 3-hydroxipropionate, methylcitrate, propionylglycine, and tiglylglycine are the major diagnostic metabolites, but a raised methylmalonate is never found because it is downstream (7,8).

1.4. Newborn screening inborn errors of metabolism

The newborn screening is the set of actions aimed at systematic detection of congenital errors of metabolism in neonatal age (26). According to Society for Neonatal Screening "The newborn screening comprises the sum of necessary actions to ensure that, as soon as possible, all the newborns from target population are tested, the necessary follow-up is realized, and all founded cases are rightly treated in a minimum and equitable period of time" (27).

It has the principal aim to identify infants with serious but treatable disorders to prevent or ameliorate the clinical consequences and facilitate future interventions (26).

Over the years, there have been some organizations, as the American College of Medical Genetics, that have established all the necessary points to include a disease in the newborn screening (28).

1.4.1. Newborn universal criteria

In 1968 Wilson and Jungner created some **criteria** for disease selection, which was included in the newborn screening. They focused on the capacity to detect it and the existence of a treatment. Other criteria have been established over the years.

Posteriorly, Andermann et al. included some criteria in 2008 (29).

The classic criteria were: **(A)** the illness must be an important health problem and it must have an acceptable treatment, **(B)** facilities of diagnostic and treatment must be available, **(C)** recognition during a latent step or early symptoms, and **(D)** there must be an agreement policy, a balanced economic **spending** and continuous case-finding (29).

Moreover, the recent norms are to have a defined population and objectives, have scientific evidence of the program effectiveness, respond to a need, have quality, evaluation, and confidentiality control, and integrate education, laboratory tests, clinical services, and organization (30).

On the other hand, in 2006 some new inclusion criteria were established in the newborn screening, such as that the illness had to lead to mortality, or physical or mental morbidity if it is not diagnosed in early steps, the incidence must be at least 1/10,000 alone or combined with other ones detected in the same analytic process. Moreover, there must be a treatment and the benefits must be higher than disadvantages, between others (8).

1.4.2. Current newborn screening in Catalonia

Nowadays 22 newborn screening centers exist in Spain, which offers as a minimum the detection of congenital hypothyroidism and phenylketonuria (28).

The criteria for the newborn screening in **Catalonia** proposed a relationship between illness, method of detection, treatment, and program (30). The illness must be an important health problem, with clear epidemiology and evolution. A biomarker is necessary to detect disease in latent period and the primary interventions but also economic. There are some requirements, such as a simple, secure, and precise test of detection, the knowledge of the distribution of test values in the population, and a political agreement for organizing and funding diagnostic tests. In addition, the preventive treatment should give better results than the latter one and be optimized to treat the newborns (30).

The screening program must include random high-quality tests to demonstrate that the screening is efficient to reduce mortality. In general, it must be clinical, social, and ethically acceptable with more benefits than physical or psychological damages (30).

It should not be forgotten that this program is universal and recommended by the socio-health evidence but is ultimately voluntary (31).

The current steps of newborn screening in Catalonia are:

1. Universal screening of all newborns in the maternal centers of Catalonia by blood spots dried.
2. Definitive diagnosis.
3. Preventive treatment.
4. Follow-up of newborns.
5. Coordination and evaluation (30).

Nowadays, over **50 inborn errors of metabolism** can be detected by screening and the latter technology has greatly changed the methods and results with rapid simultaneous analysis of multiple analytes. However, in Catalonia only the following diseases are included in the program:

(1) Amino acid metabolic disease: phenylketonuria/hyperphenylalaninemia, maple syrup urine disease, tyrosinemia type I, citrullinemia type I, and homocystinuria.

(2) Organic acid metabolic disease: glutaric aciduria type I, isovaleric acidemia, methylmalonic acidemia, 3-hydroxy-3-metilglutaril-CoA lyase deficiency, beta-ketothiolase deficiency, and propionic acidemia.

(3) Fatty acid metabolism disorders: medium and long-chain acyl-CoA dehydrogenase deficiency, mitochondrial trifunctional protein deficiency, carnitine palmitoyltransferase 1 and 2 deficiency, carnitine-acylcarnitine translocase deficiency, multiple deficiencies of acyl-CoA dehydrogenases, and carnitine cellular uptake deficiency.
(4) Other endocrine-metabolic pathologies: congenital hypothyroidism, cystic fibrosis, sickle cell anemia, and severe combined immunodeficiencies (31).

Nonetheless, there also exist other metabolic diseases that are not included in the neonatal screening but are detected anyway. One example of that situation is the biotinidase deficiency, which is detected because the C3 and C5-1 or C5OH levels would be above the cut-off (31).
Moreover, there is a group of diseases that make up the second panel of the Catalonia Newborn Screening Program, which among many others, include maternal vitamin B12 deficiencies, short-chain acyl-CoA dehydrogenase deficiencies, or riboflavin 1 and 2 deficiencies (31).
Methylmalonic acidemia, propionic acidemia, and homocystinuria are an extensive group of inherited genetic defects included in the expanded newborn screening (NBS) program in some countries (11).
Nowadays, NBS detection analyses the C3, methionine (Met), ratio C2/C3 and C3/Met, MMA, MCA, and Hcys in dried blood spots (DBS). In recent years, heptadecanoylcarnitine (C17) has been proposed as a new biomarker. Consequently, the acquired and congenital cobalamin deficiency in newborns can be detected by the NBS program. Moreover, the same tests were performed in the mothers and serum cobalamin and folate are routinely measured (25).

2. Hypothesis

Inadequate maternal nutrition, specifically the lack of aliments of food of animal origin, increases the number of acquired cobalamin deficiencies in newborns in recent years.

3. Objectives

Principal objective.

- To determine the incidence of cobalamin disorders and their biochemical alterations over the years in the reported cases in Vall d'Hebron Hospital, Barcelona, Catalonia, Spain, Europe.

Secondary objectives.

- To study the biochemical difference between primary and secondary deficiencies.
- To study the maternal influence in the secondary newborn cobalamin deficiency.
- To study and compare the effectiveness and efficacy of hydroxocobalamin intramuscular injections as the treatment for primary and secondary deficiencies in newborns.

4. Materials and methods

Due to the recent changes in the cut-offs and secondary tests in the newborn screening, an analysis based on the data obtained by the Vall d'Hebron Hospital has been carried out about the positive screening newborns from 2013 to 2020. The analysis has focused in specific and infrequent abnormalities relating to cobalamin: the incidence of the disease and the different biochemical parameters that are used to suggest a suspected disease before a confirmatory genetic test.

4.1. Heel crick test

To make these analyses possible, newborn screening tests are mainly carried out on dried blood spots on especially filter papers at 48-72 hours after birth, which is also called the **heel test**, to analyze the acylcarnitines profile, amino acids profile, and organic acids profile, as described (21).

That detection typically provides three possibilities: **(i)** the disorder may present in the first days of life, before any available screening result, which includes organic acidemias such as methylmalonic acidemia, **(ii)** disorders later presenting and have an effective treatment can be beneficial, in which the newborn screening program is most useful, and **(iii)** the disorder is benign or has a slow and/or attenuated progression, in which cases there is no benefit from early diagnosis (11).

In the newborn screening program in Catalonia, when any of the primary markers are altered, samples undergo a **second-tier test** on the first DBS in order to improve specificity (to decrease the number of false positives). In propionic/methylmalonic acidemias, primary markers are: C3, C3/C2, Met and C3/Met, which indicate alterations related to the propionic acid catabolism pathway. Secondary makers are MMA, MCA and Hcys. The algorithm (see **figure 5**) shows the classification of samples according to the concentration of second-tier tests in the first DBS. NBS is classified as normal when all biomarkers are below the cut-off value (21).

