PRDX1 gene-related epi-cblC disease is a common type of inborn error of cobalamin metabolism with mono- or bi-allelic MMACHC epimutations

Catia Cavicchi1, Abderrahim Oussalah2, Silvia Falliano1, Lorenzo Ferri1, Alessia Gozzini1, Serena Gasperini3, Serena Motta3, Miriam Rigoldi4, Giancarlo Parenti5, Albina Tummolo6, Concetta Meli7, Francesca Menni8, Francesca Furlan8, Marta Daniotti9, Sabrina Malvagia10, Giancarlo la Marca10,11, Céline Chery2, Pierre-Emmanuel Morange12, David Tregouet13, Maria Alice Donati9, Renzo Guerrini1,14, Jean-Louis Guéant2 and Amelia Morrone1,14*

Abstract

Background: The role of epigenetics in inborn errors of metabolism (IEMs) is poorly investigated. Epigenetic changes can contribute to clinical heterogeneity of affected patients but could also be underestimated determining factors in the occurrence of IEMs. An epigenetic cause of IEMs has been recently described for the autosomal recessive methylmalonic aciduria and homocystinuria, cblC type (cblC disease), and it has been named epi-cblC. Epi-cblC has been reported in association with compound heterozygosity for a genetic variant and an epimutation at the MMACHC locus, which is secondary to a splicing variant (c.515-1G > T or c.515-2A > T) at the adjacent PRDX1 gene. Both these variants cause aberrant antisense transcription and cis-hypermethylation of the MMACHC gene promoter with subsequent silencing. Until now, only nine epi-cblC patients have been reported.

Methods: We report clinical/biochemical assessment, MMACHC/PRDX1 gene sequencing and genome-wide DNA methylation profiling in 11 cblC patients who had an inconclusive MMACHC gene testing. We also compare clinical phenotype of epi-cblC patients with that of canonical cblC patients.

Results: All patients turned out to have the epi-cblC disease. One patient had a bi-allelic MMACHC epimutation due to the homozygous PRDX1:c.515-1G > T variant transmitted by both parents. We found that the bi-allelic epimutation produces the complete silencing of MMACHC in the patient’s fibroblasts. The remaining ten patients had a mono-allelic MMACHC epimutation, due to the heterozygous PRDX1:c.515-1G > T, in association with a mono-allelic MMACHC genetic variant. Epi-cblC disease has accounted for about 13% of cblC cases diagnosed by newborn screening in the Tuscany and Umbria regions since November 2001. Comparative analysis showed that clinical phenotype of epi-cblC patients is similar to that of canonical cblC patients.

*Correspondence: amelia.morrone@meyer.it; amelia.morrone@unifi.it

1 Molecular and Cell Biology Laboratory of Neurometabolic Diseases, Paediatric Neurology Unit and Laboratories, Meyer Children’s Hospital, Viale Pieraccini 24, 50139 Florence, Italy

Full list of author information is available at the end of the article
Introduction
The role of epigenetics in inborn errors of metabolism (IEMs) is poorly investigated. IEMs are a large and heterogeneous group of genetic disorders caused by single gene defects that disrupt normal metabolism. Many IEMs can be detected by newborn screening (NBS) with significant reduction of mortality and disease burden, if promptly treated [1, 2]. Besides their genetic causes, it is increasingly recognizing that epigenetic changes contribute to clinical heterogeneity of patients affected by IEMs [3–5]. Moreover, routine genetic test of genes responsible for IEMs may be inconclusive in some patients with a clear-cut clinical phenotype. Epigenetics could have an underestimated causal role in these patients. In fact, an epigenetic cause of IEMs, named *epi-cblC*, has been recently reported for methylmalonic aciduria and homocystinuria, cobalamin C type (*cblC* disease; OMIM #277400) [6].

*CblC* disease is the most common inborn error of intracellular vitamin B12 (also called cobalamin, cbl) metabolism with a worldwide prevalence ranging from 1:37,000 to 1:100,000 [7]. It is caused by a deficiency of the *MMACHC* protein, which is needed to convert vitamin B12 into adenosylcobalamin (AdoCbl) or methylcobalamin (MeCbl), the two cofactors of methylmalonyl-CoA mutase and methionine synthase, respectively [8]. This combined enzymatic defect leads to increased concentrations of methylmalonic acid (MMA) and homocysteine (Hcy) in plasma and urine with normal or decreased concentrations of methionine (Met) in plasma [8]. Propionylcarnitine (C3) and the C3/acycetilcarnitine (C2) ratio, combined with the second-tier markers MMA and total Hcy, are the tandem mass spectrometry biomarkers for the early detection of *cblC* disease in expanded NBS programs [9, 10].

*CblC* disease is clinically heterogeneous for signs and symptoms and age of presentation. Depending on the age of presentation, patients have been classified into early-onset (<1 year) and late-onset (>1 year) [11]. More recently, three clinical forms have been distinguished: prenatal, infantile and non-infantile [8].

*CblC* disease is caused by recessive pathogenic variants in the *MMACHC* gene (Gene ID: 25974; OMIM *609831, location: 1p34.1) [11] in which 115 disease-causing variants have been reported so far (http://www.biobase-international.com/product/hgmd), some of them clustering according to ethnicity [12]. The most common is c.271dupA p.(Arg91Lysfs*14) which represents at least 30% of mutant alleles in Europe [12]. Next-generation sequencing (NGS) permits differential diagnosis among the spectrum of cobalamin disorders and can also identify copy number variations (CNVs) that account for about 6% of reported *MMACHC* disease-causing variants [8]. However, some cases remain without a conclusive molecular diagnosis. Some of these cases recently have been diagnosed as affected by *epi-cblC* disease [6]. Previously reported *epi-cblC* patients are compound heterozygous for a genetic variant and a secondary epimutation at the *MMACHC* locus. The secondary epimutation results from a splicing variant in the adjacent *PRDX1* gene (peroxiredoxin 1; Gene ID: 5052; OMIM *176763*), corresponding to either a c.515-1G>T or a c.515-2A>T substitution. Both these *PRDX1* variants are located in an acceptor site and cause an aberrant antisense transcription starting from *PRDX1* and encompassing the *MMACHC* gene promoter. Antisense transcription through the promoter of the *MMACHC* gene induces cis-hypermethylation of the promoter with subsequent *MMACHC* transcriptional silencing [6].

