TO THE EDITOR:

Megaloblastic anemia, infantile leukemia, and immunodeficiency caused by a novel homozygous mutation in the DHFR gene

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Dihydrofolate reductase (DHFR) is a critical enzyme in folate metabolism that reduces folic acid to dihydrofolic and tetrahydrofolic acid and provides an important target for antineoplastic, antimicrobial, and anti-inflammatory drugs. Defective DHFR activity leads to megaloblastic anemia syndrome combined with severe cerebral folate deficiency, and cerebral tetrahydrobipterin deficiency due to a germ line missense mutation in DHFR has been reported.1,2 Folate represents a large family of water-soluble vitamins that play an important role in DNA synthesis, repair, and transmethylation pathways.3 Folate is also a substrate for purine and thymidine synthesis and a methyl donor for homocysteine to methionine conversion, with low folate status being reflected by elevated plasma homocysteine concentrations.4 Cerebral tetrahydrobipterin is required for the formation of dopamine, serotonin, and norepinephrine and the hydroxylation of aromatic amino acids as a link to neurodevelopmental symptoms.5

To date, only 6 patients have been reported with DHFR mutations who presented with a spectrum of neurological symptoms, with hematological findings noted in addition to neurological symptoms in some patients.1,2 We report on a Dutch pedigree with a novel homozygous DHFR mutation.

Patient material was obtained with informed consent by the Erasmus Medical Ethics Committee for the Dutch immunodeficiency study (NL40331.078). Whole-genome sequencing and whole-exome sequencing and mutation analysis were performed according to reported procedures.6-8 Lymphocyte phenotyping CD4+/CD8+ T cells, B cells, naïve/memory T- and B-cell subsets, and NK cells was carried out using immunostaining for flowcytometry as described.9 Enzymatic DHFR activity was defined using Epstein-Barr virus–transformed B-lymphoblastoid cell lines according to previously used methods.2

The index patient presented at 2 months of age. He was the third child of White parents. At his first immunization, he had fever and was admitted to the hospital where he received a diagnosis of moderate megaloblastic anemia and thrombocytopenia. Extensive metabolic tests of blood and urine were non-informative (Table 1). Hydroxocobalamin and folic acid suppletion remained without effect. At 4 months
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Table 1. Hematology, chemistry and metabolite parameters in blood and CSF

| Parameter                              | Case 1   | Case 2   | Case 3   | N ranges     |
|----------------------------------------|----------|----------|----------|--------------|
| ESR (mm/1st h)                         | 12       | N.D.     | N.D.     | <20          |
| CRP (mg/L)                             | <3       | 4        | 14       | <5           |
| Hb (mmol/L)                            | 5.2      | 2.0      | 4.0      | 6.0-8.9      |
| MCV (fL)                               | 89.7     | 110      | 85       | 75-95        |
| Reticulocytes (%)                      | 1.4      | 2.0      | 0.7      | 0.5-2        |
| Red cell distribution width (%)        | 15.2     | 17.2     | 22.4     | 12-16        |
| Leukocytes (10^9/L)                    | 29.3     | 68.9     | 11.8     | 6-17         |
| Thrombocytes (10^9/L)                  | 147      | 159      | 93       | 150-600      |
| % neutrophils                          | 50.4     | N.D.     | 60       | 35-75        |
| % eosinophils                          | 26.7     | N.D.     | 14       | 2-10         |
| % lymphocytes                          | 21.7     | N.D.     | 22       | 17-42        |
| % monocytes                            | 2.2      | N.D.     | 1        | 5-12         |
| % basophils                            | 0        | N.D.     | 0        | 0-2          |
| % blasts                               | 0        | N.D.     | 0        | 0-2          |
| Coombs test                            | neg      | N.D.     | N.D.     | neg          |
| LDH (U/L)                              | 2294     | 8298     | 3519     | <300         |
| Haptoglobin (mg/L)                     | 1.2      | <0.10    | <0.10    | 0-2          |
| Ferritin (µg/L)                        | 860      | N.D.     | 5200     | <250         |
| Folic acid (nmol/L)                    | 4        | N.D.     | 11.6     | 10-50        |
| VitB12 (pmol/L)                        | 359      | N.D.     | 217      | 150-700      |
| Homocysteine (µmol/L)                  | 14†      | N.D.     | 15†      | 0-9          |
| Transcobalamin normal                  | N.D.     | N.D.     | present  |              |
| Thrombopoietin (U/mL)                  | 280      | N.D.     | N.D.     | <40          |
| Creatinine (µmol/L)                    | 36       | 32       | 32       | 35-80        |
| ASAT (U/L)                             | 47       | 330      | 22       | <50          |
| ALAT (U/L)                             | 26       | 131      | 19       | <50          |
| CSF (with folic acid supplementation)  |          |          |          |              |
| S-MTHF (nmol/L; for BH4+BH2)           | N.D.     | N.D.     | 374      | 105-233      |
| Biotin (nmol/L; for BH4+BH2)           | N.D.     | N.D.     | 22       | 10-50        |