In altered samples, the newborns have a positive suspicion and are referred to a reference hospital to confirm the final diagnosis and to be treated accordingly, distributed as in Annex I.

In the group of diseases treated in that work, the initial treatment consists of 1 mg of intramuscular hydroxocobalamin injection preventively, that is, this treatment is applied before the results of the confirmatory test are known. The control analysis of the child is done two weeks after the treatment and the last control is performed when the child is 6 months old. In those controls, in order to rule out (in false-positive cases) or to guide the diagnostic, the laboratory testing in urine (MMA, MCA and amino acids), plasma (Hcys, MMA, acylcarnitines and amino acids), and serum (vitamin B12) were assessed (21).

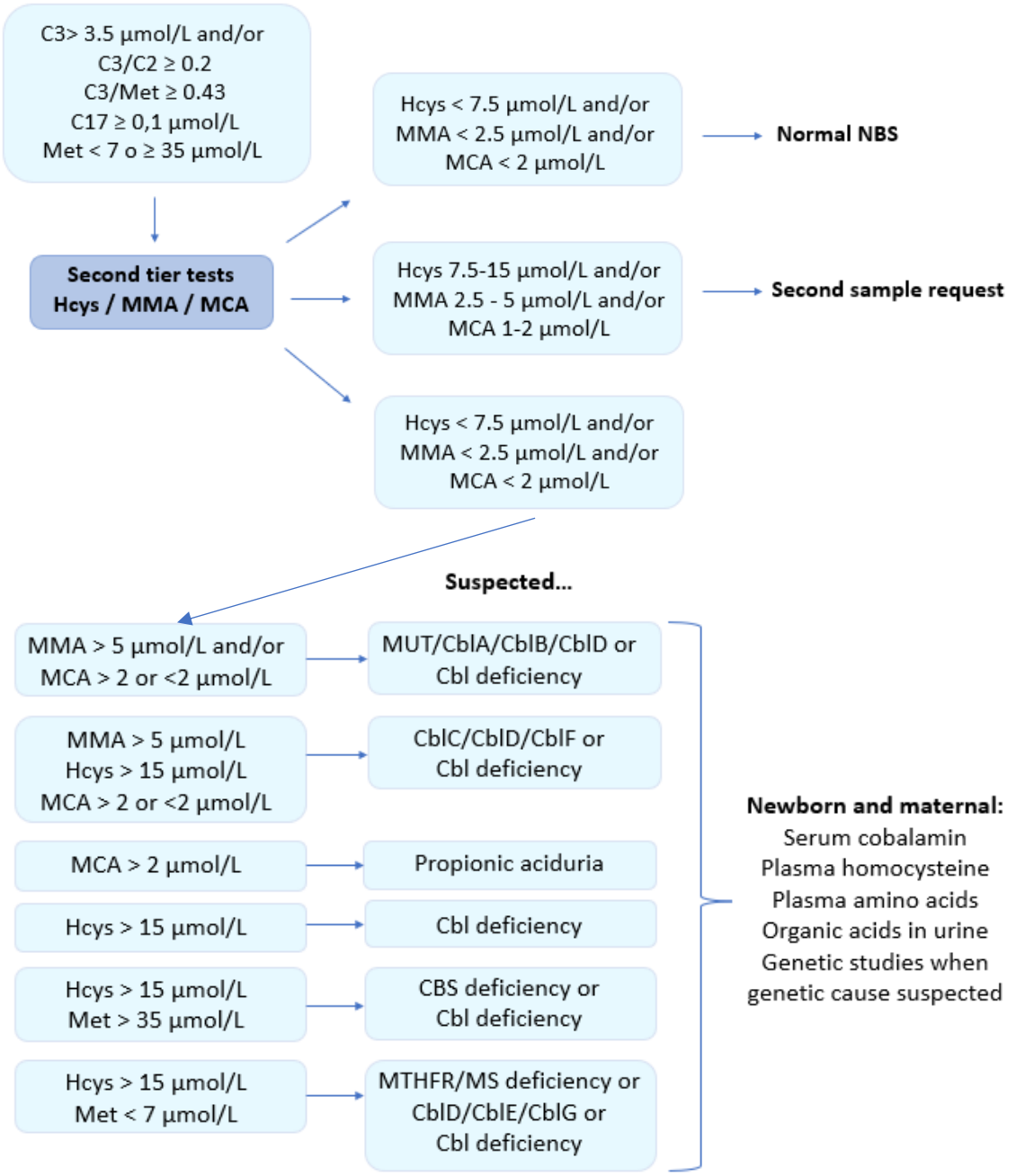

Figure 6. **Flowchart of newborn altered samples during the heel test.** See text for more details. Adapted from (21).

A discrete percentage of pre-analytical errors may be related to the type of sampling: homogeneity problems in the distribution of the blood and depending on the composition of the chosen paper. Standardization is affected by the type of paper, the volume of the blood impregnated, the hematocrit and the part of the bloodstain from which a disk is taken to perform tests. On the other hand, one time that the samples are dry, they remain stable for long periods of time, due to the degradation processes biological they usually take place wet (32).

4.2. Proves to analyze the cobalamin deficiency in the reference hospital

One time the patient with a suspicion of methylmalonic aciduria arrives at its reference hospital, two possibilities are available to analyze cobalamin: **(A)** the **direct method**, that analyzes the cobalamin concentration in fluids (plasma), which is an automatized immunoassay, and **(B) indirect method**, that analyze the concentration of the metabolites resulting from Cbl deficiency: **(i)** methylmalonic acid in plasma by gas chromatography-mass spectrometry (GC-MS) with isotopic dilution and in urine by the organic acid profile performed by GC-MS; **(ii)** homocysteine in plasma by specific automated immunoassay, and in urine by the amino acid profile, which detect homocysteine (see figure 7).

With the aim of identifying the different cobalamin deficiencies and their causes, the organic acids profile, amino acids profile, acylcarnitines profile, plasma methylmalonic acid and homocysteine are performed. And last but not least, a genetic test must be done to identify the pathogenic variant that causes the alteration.

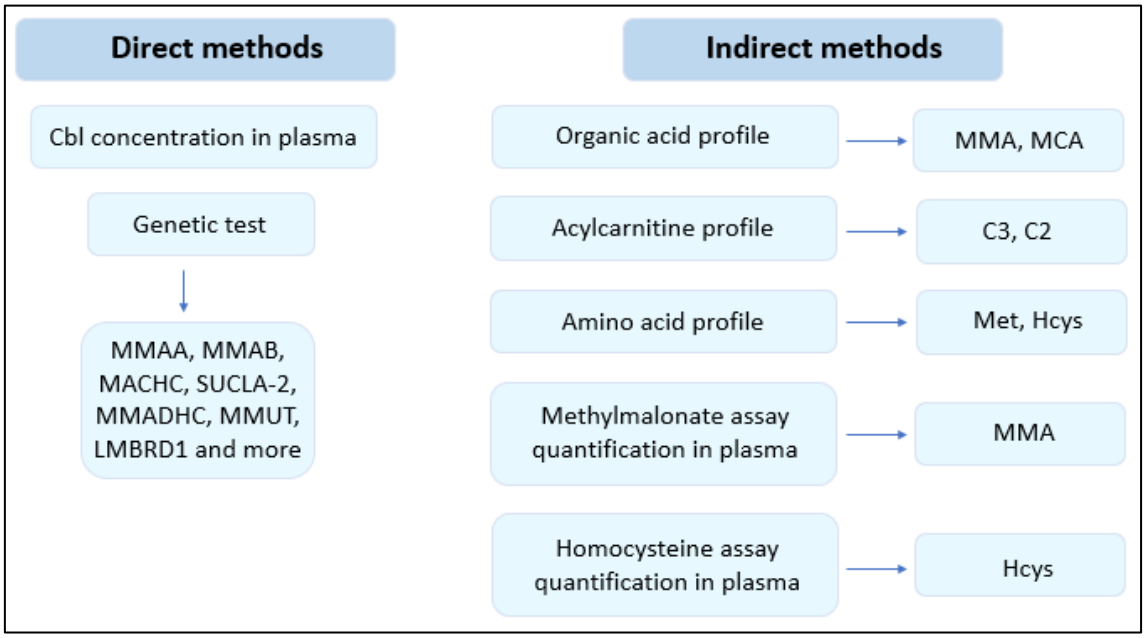

Figure 7. Flowchart of direct and indirect methods to analyze cobalamin in newborn with altered values. See text for more details.