Herein, we report the molecular analysis of 11 Italian probands, with a diagnosis of methylmalonic aciduria and homocystinuria between 2008 and 2018, who had previously been tested with *MMACHC* gene sequencing without reaching any conclusive molecular diagnosis. Among them, ten turned out to have the *epi-cblC* form of the disease, with compound heterozygosity for an epigenetic and a genetic *MMACHC* variant. One patient had a bi-allelic *MMACHC* epimutation due to the homozygous *PRDX1* c.515-1G>T variant transmitted by both parents.

**Results**
**Clinical and biochemical data**
The clinical and metabolic findings of the *epi-cblC* patients are shown in Additional file 1: Tables S2 and S3. All patients had methylmalonic acidemia and homocystinuria and an inconclusive molecular diagnosis for the *MMACHC* gene. In two patients (Pts 3 and 9), disease onset was probably in the prenatal period, as
an intrauterine growth restriction was observed. Common symptoms at onset in the young patients included failure to thrive, vomiting, hypotonia, respiratory distress and hematologic abnormalities. Recurrent infections, developmental delay, and ocular disease were frequent later manifestations. Maculopathy was ascertained in seven young patients, in five occurring within the first year of life. Haemolytic uremic syndrome occurred in two patients (Pts 8 and 9). Pt 8 developed anaemia and acute renal failure at 1 month of life which required haemodialysis. Pt 9 developed anaemia with high levels of serum lactate dehydrogenase and proteinuria in the first few days of life.

A diagnosis of combined methylmalonic acidemia and homocystinuria was made by NBS in four patients (Pts 2, 7, 9 and 11) and after a clinical/biochemical assessment in the remaining seven patients. One of the patients recognized clinically (Pt 1) was negative at NBS for metabolic disorders but underwent metabolic investigation because of hyperalaninaemia detected on his DBS at birth and early symptomatology. Pt 3 developed symptoms before his NBS results were available. Four patients (Pts 4–6 and 8) were born before the NBS was introduced and one patient was an adult when diagnosed (Pt 10). Three of the patients identified by NBS (Pts 2, 9 and 11) were promptly confirmed to have methylmalonic aciduria, thanks to a second-tier test performed within the sixth day of life which determined MMA on the DBS.

The adult patient (Pt 10) had a complex medical history, mainly characterized by antiphospholipid syndrome, renal symptoms and thrombotic events. At 53 years of age, he received a diagnosis of homocystinuria. It was only after urinary organic analysis at the age of 63 years that increased MMA was detected and that a final diagnosis of methylmalonic acidemia and homocystinuria was made. This patient never developed the typical retinal alterations of cblC disease. His only ocular signs were xerophthalmia associated with astigmatism in the context of systemic lupus erythematosus and dry eyes, and cataract, probably related to his age.

With the exception of Pt 4, who died in the second month of life during an acute metabolic crisis, all patients have been treated with hydroxocobalamin, levocarnitine, betaine and folates since diagnosis. The levels of metabolic markers in 9/10 treated patients show a good response to long-term therapy; the exception is Pt 7 who still has unsatisfactory levels of MMA and Hcy. Therapy has not, however, prevented neurological and ocular symptoms, even in patients identified by NBS and treated early. This is in line with observations in canonical cblC patients [12, 13].

Routine molecular analysis for methylmalonic acidemia and homocystinuria
Sanger sequencing of the MMACHC gene identified a heterozygous genetic variant in 10/11 patients. No pathogenetic variant was identified in the MMACHC gene of Pt 11. Molecular findings of the epi-cblC patients are summarized in Table 1. All variants identified in the MMACHC gene were previously reported [11, 14]. The c.271dupA p.(Arg91Lysfs*14) and the c.666C > A p.(Tyr222*) were found in 5 and 2 patients, respectively. Pt 10 was heterozygous for the MMACHC:c.617G > A p.(Arg206Gln) variant [14] and homozygous for the thermolabile polymorphism NM_005957.5:c.665C > T p.(Ala222Val) of the MTHFR gene [15]. NGS analysis excluded other genetic defects of cbl metabolism and CNVs in Pts 9, 10 and 11.

Biochemical phenotyping, PRDX1 sequencing and MMACHC expression analysis for a conclusive diagnosis of epi-cblC
The biochemical phenotyping of fibroblasts from Pts 1, 2, 10 and 11 suggested an intracellular defect of cobalamin metabolism. Complementation analysis clearly indicated that these four patients belonged to the cblC complementation group. Sequencing analysis of the PRDX1 gene identified the c.515-1G > T variant at a heterozygous state in all the patients (10/11) who also harboured an MMACHC genetic variant at a heterozygous level (Table 1). With the exception of the 63-year-old patient (Pt 10), MMACHC/PRDX1 gene sequencing extended to parents of these probands confirmed the allelic segregation of the mutant alleles in all of them. In Pt 11, we found the PRDX1 c.515-1G > T variant at a homozygous state with no genetic variants in MMACHC and any other genes of cobalamin metabolism (Fig. 1a). Both parents of Pt 11 were heterozygous for this PRDX1 gene variant (Fig. 1a). Family pedigree of Pt 11 is shown in Fig. 1b. Multiplex RT-PCR assays of mRNA from fibroblasts of Pt 11 did not detect any MMACHC transcript, compared to results obtained in two healthy controls (Fig. 1c). This result suggested that a bi-allelic epimutation could produce the complete silencing of MMACHC.