Values represent the cell numbers and concentrations of the indicated parameters determined during the first week upon admission. Values outside of the age-related normal reference ranges are marked in bold.

ALAT, alanine amino transferase; ASAT, aspartate amino transferase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; LDH, lactate dehydrogenase; MCV, mean corpuscular volume; S-MTHF, S-methyltetrahydrofolate.

†Value at admission at an academic center, after the start of folic acid and vitamin B12 supplementation because of anemia and MCV of 105 fL. At admission, the bone marrow biopsy still showed megaloblastic anemia (Figure 1A).

Mildly increased, not compatible with homozygous MTHFR, CBS, or MS enzyme defect.

of age, the boy’s health deteriorated, and symptoms such as failure to thrive, dyspnea, coughing, and vomiting were observed. Bronchoalveolar lavage detected Pneumocystis jirovecii, and the infection was treated with high-dose co-trimoxazole (trimethoprim-sulfamethoxazole [TMP-SMX]) and prednisolone. Because of progressive respiratory failure, the index died a week later. Blood erythrocyte sedimentation rate and C-reactive protein levels were low in the presence of leukocytosis, increased lactate dehydrogenase, ferritin, and liver enzyme tests (Table 1). We observed normal absolute lymphocyte counts, including CD4/CD8 ratio and naïve/memory proportions when compared with age-matched controls, normal lymphocyte proliferation, and normal CD40L upregulation upon in vitro activation of T cells (data not shown).

Despite normal serum folate levels, there was persistent megaloblastic anemia in the bone marrow (Figure 1A,B).

The second patient was born as the second child to healthy parents. She presented after a choking incident with hypothermia (34.5°C) at 2 months of age in a moribund state with deep anemia (hemoglobin 2.0 mmol/L). A postmortem examination revealed hepatosplenomegaly and enlarged lymph nodes. Although the diagnosis is limited because of late sampling in a very poor condition and myelodysplasia cannot be formally excluded, the diagnosis of acute myeloid leukemia (AML) was made after extensive discussion of the case, based on CD33 staining and partly myeloperoxidase-positive blasts (Figure 1C).