4.2.1. Organic acid profile

Organic acids are hydrocarbon molecules of 2-14 carbons with one to three carboxyl groups with a medium molecular weight, which are intermediates of fatty acids and amino acids catabolism and the central energetic pathways. The profile is specially used for the diagnosis of organic acidurias and fatty acid beta-oxidation defects by GC-MS as described for qualitative and quantitative identification (33).

Organic acid profile was analyzed with gas chromatography using a non-selective hydrophobic capillary column is used (HP-5MS, 95%/5% methyl/phenyl silicone, 30 m long, a diameter of 0.250 mm, and 0.50 µm of film thickness. Temperature ranges from -60ºC to 325ºC (350ºC)) from Agilent following the instructions of the manufacturer.

The group of substances of interest that is detected in an organic acid profile includes:

(1) Dicarboxylic acids derived from beta-oxidation and ketogenesis. Examples of these are the adipic acid, suberic acid, 3-hydroxy-butyric, etc.

(2) Acids derived from the catabolism of branched chain amino acids. Examples of this are the propionate, methylmalonate, 3-hydroxy-isovalerate, etc.

(3) Compounds derived from the catabolism of other amino acids. Examples of this are the glutaric acid, succinylacetone, phenolic derivatives, etc.

(4) Conjugated with acids. Examples of this are short or medium chain acylglycines.

(5) Intermediates of mitochondrial energy metabolism. Examples of this are the lactic acid, Krebs cycle metabolites, etc.

(6) Other endogenous and exogenous substances. For example, dietary derivatives, intestinal flora compounds, drugs metabolites and others.

4.2.2. Acylcarnitine profile

Acylcarnitines are intermediates in **fatty acid and amino acid breakdown,** generated from the conversion of acyl-CoA species to their correspondent carnitine esters by the action of **carnitine acyltransferase**. Their identification and quantification are useful for the diagnosis and follow-up of inherited errors of mitochondrial fatty acids beta-oxidation defects and organic acidurias (34).

The previous separation is carried out in a UPLC liquid chromatography instrument, using separation by reverse-phase analysis with relatively simple solvents, acidified with formic acid (provides acidity to improve the ionization).

After the chromatographic separation, the substances enter in the tandem mass spectrometry and are ionized in the first place in the ion source by the **electrospray technique**, which is a soft technique that fragments little and allows to obtain massive **parental ions** equivalents to molar mass or easily identifiable. The detection can be done in positive or negative mode but in that case, it is done in **positive** mode by producing ions with the formula MH+.

Parental ions are filtered in the first quadrupole (**Q1**). Later, the beam of parental ions enters in a collision cell (**Q2**), where they are bombarded with a gas (Argon) at high speed, obtaining more rich spectra of product ions. The group of **product ions** enters at the third quadrupole (**Q3**), where they are separated by their m/z ratio.

In the quadrupole mass analyzers, the ions are transiently applied different potentials of direct and alternating current that causes that only those ions that pass through it with an adequate m/z ratio have a stable trajectory to the detector. In this way, in the triple quadrupole, the molecular ions that leave the source are selected in the Q1 (precursor or pairs).

They can be subjected or not to fragmentation induced by collision in the Q2 (collision cell), after which the resulting ions (following derivatives or not of fragmentation) are selected in Q3 (product ions).

Depending on the way of acquiring or filtering the masses in the Q1 and Q3 (SIM mode, where a single mass is transmitted) or SCAN (all masses are transmitted), the equipment can operate in different detection modes depending on whether the aim is to quantify a specific substance or to know which are the substances in a sample.

(1) Product ion scan. It is used for the study of molecular structures, such as the sequencing of amino acids of a peptide molecule, or during method validation and for the study of **unexpected findings** in newborn screening.

(2) Precursor ion scan. It provides a different precursor ion spectrum that generates a selected fragment (product ion). It was classically used in acylcarnitines profile analyses

(3) Neutral loss scan. It is used to monitor the loss of a neutral fragment for a specific molecular type (similar functional groups). It is classically used in amino acid analysis and nowadays the mode MRM is increasingly used.

(4) Selective reaction monitoring (MRM or SIM-SIM). Q1 and Q3 are static for a predetermined pair of ions precursor and product. In this way, the highest specificity and sensitivity is achieved, and the technique can selectively quantify compounds within complex mixtures. Is the one used in the acylcarnitine profile. For each acylcarnitine, 1 or 2 MRM transitions are configured.

Finally, the train of product ions arrives at the detector, where the signal used for the subsequent qualitative and quantitative analysis is generated (35).

Acylcarnitine profile was performed as described (34) with minor modifications not described for confidentiality reasons. The metabolic diseases laboratory works in MRM mode. In Q3, mass 83 is selected. In Q1, it is selected sequentially by means of sequential changes of potentials, the different precursors.

The relevant species of the acylcarnitine profile are:

(1) Branched chain off numbers acylcarnitines. Examples of this are the C3 (propionyl-carnitine), C5 (isovaleryl-carnitine), C5OH (3-hydroxyisovalery-carnitine), etc. They are used for the diagnosis and monitoring of organic acidurias.

(2) Acyl-carnitines of short, medium and large linear paired chain. Examples of this are the C4 (cutyryl/isobutyril-carnitine), C6 (hexanoyl-carnitine), C8 (octanoyl-carnitine), C14, C16, C18 (long chain species) and its corresponding unsaturated or hydroxylated forms. They are used for the diagnosis and monitoring of defects of fatty acids beta-oxidation.

(3) Other species, such as glutaryl-carnitine (C5-DC), for the diagnosis of glutaric aciduria.

(4) Abundant physiological species, such as C0 (free carnitine) or C2 (acetyl-carnitine), and sometimes C4OH (3-hydroxybutyryl-carnitine), which are used to assess the level of carnitine or carnitine-using enzymes and also the presence or absence of ketogenesis.

Despite the specificity, there could be interferences by unknown drugs or substances. For example, pivalic acid and metabolites of some antibiotics result in an increase of C5.

4.2.3. Amino acids profile

Amino acids detection is useful from the nutritional point of view because it indicates the quality of the nutritional contributions, as well as for the diagnosis of inborn errors of metabolism.

Amino acids profile can be traditionally performed in plasma or urine with cation exchange chromatography and its subsequent detection. In the Vall d'Hebron metabolic laboratory, a variant of the technique is performed, which uses reverse phase chromatography after a previous transformation of amino acids. Charges are removed by buffering, conjugation and derivatization with some reagents (in this case, a reagent that conjugates amino groups and adds a useful chromophore to detect them spectrophotometrically at 254 nm) (21).