Epigenome-wide association study
All the DNA methylome profiles of the analysed subjects were of high quality and were used in statistical analyses (Additional file 1: Fig. S1). As shown in the epi-Manhattan plot (Fig. 2a), the epigenome-wide association study retrieved a top significant locus in chromosome 1 at the CpG island CpG:33 on the CCDC163/MMACHC bidirectional promoter. All the CpG probes located in CpG island CpG:33 were fully unmethylated among controls.
and hemimethylated among the epi-cblC patients carrying the heterozygous PRDX1:c.515-1G>T variant (Table 2 and Fig. 2b). Instead, the epi-cblC proband with the bi-allelic epimutation of the CpG island CpG:33 caused by the homozygous PRDX1 c.515-1G>T splice variant (Pt 11) exhibited a full-methylated profile of all the CpG probes, while his parents exhibited a hemimethylated profile (Fig. 2c).

Estimation of epi-cblC prevalence and PRDX1 c.515-1G>T allele frequency

From November 2001 to date, 600,387 newborns have been analysed by the Tuscany-Umbria NBS program at Meyer Children’s Hospital. Twenty-three newborns were diagnosed with cblC disease, including three (13%) who had an epi-cblC disease (Pts 1, 2 and 11). Hence, the birth prevalence of cblC and epi-cblC diseases in Tuscany and Umbria could be estimated at around 1:26,000 and 1:200,000, respectively (Additional file 1: Fig. S2a). In the cohort of total cases with cblC phenotype and conclusive molecular diagnosis (104 probands), epi-cblC disease accounted for 11% of cases (11/104 probands) (Additional file 1: Fig. S2b). In this cohort, 23 different genetic variants in the MMACHC gene have been identified. The allele frequency of the

### Table 1 Pathogenic variants identified in the MMACHC/PRDX1 genes of epi-cblC patients

| Pt | Italian origin | Diagnosis (age) | Variant 1 | Origin of variant 1 | Variant 2 | Origin of variant 2 | Epimutation in MMACHC | Age at epi-cblC molecular diagnosis | Onset |
|----|----------------|----------------|----------|---------------------|----------|---------------------|-----------------------|------------------------------------|-------|
| 1  | Central        | C/B (10 d)     | MMACHC:c.271dupA p.(Arg91Lysfs*14) | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 10 y                              | Early  |
| 2  | Central        | NBS (6 d)      | MMACHC:c.666C>A p.(Tyr222*)        | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 8 y                                | Early  |
| 3  | Southern       | C/B (16 d)     | MMACHC:c.271dupA p.(Arg91Lysfs*14) | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 11 y                              | Early  |
| 4  | Southern       | C/B (2 m)      | MMACHC:c.666C>A p.(Tyr222*)        | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 10 y                              | Early  |
| 5  | Northern       | C/B (2 m)      | MMACHC:c.331C>T p.(Arg111*)        | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 6 y                                | Early  |
| 6  | Northern       | C/B (6 m)      | MMACHC:c.481C>T p.(Arg161*)        | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 7 y                                | Early  |
| 7  | Southern       | NBS (1 m)      | MMACHC:c.271dupA p.(Arg91Lysfs*14) | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 18 y                              | Early  |
| 8  | Southern       | C/B (1 m)      | MMACHC:c.271dupA p.(Arg91Lysfs*14) | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 2 y                                | Early  |
| 9  | Northern       | NBS (4 d)      | MMACHC:c.271dupA p.(Arg91Lysfs*14) | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 75 y                              | Late   |
| 10 | Northern       | C/B (63 y)     | MMACHC:c.617G>A p.(Arg206Gln)      | ND       | PRDX1:c.515-1G>T    | ND       | Het                 | 7 y                                | Early  |
| 11 | Central        | NBS (4 d)      | PRDX1:c.515-1G>T                   | Paternal | PRDX1:c.515-1G>T    | Maternal | Het                 | 7 y                                | Early  |

C/B, diagnosis of methylmalonic aciduria and homocystinuria performed after a clinical/biochemical assessment; d, days; m, months; het, heterozygous; hom, homozygous; NBS, diagnosis of methylmalonic aciduria and homocystinuria performed by expanded newborn screening; ND, not determined; y, years

*a* We identified the same genotype of Pt 4 in an abortion specimen of this family. Such foetus was biochemically affected

*b* Methylation analysis of this patient has been previously reported by Gueant et al. [6]
common variants is shown in Additional file 1: Fig. S2c. As expected, **MMACHC**:c.271dupA was the most common variant, accounting for about 54% of mutated alleles causing **cblC**. **PRDX1**:c.515-1G > T is the second most frequent disease-causing variant in our cohort with an estimated allele frequency of about 6% (12/208 alleles) (Additional file 1: Fig. S2c).

**Comparison of clinical phenotype between epi-cblC and canonical cblC patients**

Clinical comparison of our epi-cblC patients and canonical cblC patients, belonging to a larger cohort reported by Huemer et al., showed that the clinical manifestations of epi-cblC patients were similar to those of canonical cblC patients (Table 3). However, by restricting the comparison to our MMACHC:c.271dupA homozygotes from NBS, we found that the clinical phenotype of the epi-cblC homozygote appeared to be more severe than the c.271dupA homozygotes (Additional file 1: Table S4). Metabolic findings in the epi-cblC homozygote resembled those of the MMACHC:c.271dupA homozygotes, although a lower value of plasma methionine was detected in the epi-cblC homozygote (2.6 μmol/l compared to the mean value of 7.1 in the c.271dupA homozygotes) (Additional file 1: Table S4).
MMACHC gene and one patient with compound heterozygosity for the epimutation and the related c.515-1G > T variant leads to a loss of MMACHC protein expression through the second 1G > T allele [6, 18].

Discussion

Due to its prevalence, methylmalonic aciduria combined with homocystinuria type cblC is frequently identified by NBS[10]. Early detection of the disease allows prompt treatment and surveillance of affected newborns, which significantly reduces mortality and disease burden. For these reasons, cblC has been included in the Recommended Universal Screening Panel (RUSP) of the USA since 2006[16]. In Italy, NBS for metabolic disorders has been recently regulated and made mandatory in all Italian regions (Law n. 267/2016). As a result, the number of cblC cases detected by NBS has increased.