The third patient was born to the same parents described in the previous instance. At 6 weeks of age, he was treated for herpes stomatitis and developed unexplained anemia for which he received red blood cell transfusions. A bone marrow smear at 7 weeks of age showed decreased erythropoiesis with megaloblasts and dysplastic myelopoiesis with hypersegmented neutrophils. At 13 weeks of age, he was admitted with fever, anemia, hepatomegaly, and dyspnea due to P. jirovecii infection. We again observed normal absolute lymphocyte counts, including CD4/CD8 ratio, subsets, and immunoglobulin G (IgG), IgA, and IgM plasma levels (data not shown). High-dose TMP-SMX and prednisolone therapy was initiated, and extracorporeal membrane oxygenation (ECMO) support was provided. An MRI of the brain showed signs of cortical laminar necrosis, hemorrhagic leukomalacia, vermis inferior hypoplasia, and diffuse tissue loss of the supra and infratentorial parenchyma with normal myelinization (Figure 1D), which could be related to not only DHFR deficiency but also ECMO. While the patient was on ECMO support, supplementation of IV folic acid was initiated after (genetic) diagnosis. The child’s condition improved, and after initial improvement and detubation, pulmonary hypertension and respiratory insufficiency recurred, and the patient died of these pulmonary complications at the age of 4 months.

We identified a novel homozygous DHFR mutation in 3 infants by next-generation sequencing, cosegregating with the phenotype across 11 sequenced individuals (Figure 2A) and predicted to have a deleterious effect on protein function, as was biochemically confirmed (Figure 2B,C).

Family members were recruited for whole-genome sequencing. Because of rapid deterioration in the health of the third patient, a clinical single nucleotide polymorphism array analysis and ultra-rapid exome sequencing were performed in the meantime. Both approaches independently yielded a homozygous novel missense variant in the DHFR gene (NM_000791.3; c.61G>A; p.Gly21Arg), cosegregating with disease status under a recessive mode of inheritance and confirmed by Sanger sequencing (Figure 2). There were no abnormal hematology parameters in the heterozygous carriers (data not shown).

The phenotypes reported thus far were focusing on the neurological aspects of the disease. The first 3 patients reported had severely delayed psychomotor development, generalized seizures, and cerebral and cerebellar atrophy, whereas the other 3 siblings were still asymptomatic or had childhood absence epilepsy with eyelid myoclonus and mild learning disabilities.
not have neurological symptoms at the start. The secondary microcephaly reported in the 2 previous publications was absent in our cases.

As expected, neither vitamin B12 nor folic acid in patient 1 improved any of the clinical and laboratory manifestations. In patient 3, we demonstrate that although hematological recovery was seen with treatment administered at the time, ECMO could be stopped. In addition, the supplementation of folinic acid did not prevent the following detrimental course of the disease. Cerebrospinal fluid (CSF) measurement of folate metabolites in this patient showed that folinic acid supplementation led to increased levels of 5-methyl-tetrahydrofolate (THF) in CSF, but a beneficial effect on the neurological outcome could not be acclaimed. This may be related to the complete absence of DHFR activity in this patient (Figure 2C), whereas in previously reported patients, some DHFR activity at rest could still be detected.1,2 A more severe neurological phenotype is less likely to be attenuated by folinic acid supplementation. Therefore, it remains to be shown to what extent folinic acid can affect the course of disease in every case of DHFR deficiency.

The discrepancy in complete recovery of blood but not of cerebral levels has been noted previously.1 The CSF-to-blood folate ratio in healthy humans is 3:1.10 In hereditary folate malabsorption, CSF folate is very low or undetectable, even when blood folate levels in patients are restored to normal or elevated levels through folate supplementation. In addition, in children affected with FOLR1 mutations, a low level of 5-methyl-THF in the CSF with normal serum and erythrocyte folate levels are present.

The active isomers of 5-formyl-THF (leucovorin) or 5-methyl-THF (metafolin) are preferred forms to cross the blood-brain barrier to enter the brain because their affinity for the solute transporter is more than 2 orders of magnitude greater than the affinity of folic acid for this transporter.10

Folate deficiency can cause uracil misincorporation into DNA, leading to chromosome breakage,11 which was proposed to contribute to the increased risk of cancer, consistent with the possible and seemingly congenital AML in the second case in our pedigree.