For the group of diseases objected in this work, the most interesting amino acids are the family of sulfur-containing amino acids: methionine, homocysteine/homocystine, and the propionic acid precursors (valine and isoleucine especially, but also threonine). Nonetheless, Hcys is prone to oxidation because has free sulfur groups. This makes it easier to form disulfide bridges with the cysteine or homocysteine, making them not visible on the chromatogram. Therefore, it is necessary to use a protector and deprotonate the sample or detect the Hcys by immunoassay because all amino acids with free sulfur groups (SH) are prone to oxidation and the formation of other amino acids, so the Hcys quantification is altered (36).

4.2.4. Methylmalonate assay quantification in plasma

Methylmalonate levels can be studied in urine by the organic acid profile or in plasma by a methylmalonic specific assay. The sample preparation follows the same procedure as in organic acid profile. The detection and quantification are based in CG-MS in **SIM** mode using as internal standard the deuterated methylmalonate. Thanks to this method, the sensibility and variability of the quantification are improved.

4.2.5. Homocysteine assay quantification in plasma

Homocysteine levels can be detected with the amino acid profile. To improve the sensitivity of detection and quantification, an **immunometric method** is used for specific total homocysteine performed with Agilent 6460 Triple Quadrupole LC/MS following the instructions of the manufacturer because the sulfur groups from amino acids are prone to form other amino acids (3).

4.2.6. Genetic tests

To obtain the genetic profile of a patient, a sample of its DNA must be sequenced by **Sanger** or **Next generation sequencing** (NGS) methods. The Sanger technique, which is the traditional reference, is used when the variant or the candidate gene is known with certainty and is methodologically available (size, etc.) (37).

Nowadays, the Next Generation technique is increasingly being imposed, which allows chain-by-chain visualization and allows many genes to be studied at the same time (37). The objective of this technique is to have the biggest horizontal (largest number of genes and regions) and vertical (highest number of reads per bp) coverage.

To obtain reliable results the coverage and quality of the profile must be the highest possible, but it is also necessary different filtering stages and errors to avoid non-significant variants (SNPs) and finally, an analysis of the possible pathogenic variants.
These are bioinformatic studies of variant analysis, in which quality and error filtering is performed first and after filtering of pathogenic or benign variants. Depending on the pathogenicity, different genotypes can exist. Usually, a non-sense or a splicing or a severe missense variant are more pathogenic than a variant (usually missense) of unknown significance (VUS) (38).

5. Results

5.1. Positive newborn screening

In newborn screening, the group of positive cases for cobalamin deficiency is the most frequent. As shown in **figure 8**, the average ratio of positive screening of suspected cobalamin deficiency over all the positives is around 43% ± 1.1%, and it has constantly increased over the years.

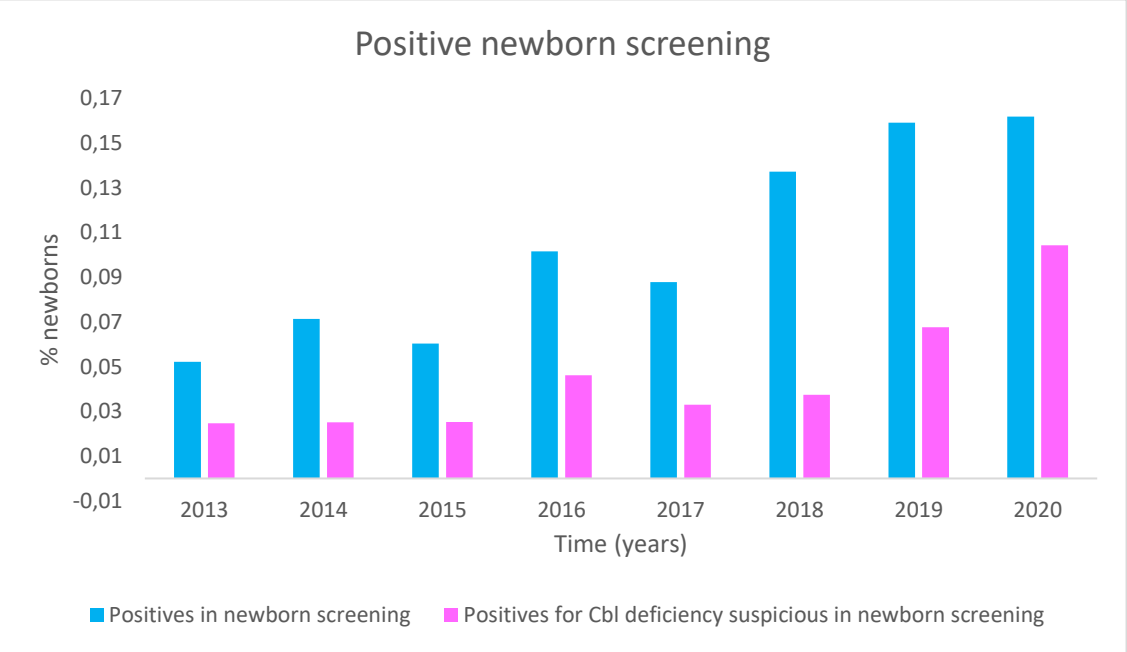

Figure 8. Increasing of positives cobalamin suspicious deficiencies along the years in newborn screening. Data provided by the Metabolic diseases unit of Vall d'Hebron Hospital.

What is more, in the year's series the amount of transitory vitamin B12 acquired deficiency has grown substantially, while genetically confirmed cases have remained constant, as shown in **figure 9**.

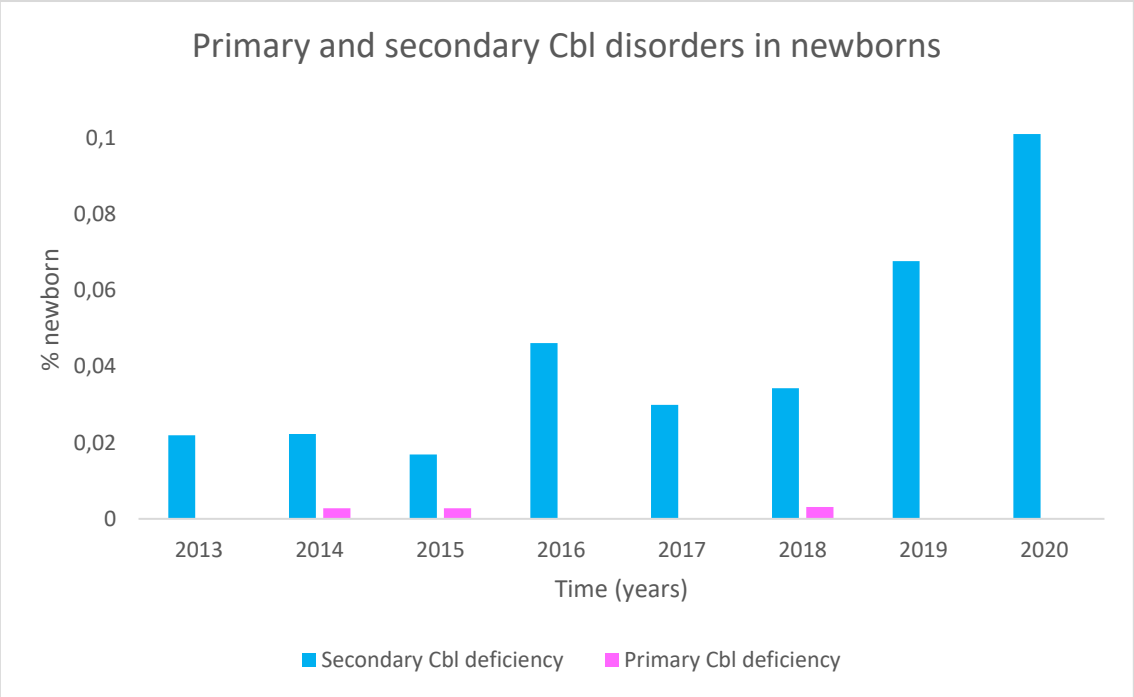

Figure 9. Increasing secondary cobalamin deficiency in newborn screening. Data provided by Metabolic diseases unit of Vall d'Hebron Hospital.