When a combined methylmalonic aciduria with homocystinuria is suspected, genetic testing is needed to make differential diagnoses of the inherited cbl disorders which have been associated with at least 12 genes so far [17]. MMACHC is the most frequently mutated gene. However, a single heterozygous variant or no variants at all can be found in some patients with a cblC phenotype.

Herein, we report the clinical, biochemical and molecular study of 11 epi-cblC probands who did not have a conclusive molecular diagnosis after routine MMACHC gene sequencing. In all patients the molecular diagnosis of epi-cblC disease was established by the identification of MMACHC epimutation and the related c.515-1G > T variant in the PRDX1 gene. We classified ten patients with compound heterozygosity for the epimutation and a genetic variant of the MMACHC gene and one patient with a bi-allelic homozygous MMACHC epimutation due to the homozygous PRDX1:c.515-1G > T.

Until now, nine epi-cblC patients have been reported, all of whom with a mono-allelic MMACHC epimutation and a MMACHC genetic variant affecting the other allele [6, 18]. The PRDX1:c.515-1G > T variant was found in patients with Caucasian origin [6, 18] whereas the PRDX1:c.515-2A > T variant was only detected in one patient of Japanese-Korean ancestry [6]. To our knowledge, we are describing the first instance of epi-cblC due to a bi-allelic MMACHC epimutation.

Because of the limited number of epi-cblC patients described in the literature so far, epidemiological data about epi-cblC disease are lacking. Our retrospective study indicates that epi-cblC disease is more frequent than thought. It accounts for about 13% of NBS cblC patients diagnosed in Tuscany and Umbria (birth prevalence ~ 1:200,000) and for 11% of Italian cblC probands genetically characterized in our Unit. The PRDX1 c.515-1G > T allele frequency currently reported in gnomAD (Genome Aggregation Database: https://gnomad.broadinstitute.org/) is 4.01e-5 (10/249150 European non-Finnish alleles, 5/10 Southern European). Our data show that this frequency is undoubtedly underestimated.

Genotype–phenotype correlations are possible in our patients. The PRDX1:c.515-1G > T variant leads to a loss of MMACHC protein expression through the secondary MMACHC epimutation triggered by its aberrant transcription [6]. Thus, an early- or late-onset phenotype in epi-cblC patients harbouring the heterozygous epimutation depends on the pathogenicity of the second MMACHC genetic variant. Consistently, our early onset epi-cblC cases had MMACHC truncating variants.
Table 3  Comparison of signs and symptoms in epi-cblC and canonical-cblC patients

|                                | Epi-cblC | Canonical-cblC* |
|--------------------------------|----------|-----------------|
| Number of reported cases       | 11 (this study) | 169             |
| Eating disorders/failure to gain weight |          |                 |
| Small for gestational age       | ++ [2 Pts] | +               |
| Feeding difficulties, failure to thrive | +++++ [9 Pts] | +++             |
| Nervous system                  |          |                 |
| Decreased consciousness and/or apnoea | +++++ [9 Pts] | ++              |
| Seizures                        | +++ [5 Pts] | +++             |
| Ataxia                          | −         | +               |
| Movement disorder and/or abnormal muscle tone | +++++ [9 Pts] | +++             |
| Peripheral neuropathy/subacute degeneration of spinal cord | (+) | ++ |
| Hydrocephalus                   | −         | ++              |
| Visual impairment (retinopathy, optic atrophy) | +++++ [9 Pts] | +++ |
| Developmental disorder/cognitive impairment | +++++ [8 Pts] | +++ |
| Behavioural/mental disorders    | ++ [2 Pts] | +               |
| Microcephaly                    | (+)      | ++              |
| Blood and bone marrow           |          |                 |
| Megaloblastic anaemia           | +++ [5 Pts] | ++             |
| Pancytopenia/neutropenia        | (+)      | ++              |
| Recurrent severe infections     | +++++ [4 Pts] | (+) |
| Kidneys                         |          |                 |
| Haemolytic uraemic syndrome     | ++ [2 Pts] | ++             |
| Glomerulopathy                  | −         | +               |
| Tubulointerstitial nephropathy  | −         | +               |
| Cardiopulmonary                 |          |                 |
| Cardiac malformation            | ++ [2 Pts] | +             |
| Cardiomyopathy                  | (+)      | ++              |
| Interstitial pneumonia          | −         | +               |
| Pulmonary hypertension          | −         | +               |
| Vascular                        |          |                 |
| Stroke                          | (+)      | (+)             |
| Venous thrombosis/embolism      | (+)      | +               |
| Malformations                   |          |                 |
| Facial dysmorphism              | ++ [2 Pts] | +             |
| Skeletal deformity              | +++++ [3 Pts] | (+) |
| Gastrointestinal                |          |                 |
| Cheilitis/gastritis             | (+)      | −               |
| Liver steatosis                 | −         | +               |
| Skin                            |          |                 |
| Dermatitis/rash/hyperpigmentation | −          | +             |
| Other                           |          |                 |
| Hydrops foetalis                | −         | +               |
| Metabolic acidosis and/or hyperammonaemia | +++++ [5 Pts] | + |
| Temperature instability/hypothermia | (+)      | +               |

++++, very frequently (> 50% of cases); ++++, frequently (25–50% of cases); ++, infrequently (10–25% of cases); +, occasionally seen (<10% of cases); (+) single case reports, probably disease-related conditions; -, absent/not reported; Pts, patients. The number of our epi-cblC patients who exhibited that sign/symptom is indicated in square brackets.