5.2. Cobalamin transitory alterations in newborn screening

From 2015 to 2020, not only second-tier test was performed, but also cut-off C3 was lowered. During these years, a total of 422,044 newborns were screened. From those ones, only 326 (0.15%) were referred to Vall d'Hebron Hospital to do the metabolic study because had altered primary and secondary biomarkers. Of those ones, 64 were acquired vitamin B12 deficiency, 6 were diagnosed with genetic defects related to Cbl or MCM deficiency and 7 were false positives.

To analyze the most frequent primary and secondary markers, **figure 10** shows the percentage of increased C3, C17, MMA, MCA and Hcys from the heel test screening center around the respect all the positive screenings cases of transitory cobalamin deficit. C3 is altered in all the newborns, but Hcys and MMA are also altered in many cases.

Figure 11 shows the most significant metabolic alteration in confirmatory studies at the Vall d'Hebron Hospital.

The presence of increased secondary screening test markers MMA, MCA and Hcys are also present in a high proportion of transitory alterations but has been varying over the years.

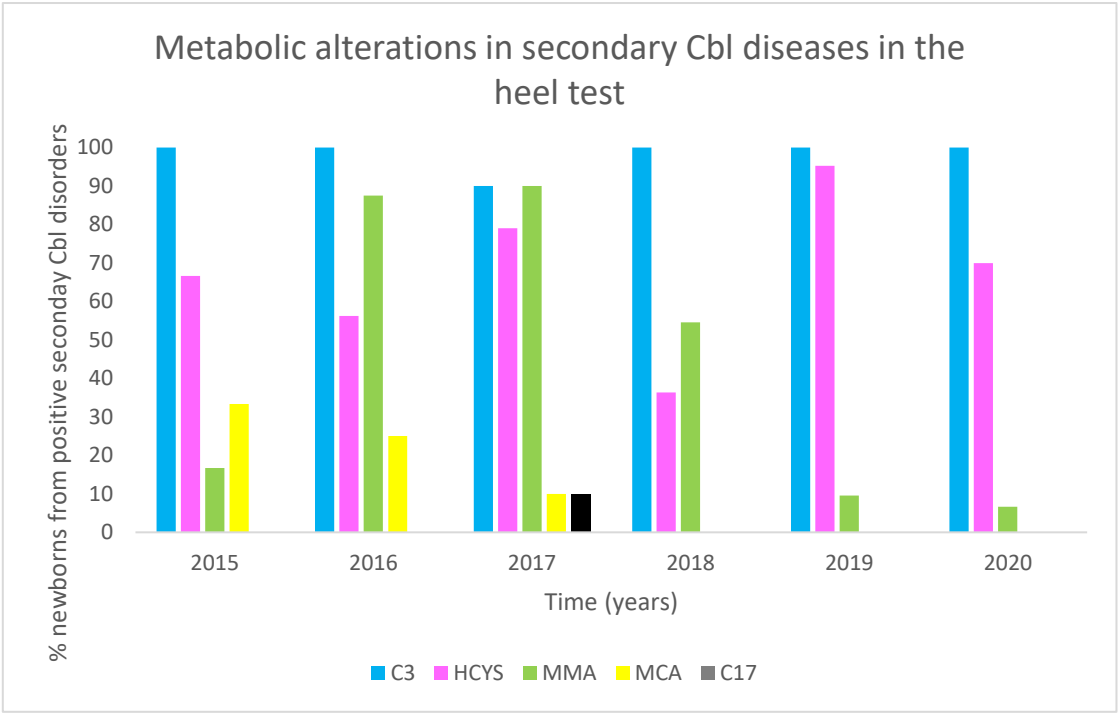

Figure 10. Propionylcarnitine is the most constant marker in transitory deficiencies over the years from positive cobalamin disorder in the heel test. Data provided by the Metabolic diseases unit of Vall d'Hebron Hospital.

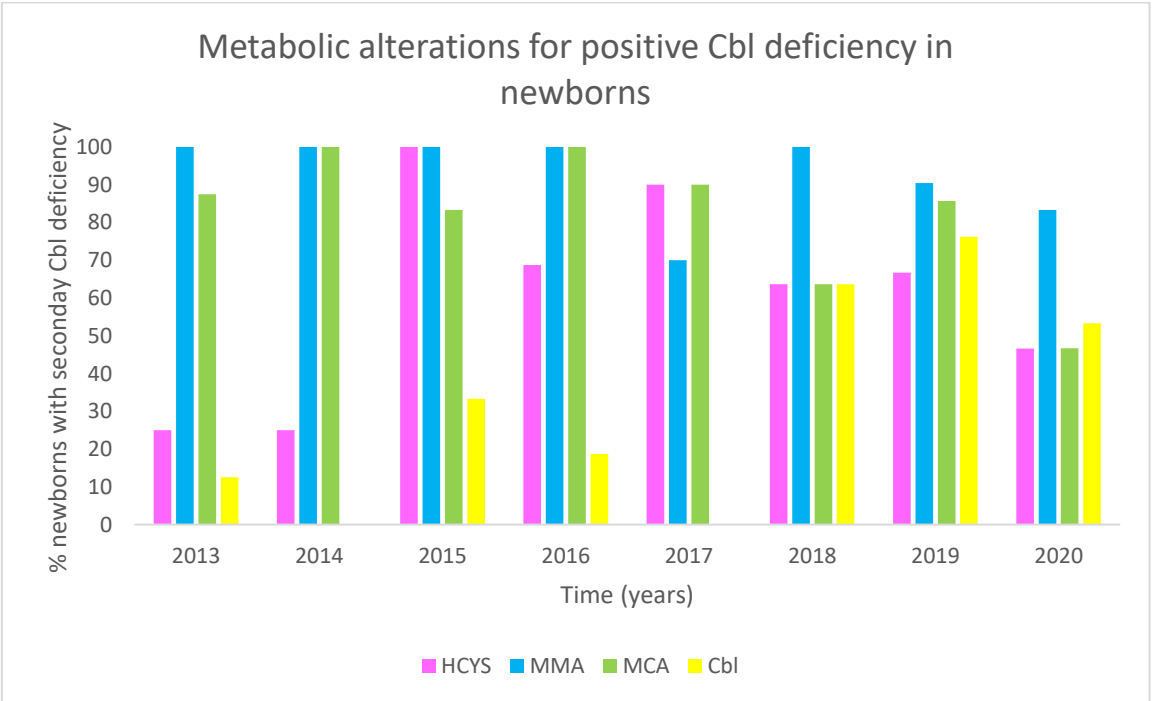

Figure 11. Methylmalonic acid and Methylcitric acid in cobalamin transitory deficiencies are constant over the years in positive cobalamin deficiencies. Data provided by the Metabolic diseases unit of Vall d'Hebron Hospital.
**C3 biomarker was incorporated in 2019 in the Vall d'Hebron metabolic laboratory.*

5.3. Maternal alterations with suspicious Cbl newborn deficiencies

The large percentage of mothers with cobalamin related deficiencies must be highlighted. From 2015 to nowadays recommendations to monitor and supplement cobalamin during pregnancy follow-up have been sent to the community health authorities due to the evidence that about a 25-35% of analyzed mothers have cobalamin deficiency direct or indirectly because of a poor cobalamin diet. Moreover, low folate can cause a cobalamin deficiency due to the conversion of **MTHF** is catalyzed by the methionine synthase and **MeCbl**.

In addition, another evidence of low protein intake of animal origin is the iron deficiency (showed as high transferrin and low ferritin levels). Nevertheless, the transitory deficiencies in babies that do not have a maternal origin can be caused because babies use to have higher nutritional and metabolic requirements.

Figure 12 shows the altered biochemical parameters in mothers whose newborns are positive for cobalamin deficiency in the screening. Those are the low levels of cobalamin and folate, and high levels of transferrin and/or low levels of ferritin.

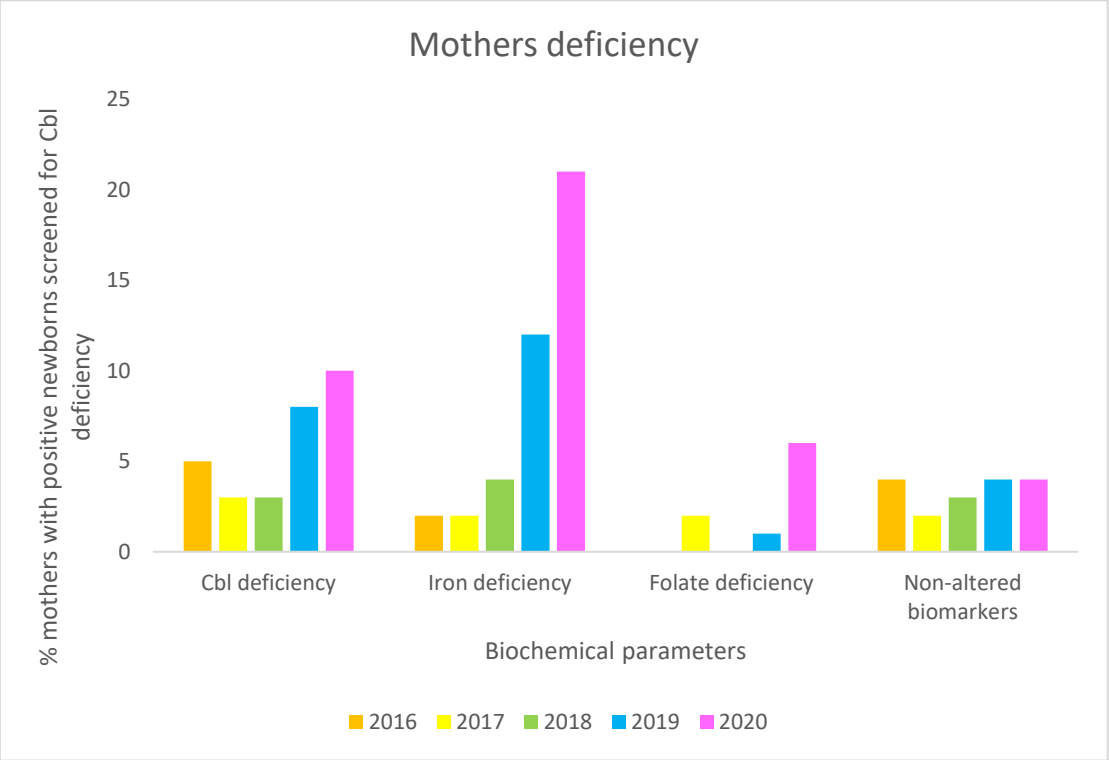

Figure 12. 25-35% of mothers from newborns with suspicion of cobalamin secondary deficiency have biochemical alterations. Data provided by Metabolic diseases unit of Vall d'Hebron Hospital.

5.4. Treatment for newborns with positive secondary markers for cobalamin or methylmalonyl CoA mutase deficiency

Due to methylmalonic aciduria involves CblA, CblB and MCM deficiencies, the report of primary cobalamin deficiencies has included the MCM deficiencies.

When the second-tier tests are positive for vitamin B12 deficiency, patients are sent to their reference hospital to be studied and to ensure that their metabolite profile is normalized with the appropriate supplements. Patients are initially treated with 1mg intramuscular hydroxocobalamin. The levels of metabolites in urine, blood and plasma are studied until they normalize, or, contrary, the genetic test detects a pathogenic variant. In all the cases the metabolites from acquired deficiencies fell to normophysiological levels (21).

In genetic deficiencies, the levels of MMA and MCA decrease substantially in all the cases after the initial treatment with hydroxocobalamin, especially in combined deficiencies.
In some cases, the toxic metabolites may drop to physiologically normal levels, but in others, as in most *mut*^{0} cases, the urine MMA concentration never falls below 2,900 mmol/mol creatinine (N<8).
In most cases, the MUT^0 and CblA patients, despite having oscillations depending on the treatment and catabolic crises, the concentration of the specific metabolites is usually persistently high.
In some cases with more benign mutations, the excretion of metabolites may be lower. In addition, in this type of disease (CblC), there are specific cases in which the treatment is very favorable with respect to the excretion of toxic metabolites (**figure 13**).
The methylcitrate monitoring is more difficult to understand than the methylmalonate one owing to its additional relationship with the central energy pathways and to be dependent on enough replenishment (anaplerosis) of central energy intermediates.

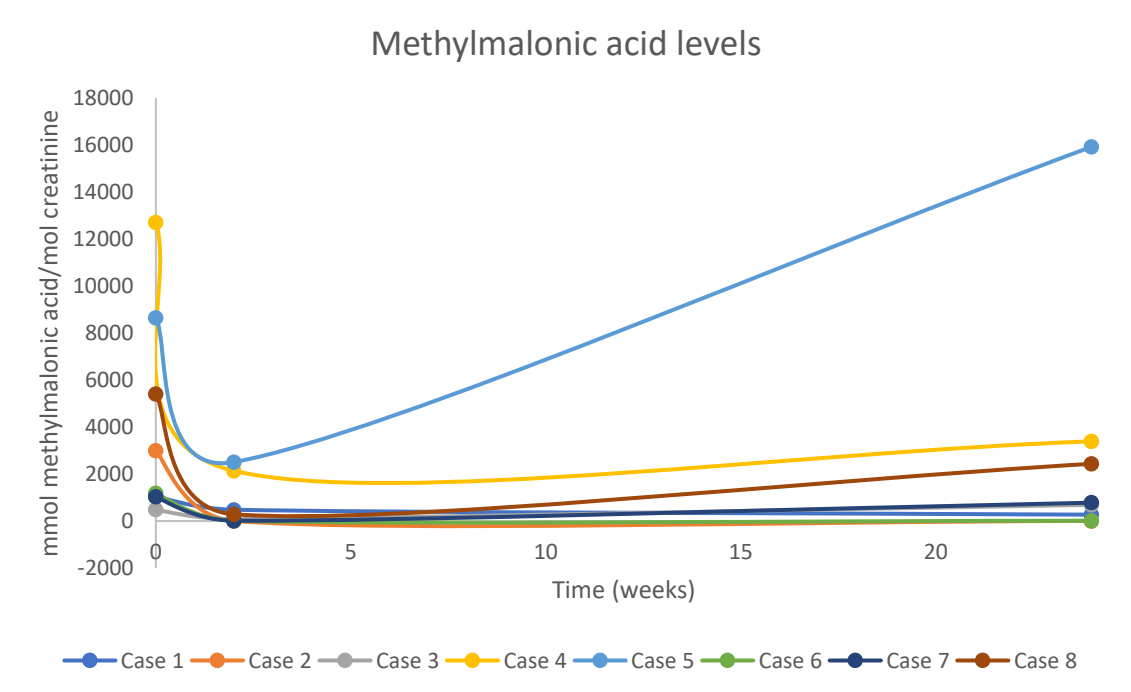

Figure 13. Methylmalonic acid levels in genetic deficiencies decrease temporarily after the first hydroxocobalamin intramuscular injection (2 weeks). Data provided by Metabolic diseases unit of Vall d'Hebron Hospital. **Case 4: Exitus in 2016*

5.5. Confirmatory biomarkers from cobalamin and methylmalonic aciduria genetic diseases

The main markers in Vall d'Hebron metabolic diseases laboratory to confirm the cobalamin or methylmalonic aciduria genetic disease are the MMA, MCA and Hcys.