* Clinical data for canonical-cblC patients (signs, symptoms and their frequencies) are derived from Huemer et al. [12]
Our findings indicate that the clinical signs and symptoms observed early in life are consistent with the silencing of transcription of the *MMACHC* gene. The newborn had a c.271dupA genotype. As an homozygous patient, this observation is based on a unique epi-cblC homozygous genotype associated with a more severe clinical phenotype than the p.Arg206Gln mutation. In fact, it has been reported that a PRDX1 deletion leads to damage of telomeric DNA upon oxidative stress [29]. Although PRDX1 gene variants have not been identified in human tumours [21], it is known that a tumorigenic human melanoma cell line, named MeWo-LC1, has a cellular phenotype identical to that of *cbIC* patients' cells. The growth of this cell line is methionine dependent, as a cellular phenotype identical to that of patients' cells is observed [20]. The molecular mechanisms of PRDX1-induced carcinogenesis are still not fully understood, although it has recently been reported that a PRDX1 deletion leads to damage of telomeric DNA upon oxidative stress [29]. Although PRDX1 gene variants have not been identified in human tumours [21], it is known that a tumorigenic human melanoma cell line, named MeWo-LC1, has a cellular phenotype identical to that of *cbIC* patients’ cells. The growth of this cell line is methionine dependent, as a consequence of the epigenetic inactivation of *MMACHC* gene through PRDX1 aberrant transcription [30]. For these reasons, we recommend periodically monitoring subjects bearing the c.515-1G>T variant to prevent the potential risk of cancer.

According to the criteria that define the types of epimutations [31, 32], *epi-cblC* results from a secondary constitutional epimutation, which produces the phenotype of the disease. The epimutation is the consequence of a nearby PRDX1 sequence alteration that produces an aberrant antisense transcription encompassing the *MMACHC* promoter [6]. An epigenetic silencing caused by aberrant transcription (i.e. transcriptional interference) has been previously described for Lynch syndrome (*MSH2* gene) [33] and α-thalassaemia (*HBA2* gene) [34]. Transcriptional interference arises at tandem and convergent promoters and involves different mechanisms, e.g. promoter occlusion, roadblocking and RNA polymerase collision [35]. The result of these events is DNA methylation, but the involved processes and factors are not
entirely clear [36]. In these three diseases, the abolishment of transcription termination and concomitant transcriptional read-through lead to methylation of the CpG island and silencing of the downstream gene, irrespective of the orientation of the latter. In epi-cblC disease, the loss of poly(A) signal is due to a PRDX1 splice site variant which causes skipping of its last exon and aberrant antisense transcription [6]. In both α-thalassaemia and Lynch syndrome, the causes of transcriptional elongation are genomic deletions. Specifically, in α-thalassaemia the genomic deletion in the 3′-end of forward LUC7L gene produces an elongated transcript that overlaps the HBA2 reverse gene [34]. In Lynch syndrome, terminal deletions of forward EPCAM gene result in transcriptional read-through across the MSH2 promoter in sense direction [33]. A difference between epimutations of MMACHC and HBA2 concerns their maintaining or not in germ cells. In sperm, the MMACHC secondary epimutation is maintained [6], while the epigenetic silencing of HBA2 is erased [34, 37].

The secondary epimutation of epi-cblC patients is located in a reverse CCDC163P-forward MMACHC-reverse PRDX1 trio of genes (R1-F2-R3) [6]. Gueant et al. [6] reported that similar configuration of gene trios exists in other regions of the human genome and provided a list of such trios. Thus, on a broader perspective, this epigenetic mechanism could be associated with other IEMs, as for example: Sandhoff disease (OMIM #268800), ACAT2 deficiency (OMIM #203750), Mucolipidosis III gamma (OMIM #252605), cystinosis (OMIM #219800, 219750 and 219900) and galactosialidosis (OMIM #256540). This finding should be kept in mind when routine molecular analysis results inconclusive in patients with a clear-cut clinical/biochemical phenotype.

Conclusions
In summary, we found that epi-cblC disease caused by the MMACHC epimutation secondary to the PRDX1:c.515-1G>T variant is a relatively frequent inborn error of cobalamin metabolism and report the first instance of epi-cblC due to a bi-allelic MMACHC epimutation. MMACHC epimutation/PRDX1 mutation analyses should be part of routine genetic testing for all patients presenting with a metabolic phenotype that combines methylmalonic aciduria and homocystinuria. Future research could elucidate the epidemiology, pathophysiology and prognosis of epi-cblC patients.

Methods
Patients
Eleven patients referred from several Italian regions to our diagnostic laboratory between 2008 and 2018 were included in this study. All patients received a diagnosis of methylmalonic aciduria and homocystinuria by NBS or after a clinical/biochemical assessment. At the time of diagnosis, seven patients were neonates (up to 1 month of age), three were young infants (2–6 months old) and one was an adult (63 years old). Two patients (Pts 1 and 2) had previously been described due to their ocular manifestations [38]. Mutation and epimutation analysis of the MMACHC gene for the adult patient (Pt 10) was previously reported [6]; herein, we describe his medical history and genotype–phenotype correlation. The study was performed in line with the principles of the 1964 Helsinki Declaration and approval was granted by the Ethics Committee of the Tuscany Region (No. CS_01/2021). Informed consent was obtained from all individual participants included in the study.

Biochemical analysis
For the cases detected by NBS, diagnosis was initially made by identifying increased levels of C3 and reduced Met. As for clinically diagnosed patients, confirmatory laboratory testing was performed on all positive NBS cases. Tests we carried out included plasma and/or urinary amino acid analysis, determination of plasma homocysteine, plasma and/or urinary organic acid analysis and/or plasma acylcarnitine analysis. In four patients (Pts 1, 2, 10, and 11) specialized biochemical assays for the cobalamin metabolic pathway were performed on cultured fibroblasts in the laboratory of Prof. Matthias Baumgartner (University Children's Hospital, Zurich, Switzerland).

Sequencing analysis
Genomic DNA of the patients and their parents was extracted from peripheral blood using a QIASymphony instrument (Qiagen, Hilden, Germany). We performed Sanger sequencing of the MMACHC gene in all patients, as previously reported [38]. In patients 9–11, we also performed targeted NGS of 40 genes linked to methylmalonic aciduria, hyperhomocysteinemia and disorders of cobalamin and folate metabolism using a custom-designed panel (Illumina, San Diego, CA). We prepared libraries using the Nextera rapid capture enrichment kit (Illumina) according to the manual instructions. The libraries were sequenced by a paired-end 2 × 150 bp protocol on a MiSeq System (Illumina, San Diego, CA) to obtain an average coverage of above 100x, with >95% of target bases covered at least 15x. For data analysis, we used the BWA, Picard and GATK tools. Variant annotation was performed by the ANNOVAR tool. To identify possible CNVs, the sequencing data were analysed by the CoNVaDING tool [39]. Screening of the known c.515-1G>T and c.515-2A>T variants in the PRDX1 gene was performed on genomic DNA by Sanger sequencing.
of the specific intron–exon boundary (intron 6-exon 7). This boundary was amplified by PCR using the primer pair PRDX1-7fw and PRDX1-7rev (Additional file 1: Table S1), appropriately designed to avoid an artefact due to the presence of a poly-T after the termination codon in sequencing analysis. PCR products were sequenced with the same primers by using the BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 3130 GeneticAnalyser (Applied Biosystems, Foster City, CA, USA). The reference sequences used for nomenclature of the MMACHC and PRDX1 variants were NM_015506.3 and NM_002574.3, respectively.

MMACHC expression analysis in the homozygous PRDX1 patient
To demonstrate a transcriptional silencing of the MMACHC gene caused by the PRDX1:c.515-1G > T variant in the homozygous patient (Pt 11), we performed a non-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was obtained from cultured fibroblasts of the patient and two normal controls with RNeasy Protect Mini Kit (Qiagen, Hilden, Germany). cDNA was produced using the random hexamers and the TaqMan Reverse Transcription kit (Applied Biosystems). A multiplex PCR assay was performed to simultaneously amplify a fragment of 512 bp for the MMACHC gene and a fragment of 174 bp for the ACTB gene, used as a housekeeping gene. Primers for multiplex RT-PCR and PCR conditions are listed in Additional file 1: Table S1. PCR products were checked by agarose gel electrophoresis.

DNA methylome analysis
To confirm that the PRDX1:c.515-1G > T variant identified in our patients produces an epimutation in the MMACHC promoter, we performed an epigenome-wide DNA methylome analysis, as previously described [6], in the epi-cblC patients carrying the heterozygous PRDX1:c.515-1G > T variant. The same analysis was performed in the proband (Pt 11) carrying the PRDX1:c.515-1G > T mutant allele among the MMACHC homozygous patient and his parents. We carried out a bisulphite conversion of 600 ng of DNA extracted from whole blood using the EZ DNA Methylation kit (Zymo Research, Proteigene, Saint-Marcel, France). Genome-wide profiling of DNA methylation was determined using the Infinium MethylationEPIC BeadChip array (Illumina, Paris, France), according to the manufacturer’s instructions. The Infinium MethylationEPIC BeadChip provides a coverage of 850,000 CpG probes in enhancer regions, gene bodies, promoters, and CpG islands. The arrays were scanned on an Illumina iScan® system, and raw methylation data were extracted using the Illumina GenomeStudio Methylation Module. For each CpG probe, the methylation level was described as a β value, ranging between 0 (fully unmethylated CpG probe) and 1 (fully methylated CpG probe). Background correction and normalization was implemented using the SWAN method (R Package Minfi) [40]. We visually inspected the whole-genome distribution of the CpG probes according to their β values. In the epigenome-wide association study, we compared the whole blood DNA methyle profile of subjects with epi-cblC with 350 controls from the MARTHA cohort [41]. For each CpG probe, we compared the mean β values between epi-cblC subjects and controls using a t-test with Bonferroni and false discovery rate corrections to account for multiple testing. Due to the low sample size, and considering the exploratory approach of our analysis, we used the smoothed P-value transformation by converting nominal P-values obtained from the t-test to smoothed P-values using a window radius of 3, as previously reported [6]. All statistical analyses were performed using the SNP & Variation Suite (v8.8.1; Golden Helix, Inc., Bozeman, MT, USA).

Estimation of epi-cblC prevalence and PRDX1 c.515-1G > T allele frequency
The NBS-case cohort of the Tuscany and Umbria regions was used to estimate the birth prevalence of epi-cblC disease. Instead, to estimate the frequency of the PRDX1:c.515-1G > T mutant allele among cblC patients, we used the total cblC-case cohort which includes all patients with a confirmed molecular diagnosis analysed in our laboratory since 2006. We divided the cblC patients into three groups: MMACHC-cases or “canonical-cblC” with bi-allelic genetic MMACHC variants, MMACHC/PRDX1-cases with compound heterozygosity for a MMACHC genetic variant and an epigenetic variant, and PRDX1-cases or “homozygous epi-cblC” with the PRDX1:c.515-1G > T variant at a homozygous level leading to bi-allelic MMACHC epimutation.

Comparison of clinical phenotype between epi-cblC and canonical cblC patients
To evaluate if the clinical phenotype of epi-cblC is similar to that of canonical cblC, we first compared signs and symptoms between epi-cblC and cblC patients according to a detailed review on cblC presentation [12]. Then, we compared NBS data and clinical manifestations of our epi-cblC homozygous patient with MMACHC:c.271dupA homozygous patients from the Tuscany-Umbria NBS cohort, who we deemed representative of early onset canonical cblC.
Contributions

All authors commented on previous analysis of the control population. CC, AM and JLG drafted the manuscript. AO and JLG performed methylome analysis. PEM and DT performed methylome AG performed sequencing, expression and epidemiological analyses. AO, CCh provided samples. SM and GlM performed biochemical analysis. CC, SF, LF and MR, GP, AT, CM, FM, FF, MD, MAD and RG clinically ascertained patients and contribution to the study design. AM and JLG supervised the study. SG, SM, Lerner-Adler, Cavicchi, CCh, MF, GL, IM, J, et al. Efficacy of early treatment in patients with cobalamin C disease. I. Clinical presentations, diagnosis and outcomes. J Inherit Metab Dis. 2012;35(1):103–14.

Carrillo-Carrasco N, Pandiella A, Lodish H, et al. Combined methylmalonic acidemia and homocystinuria, cblC type. II. Complications, pathophysiology, and outcomes. J Inherit Metab Dis. 2012;35(1):103–14.

Carrillo-Carrasco N, Pandiella A, Lodish H, et al. Combined methylmalonic acidemia and homocystinuria, cblC type. I. Clinical presentations, diagnosis and outcomes. J Inherit Metab Dis. 2012;35(1):91–102.