Generally, in primary disorders, MMA and MCA in urine are considerably elevated. While in transitory diseases MMA might be elevated with values of 8-150 mmol/mol creatinine, in primary diseases MMA is found in levels between 243-12,710 mmol/mol creatinine, which is the first visible difference.
Moreover, usually plasmatic MMA in primary diseases is elevated, while in transitory alterations it is used to be normal or slight to moderate level.
When the alteration is a combined deficiency, the biochemical results show also high levels of homocysteine in plasma, which is not seen in the other isolated alterations.

Table 3. Methylmalonic acid and methylcitric acid are elevated in cobalamin genetic and methylmalonyl CoA mutase deficiency, but homocysteine only in cobalamin type C deficiencies.

Disease (case)	MMUT (1)	CblC (2)	MMUT (3)	CblB (4)	MMUT (5)	MMUT (6)	CblC (7)	MMUT (8)
MMA urine; N<8 (mmol/mol creatinine)	243,3	2993	485,5	12710	8648,4	1180	1028	5404
MCA urine; N<3 (mmol/mol creatinine)	9	15,4	18,3	179	88	54,7	10,9	80
MMA plasma; N<0.5 (µmol/L)	8,03	16	8,6	736,6	679,2	*	*	166
Hcys plasma; N<12 (µmol/L)	5	54	5,4	6,4	*	*	55,3	*

Data provided by Metabolic diseases unit of Vall d'Hebron Hospital.
(*) Not performed.

5.6. Reported cases of genetic deficiencies from newborn screening that affect the cobalamin

Since 2013, 8 genetic defects have been detected in Vall d'Hebron Metabolic Laboratory, including one false-negative case before the implementation of second-tier tests.
Among those 8 genetic defects, there were five isolated methylmalonic acidurias (four *mut*^{0} in *MMUT* gene and one CblB in *MMAB* gene) and two methylmalonic acidurias with homocystinuria (CblC).

5.6.1. Patients from newborn screening diagnosed with methylmalonic acidemia and homocystinuria CblC disease

In the **CblC** disease, which is a combined disease, the screening showed high levels of C3, methylmalonic acid and homocysteine. In the studied case, there were a high elevation of MMA and methylcitrate acid, and also homocysteine. Surprisingly, the patient had a bit elevated concentration of cobalamin. It is due to cobalamin cannot become in its active form and for that reason it keeps in the cytoplasm without being used. In mother analysis, high levels of homocysteine and C3-C and low levels of folate were found.

In an independent case of the screening, which is followed in the Vall d'Hebron Hospital, the patient was diagnosed with hemolytic uremic syndrome. The patient has movement retard, cortical atrophy, microcephalia, visual deficit, cognitive retard, uncoordinated ocular movements, and convulsions.
Biochemically, high levels of methylmalonic, methylcitrate, homocysteine and C3/C0 ratio were found, as in any CblC case. Nonetheless, cobalamin serum levels are normal.
In this case, the treatment consists of the external addition of hydroxocobalamin, carnitine, folic acid, betaine, and cholecalciferol (14,15).

5.6.2. Patients from newborn screening diagnosed with methylmalonic acidemia CblB disease

Neonatal screening also revealed isolated methylmalonic aciduria, CblB type, in which the *MMAB* gene has an alteration.
Most patients have an acidotic crisis in the 1st year of life, which causes bone marrow abnormalities and anemia, leukopenia, and thrombocytopenia. And usually, the acylcarnitines show an elevation of C3, C3/C2 and C3/C16.
Despite the newborn screening only showed high levels of C3 (did not have time to perform a second-tier test), the clinical onset happened on the 3rd day of life. In posterior metabolic studies, high levels of methylmalonic acid, methylcitrate acid and 3-hydroxy-propionate were found.
In this case, parents had sickle cell anemia in addition, -so, the most probably- is that the presentation was exacerbated due to this parental deficiency.

5.6.3. Patients from newborn screening diagnosed with methylmalonic acidemia CblA disease

The defect in **CblA** disease results from mutations in the *MMAA* gene, whose protein plays a role in the transfer of AdoCbl from adenosyltransferase to methylmalonyl-CoA mutase and keeping the active form of the mutase-bound AdoCbl.
In the urine diagnostic test, methylmalonic and methylcitric acid levels were elevated, but homocysteine levels were normal. In addition, serum Cbl is usually normal because it cannot be transformed in active cobalamin.

5.6.4. Patients from newborn screening diagnosed with methylmalonyl CoA mutase deficiency

The **MCM** is a mitochondrial homodimeric enzyme that catalyzes the conversion of methylmalonyl coenzyme A (CoA) to succinyl CoA, and its deficiency is caused by an alteration in *MMUT* gene.

An exceptional case of homozygosis in ***MMUT*** gene and simple heterozygosis in ***MACHC*** gene was detected. The fact of being homozygous means that the newborn has the same alteration in the alleles received from both parents.

The newborn screening showed high levels of C3, C3/C2 and methylmalonic acid. In posterior metabolic studies, high levels of methylmalonic and citric acid were found. Surprisingly, cobalamin serum levels were in the normal ranges.

One of the patients had the first sharp debut in the first 36 hours of life with vomiting, drowsiness, hypoglycemia, metabolic acidemia, and hyperammonemia.

The studies revelated high levels of methylmalonic and methylcitrate acid, but also 3-hydroxy-propionate and propionyl-glycine (secondary upstream, metabolites).

The third patient with a mutation in ***MMUT*** gene had his first debut 3 days after the birth, in which he had a cardiac alteration and intense methylmalonic acidemia.

In the screening test appeared high levels of C3 and methylmalonic acid, and in posterior studies added high levels of methylcitrate and low levels of C0.

And the last patient with ***MMUT*** gene mutation was asymptomatic with high levels of C3 and methylmalonic acid in the first screening. In addition, high levels of methylmalonic and methylcitric acids were found. On the other hand, the mother analysis did not reveal any deficiency.

6. Discussion

Despite the increase of secondary Cbl deficiencies, the primary deficiency of vitamin B12 does not increase over the years. This is because the primary deficiencies do not depend on the social movements, otherwise of genetic features (**figures 8 and 9)**. This data is correlated with (1), in which some of the deficient Cbl newborns had mothers with normal serum levels of vitamin B12. A possible explanation is that the currently low limit for Cbl serum is not sensitive enough to point out a deficiency.
This growing trend for secondary Cbl deficiencies could be influenced by a higher presence of the population with characteristic cultural habits in our population (native population of the Indian subcontinent). It means, people who do not eat red meat, or eat it less than necessary for cultural or religious features.

High levels of **C3** appear in practically 100% of the primary and transitory cases. For that reason, it could be said that C3 biomarker is **unspecific** for acquired or genetic deficiencies (**figure 10**).
The percentage of increased Hcys remains high over the years, while that of MMA seems to decrease. It appears to be more sensitive, and the reason could be related to additional requirements of folate and iron -in the mother during pregnancy- which especially affects the remethylation pathway, both in primary or secondary diseases (**figure 10**).
In addition, the recent second-tier test C17 has resulted useless since it has only appeared in one person with transitory alterations and in none with of primary diseases (**figure 10**).

The use of second-tier tests has allowed to decrease the cut-off values to avoid false-negative results, without excessively increasing the number of false-positive cases (**figure 11**), which was the purpose of the newborn screening expansion in 2013.

The levels of metabolites could help to orientate between a primary and a secondary deficiency because in primary diseases the metabolite levels are higher, regardless of whether patients have received a dose of hydroxocobalamin (**table 3**).
The difference between MMA concentration in primary methylmalonic aciduria and acquired cobalamin deficiency is significant in most cases. This can happen because newborns with a secondary deficiency have low levels of Cbl, but they are able to metabolize it when it is ingested, whereas newborns with a genetic deficiency do not.
Nonetheless, for reliable results, the study should be expanded, since the number of births per year may vary.

During pregnancy higher levels of vitamins are required (especially the B group, related to metabolic functions) because the nutritional requirements for the fetus increase. Despite the hypothesis, a high percentage of mothers does not reveal any alteration related to cobalamin (**figure 12**), maybe because the nutritional requirements are sufficient for the mother, but cannot satisfy the vitamin needs of the fetus.

It is important to correlate the iron deficiency from mothers with the low intake of red meat. As appears in **table 1**, the higher sources of cobalamin are meat and not non-meat animal foods.
Moreover, the methyl-trap could also occur in B12 deficient pregnant women who exceed folic acid levels. If the analysis shows low levels of cobalamin, the folate would be inactivated in the form of 5-methyl-tetrahydrofolate (see **figure 5**) (36).

For that reason, a new controlled intervention study should be necessary to accept or discard the hypothesis. One group of mothers with a low intake of meat diet should be supplemented with Cbl, while a second group should continue with the usual diet.

Once the cause of cobalamin deficiency is known, mothers should be treated. In case that the fetus has a deficiency due to a maternal deficit, pregnant women should be supplemented with cobalamin. Furthermore, the levels of cobalamin and folate should be periodically checked so that they are within the minimum and maximum ranges so as not to trigger any pathology.

The effect of treatment does not have the same corrective magnitude in all patients with the same affected gene because the mutation type is decisive and can make that the hydroxocobalamin supplement have more or less effect: the response time and the concentration of methylmalonate in urine can vary from 7 to 3,000 mmol/mol creatinine (**Figure 13**).
An important fact is the family history and the clinical symptomatology. In all the transitory defects the newborns were asymptomatic. Otherwise, the primary deficiencies use to have an early onset, such as metabolic acidosis, hypoglycemia, somnolence, and neurological impairment. Positive familiar history could also help to suspect a primary deficiency.

7. Conclusions

Secondary deficiencies have increased over the years. The main reason for a secondary cobalamin deficiency is poor intake due to different social or economic reasons.

The biochemical findings are useful to suspect the diagnosis both in primary or secondary alterations, even if they are not huge increases. An appropriate quantification of these metabolites together with the corresponding genetic study, can guide the final diagnosis.
The results presented in this work reinforce the idea that for transitory deficiencies, MMA urine levels are lower (between 8 to 150 mmol/mol creatinine), while the methylmalonic acidurias seen are above 243 mmol/mol creatinine to several thousand.
Nevertheless, the increases in Hcys seem to be more sensitive than MMA for secondary diseases due to some additional maternal deficiencies, like folate one.
A completely different scenario appears in classical homocystinuria, because Vall d'Hebron Hospital does not have enough positive cases in newborn screening for CBS or methylcobalamin deficiency disease (surprisingly, probably due to populational bias, 0 cases in 8 years).

In addition, from all the newborns with secondary alterations, only 25-35% of mothers have any alteration in the cobalamin intake, transport or metabolization. As consequence, huge consumption of economic and health resources, even though the temporary deficiency of cobalamin is a minor metabolic problem.
Besides, exist 65-75% of mothers who do not have any alteration related to cobalamin, but their sons yes. As the hypothesis cannot be affirmed or refuted, to know if a deficiency in newborns is caused by poor maternal nutrition, further research with more data is necessary.

The current treatment is highly effective for transitory Cbl deficiencies. In inborn Cbl diseases, although it helps to reduce methylmalonate and methylcitrate levels, in most of the cases they do not tend to decrease enough to be considered normal levels. The research in gene therapy and new treatments remains active.

One of the limitations in these results and conclusions is that populational biases can influence the presence of greater or lesser detection or primary or secondary defects.
Moreover, the statistical calculations have been done with the assumption that the 50% of positive cases correspond to each clinical reference center (Vall d'Hebron Hospital and Sant Joan de Déu Hospital).
In the end, because of the low prevalence of these diseases, the number of samples is also low: only 3 genetic defects have been reported in 8 years. For that reason, to make any change in the newborn detection and management criteria, it would be necessary to study more cases.

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Annex I

Referred hospital for metabolic diseases from newborn heel test in Catalonia.

Hospital General de la Vall d'Hebron

- Hospital de Palamós
- Hospital d'Olot
- Clínica Santa Creu (Figueres)
- Hospital de Figueres
- Hospital de Campdevànol
- Hospital Comarcal de La Selva
- Hospital de Santa Caterina
- Hospital Universitari de Girona Dr. Josep Trueta
- Clínica Girona
- Clínica Bofill (Girona)
- Clínica Quirúrgica Onyar (Girona)
- Casa de naixements Migjorn
- Hospital General de Vic
- Consorci Sanitari Parc Taulí de Sabadell
- Hospital de Mollet-Societat de Socors Mutus
- Hospital de Mataró
- Clínica del Vallés
- Hospital Sant Joan de Déu de Manresa
- Althaia. Clínica Sant Josep de Manresa
- Hospital General d'Igualada
- Hospital General de Granollers
- Hospital Sant Jaume de Calella
- Hospital Sant Bernabé de Berga
- Hospital Universitari Germans Trias i Pujol
- Hospital de l'Esperit Sant
- Hospital de la Santa Creu i Sant Pau
- Hospital del Mar
- Clínica Quirón
- Clínica Nostra Senyora el Pilar
- Clínica Corachán

Hospital Sant Joan de Déu d'Esplugues

- Hospital de Tortosa Verge de la Cinta
- Hospital ed Móra d'Ebre
- Clínica Terres de l'Ebre (Tortosa)
- Serveis Assistencials d'Amposta
- Hospital de El Vendrell
- Pius Hospital de Valls
- Hospital Sant Joan de Reus
- Hospital Mèdic Quirúrgic de Reus
- Hospital Sant Pau i Santa Tecla
- Hospital Universitari de Tarragona Joan XXIII
- Clínica Monegal
- Hospital Vall d'Aran
- Hospital La Seu d'Urgell
- Hospital Comarcal del Pallars
- Hospital Universitari Arnau de Vilanova
- Clínica Perpetuo Socorro (Lleida)
- Clínica de Ponent (Lleida)
- Hospital de Puigcerdà
- Hospital Sant Camil (Sant Pere de Ribes)
- Clínica Mútua de Terrassa
- Hospital Comarcal de l'Alt Penedès
- Hospital Mútua de Terrassa
- Hospital de Terrassa
- Capio Hospital General de Catalunya
- Hospital Comarcal de Sant Boi de Llobregat
- Hospital Sant Joan de Déu de Martorell
- Hospital de la Creu Roja de l'Hospitalet de Llobregat
- Clínica Diagonal d'Esplugues de Llobregat
- Centre Mèdic Tèknon
- Hospital de Barcelona (SCIAS)
- Clínica Tres Torres
- Clínica Sant Jordi
- Clínica Sagrada Família
- Clínica Nostra Senyora del Remei
- Hospital casa de Maternitat
- Institut Universitari Dexeus
- Centre Mèdic Delfos
- Hospital Universitari Sagrat Cor