La Marca G, Malvagia S, Pasquini E, Innocenti M, Donati MA, Zammarchi E. Rapid 2nd-tier test for measurement of 3-OH-propionic and methylmalonic acids on dried blood spots: reducing the false-positive rate for propionylcarnitine during expanded newborn screening by liquid chromatography-tandem mass spectrometry. Clin Chem. 2007;53(7):1364–9.

Huemer M, Kolrich V, Rinaldo P, Baumgartner MR, Merinero B, Pasquini M, et al. Newborn Screening, Biochemistry and Pharmacology Laboratory, Meyer Children’s Hospital, Florence, Italy. 11 Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy. 12 Aix-Marseille University, INRAE, INSERM, C2VN, Marseille, France. 13 INSERM, UMR_S937, ICAN Institute, Université Pierre et Marie Curie, Paris, France. 14 Department of NEUROFARBA, University of Florence, Florence, Italy.

Acknowledgements

We would like to thank the AMMeC (Associazione Malattie Metaboliche Congenite, Italia) for continuing support.

Authors’ contributions

CC and AM conceived and designed the study design. JLG made substantial contribution to the study design. AM and JLG supervised the study. SG, SM, MR, GP, AT, CM, FM, FF, MD, MAD and RG clinically ascertained patients and provided samples. SM and GI performed biochemical analysis. CC, SF, LF and AG performed sequencing, expression and epidemiological analyses. AO, CCh and JLG performed methylome analysis. PEM and DT performed methylome analysis of the control population. CC, AM and JLG drafted the manuscript. AO contributed in drafting specific parts. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

The methylome analyses were funded by FHU ARRIMAGE and the French Agence Nationale de la Recherche, PIA project “Lorraine Université d’Excellence” (ANR-15-IDEX-04-LUE).

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files, or they are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was performed in line with the principles of the 1964 Helsinki Declaration. Approval was granted by the Ethics Committee of the Tuscany Region (CS_01/2021). Informed consent was obtained from all individual participants included in the study. This article does not contain any studies with animal subjects performed by any of the authors.

Consent for publication

Participants consented to publication of their data.

Competing interests

The authors declare that they have no competing interests.

Author details

1 Molecular and Cell Biology Laboratory of Neurometabolic Diseases, Paediatric Neurology Unit and Laboratories, Meyer Children’s Hospital, Viale Pieraccini 24, 50139 Florence, Italy. 2 INSERM, UMR_S1256 Nutrition-Genetics-Environmental Risk Exposure and Reference Centre of Inborn Metabolism Diseases, University of Lorraine and University Hospital Centre of Nancy (CHRU Nancy), Nancy, France. 3 Rare Metabolic Disease Unit, Department of Paediatrics, Fondazione MBBM, Monza, Italy. 4 Mario Negri Institute for Pharmacological Research IRCCS, Bergamo, Italy. 5 Metabolic Unit, Federico II Hospital, Napoli, Italy. 6 Metabolic Disease Unit, Giovanni XXIII Hospital, Bari, Italy. 7 Rare Metabolic Disease Unit, G. Rodolico Hospital, Catania, Italy. 8 Foundation IRCCS Ca’Granda Ospedale Maggiore Policlinico, Paediatric Highly Intensive Care Unit, Milan, Italy. 9 Metabolic and Muscular Unit, Meyer Children’s Hospital, Florence, Italy. 10 Newborn Screening, Biochemistry and Pharmacology Laboratory, Meyer Children’s Hospital, Florence, Italy. 11 Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy. 12 Aix-Marseille University, INRAE, INSERM, C2VN, Marseille, France. 13 INSERM, UMR_S937, ICAN Institute, Université Pierre et Marie Curie, Paris, France. 14 Department of NEUROFARBA, University of Florence, Florence, Italy.

Received: 22 February 2021  Accepted: 16 June 2021

Published online: 02 July 2021

References

Fuller M. Laboratory diagnosis of lysosomal diseases: newborn screening to treatment. Clin Biochem Rev. 2020;41(2):53–66.

Vernon HJ. Inborn errors of metabolism: advances in diagnosis and therapy. JAMA Pediatr. 2015;169(8):778–82.

Di Risi T, Vinciguerra R, Cuomo M, Della Monica R, Rocco E, Cocozza S, et al. DNA methylmethionin on Fabry disease. Clin Epigenetics. 2021;13(1):24.

Hasan S, Sidransky E, Tayebi N. The role of epigenetics in lysosomal storage disorders: uncharted territory. Mol Genet Metab. 2017;122(3):10–8.

Rutten MGS, Rots MG, Oosterveer MH. Exploiting epigenetics for the treatment of inborn errors of metabolism. J Inherit Metab Dis. 2020;43(1):63–70.

Gueant JL, Chery C, Oussalah A, Nadaf J, Cohelo D, Josse T, et al. APRDX1 mutant allele causes a MMACHC secondary epimutation in cblC patients. Nat Commun. 2018;9(1):67.

Carrillo-Carrasco N, Venditti CP. Combined methylmalonic acidemia and homocystinuria, cblC type. I. Clinical presentations, diagnosis and management. J Inherit Metab Dis. 2012;35(1):91–102.

La Marca G, Malvagia S, Pasquini E, Innocenti M, Donati MA, Zammarchi E. Rapid 2nd-tier test for measurement of 3-OH-propionic and methylmalonic acids on dried blood spots: reducing the false-positive rate for propionylcarnitine during expanded newborn screening by liquid chromatography-tandem mass spectrometry. Clin Chem. 2007;53(7):1364–9.

Huemer M, Kolrich V, Rinaldo P, Baumgartner MR, Meninero B, Pasquini E, et al. Newborn screening for homocystinurias and methylation disorders: systematic review and proposed guidelines. J Inherit Metab Dis. 2015;38(6):1007–19.

Lerner-Ellis JP, Tirone JC, Pawelek PD, Dore C, Atkinson JL, Watkins D, et al. Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. Nat Genet. 2006;38(1):93–100.

Huemer M, Diodato D, Schwahn B, Schiff M, Bandeira A, Benoist JF, et al. Guidelines for diagnosis and management of the cobalamin-related remethylation disorders cblC, cblD, cblE, cblF, cblG, MTHFR deficiency. J Inherit Metab Dis. 2017;40(1):21–48.

Arens-Nicklas RC, Whitaker AM, Kaplan P, Cuddapah S, Burfield J, Blair J, et al. Efficacy of early treatment in patients with cobalamin C disease.
identified by newborn screening: a 16-year experience. Genet Med. 2017;19(8):926–35.

14. Granaup N, Sulem P, Sandholt CH, Thorleifsson G, Ahiuwalla TS, Stenfors-Dottir V, et al. Genetic architecture of vitamin B12 and folate levels uncovered applying deeply sequenced large datasets. PLoS Genet. 2013;9(6):e1003530.

15. Froost P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet. 1995;10(1):111–3.

16. McCandless SE, Wright EJ. Mandatory newborn screening in the United States: history, current status, and existential challenges. Birth Defects Res. 2020;112(4):350–66.

17. Huemer M, Baumgartner MR. The clinical presentation of cobalamin-related disorders: from acquired deficiencies to inborn errors of absorption and intracellular pathways. J Inherit Metab Dis. 2019;42(4):686–705.

18. Pollini L, Tolwe M, Nardocchia F, Galosi S, Carducci C, di Carlo E, et al. Multiple sclerosis and intracellular cobalamin defect (MMACHC/PRDX1) comorbidity in a young male. Mol Genet Metab Rep. 2020;22:100560.

19. Lerner-Ellis JP, Anastasio N, Liu J, Coelho D, Suormala T, Stucki M, et al. Spectrum of mutations in MMACHC, allelic expression, and evidence for genotype-phenotype correlations. Hum Mutat. 2009;30(7):1072–81.

20. Froese DS, Krojer T, Wu X, Shrestha R, Kiyani W, von Delft F, et al. Structure switching from a peroxidase to a molecular chaperone function. Cell. 2004;117(5):625–35.

21. Ding C, Fan X, Wu G. Peroxiredoxin 1—an antioxidant enzyme in cancer. J Cell Mol Med. 2017;21(1):193–202.

22. Lederwood EC, Marshall JW, Weijman JA. The role of peroxiredoxin 1 in oxidative sensing and transducing. Arch Biochem Biophys. 2017;676:1–7.

23. Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, et al. Two enzymes interacts with the Myc Box II domain of c-Myc and selectively alters its biological function and target gene expression. J Biol Chem. 2002;277(45):43175–84.

24. Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, et al. Essential role for the peroxiredoxin Prdx1 in erythroid antioxidant defence and tumour suppression. Nature. 2003;424(6948):561–5.

25. Egler RA, Fernandes E, Rothermund K, Sereika S, de Souza-Pinto N, Jaruga P, et al. Regulation of reactive oxygen species, DNA damage, and c-Myc function by peroxiredoxin 1. Oncogene. 2005;24(54):8038–50.

26. Abeby E, Ahmed W, Redon S, Simian V, Lingner J. Peroxiredoxin 1 protects telomeres from oxidative damage and preserves telomeric DNA for extension by telomerase. Cell Rep. 2016;17(12):3107–14.

27. Loewy AD, Niles KM, Anastasio N, Watkins D, Lavoie J, Lerner-Ellis JP, et al. Epigenetic modification of the gene for the vitamin B12 chaperone MMACHC can result in increased tumorigenicity and methionine dependence. Mol Genet Metab. 2009;96(6):261–7.

28. Hitchins MP. Constitutional epimutation as a mechanism for cancer causality and heritability? Nat Rev Cancer. 2015;15(10):625–34.

29. Sloane MA, Ward RL, Hesson LB. Defining the criteria for identifying constitutional epimutations. Clin Epigenetics. 2016;8:39.

30. Litgenberg MJ, Kuiper RP, Geurts van Kessel A, Hoogerbrugge N. EPICAM deletion carriers constitute a unique subgroup of Lynch syndrome patients. Fam Cancer. 2013;12(2):169–74.

31. Tufarelli C, Stanley JA, Garrick D, Sharpe JA, Ayyub H, Wood WG, et al. Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat Genet. 2003;34(2):157–65.

32. Shearwin KE, Callen BP, Egan JB. Transcriptional interference—a crash course. Trends Genet. 2005;21(6):339–45.

33. Kühnel T, Heinz HSB, Utz N, Božič T, Horsthemke B, Steenpass L. A human somatic cell culture system for modelling gene silencing by transcriptional interference. Heliyon. 2020;6(1):e03261.

34. Hitchins MP. Inheritance of epigenetic aberrations (constitutional epimutations) in cancer susceptibility. Adv Genet. 2010;70:201–43.

35. Bacci GM, Donati MA, Pasquini E, Munier F, Cavicchi C, Morrone A, et al. Optical coherence tomography morphology and evolution in cblC disease-related maculopathy in a case series of very young patients. Acta Ophthalmol. 2017;95(8):e776–82.

36. Hitchins MP. Constitutional epimutation (constitutional epimutations) in cancer susceptibility. Adv Genet. 2010;70:201–43.

37. Bacci GM, Donati MA, Pasquini E, Munier F, Cavicchi C, Morrone A, et al. Optical coherence tomography morphology and evolution in cblC disease-related maculopathy in a case series of very young patients. Acta Ophthalmol. 2017;95(8):e776–82.

38. Johansson LF, van Dijk F, de Boer EN, van Dijk-Bos KK, Jongbloed JD, van der Hout AH, et al. CoNVaDING: single exon variation detection in Infinium DNA methylation microarrays. Bioinformatics. 2014;30(10):1363–9.

39. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2010;26(21):2591–9.

40. Dick KJ, Nelson CP, Tsaprouni L, Sandling JK, Aïssi D, Wahl S, et al. DNA methylation and body-mass index: a genome-wide analysis. Lancet. 2014;383(9933):1990–8.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.