The increased risk of infections due to an underlying immunodeficiency has been noted before in case of defective folate uptake.10,12,13 Similar to cases 1 and 3 described here, *P jiroveci* pneumonia has been reported under these folate-deficient conditions.10,12,13 Systemic folate deficiency can be easily mistaken for severe combined immunodeficiency with normal T- and B-cell functions.
counts and differentiation, which may develop before any apparent neurological symptoms to further delay the final diagnosis of an underlying metabolic disorder. However, the high-dose TMP-SMX treatment for *P jiroveci* may have affected the clinical course in DHFR deficiency because trimethoprim inhibits DHFR. The negative contribution of TMP-SMX cannot be excluded, despite the intravenous supplementation of folic acid and hematological recovery in patient 3.

In summary, we describe a pedigree with previously unrecognized clinical features of DHFR deficiency, an ultrarare inborn error of folate metabolism. The clinical spectrum was characterized by infantile-onset megaloblastic anemia and pneumocystis infection, AML, and pancytopenia with subsequent neurodevelopmental delay unresponsive to treatment. Depending on early diagnosis and the severity of DHFR deficiency, adequate treatment with (parenteral) high-dose 5-formyl-THF may attenuate neurologic features of
the disease but could be less effective in the complete absence of any remaining DHFR activity.

Acknowledgments: The authors are very grateful to King Lam, hemopathologist at Erasmus MC hospital, for sharing the available immunohistochemistry picture of patient 2. The authors also thank the family for supporting and approving the publication to properly describe and illustrate this often unrecognized and unknown disorder. The authors also thank NHIR BioResource volunteers for their participation, and gratefully acknowledge NHIR BioResource centres, NHS Trusts and staff for their contribution. The authors also thank the National Institute for Health Research and NHS Blood and Transplant. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care.

This study was supported by The National Institute for Health Research England (grant number RG65966) and the Center of Immunodeficiencies Amsterdam (CIDA-2015).

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Conflict-of-interest disclosure: The authors declare no competing financial interests.

A complete list of the members of the NBR-RD PID Consortium appears in “Appendix.”

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References

1. Banka S, Blom HJ, Walter J, et al. Identification and characterization of an inborn error of metabolism caused by dihydrololate reductase deficiency. Am J Hum Genet. 2011;88(2):216-225.
2. Cario H, Smith DE, Blom H, et al. Dihydrofolate reductase deficiency due to a homozygous DHFR mutation causes megaloblastic anemia and cerebral folate deficiency leading to severe neurologic disease. Am J Hum Genet. 2011;88(2):226-231.
3. Zheng Y, Cantley LC. Toward a better understanding of folate metabolism in health and disease. J Exp Med. 2019;216(2):253-266.
4. Blom HJ, Smulders Y. Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. J Inherit Metab Dis. 2011;34(1):75-81.
5. Watkins D, Rosenblatt DS. Lessons in biology from patients with inherited disorders of vitamin B12 and folate metabolism. Biochimie. 2016;126:3-5.
6. Arts P, Simons A, AlZahrani MS, et al. Exome sequencing in routine diagnostics: a generic test for 254 patients with primary immunodeficiencies. Genome Med. 2019;11(1):38.
7. Thaventhiran JED, Lango Allen H, Burren OS, et al. Whole-genome sequencing of a sporadic primary immunodeficiency cohort. Nature. 2020;583(7814):90-95.
8. Turro E, Astle WJ, Megy K, et al. Whole-genome sequencing of patients with rare diseases in a national health system. Nature. 2020;583(7814):96-102.
9. Tijm tenburg P, Lango Allen H, Burns SO, et al; NIHR BioResource-Rare Diseases Consortium. Loss-of-function nuclear factor KB1 (NFKB1) variants are the most common monogenic cause of common variable immuno deficiency in Europeans. J Allergy Clin Immunol. 2018;142(4):1285-1296.
10. Zhao R, Min SH, Oiu A, et al. The spectrum of mutations in the PCFT gene, coding for an intestinal folate transporter, that are the basis for hereditary folate malabsorption. Blood. 2007;110(4):1147-1152.
11. Blount BC, Mack MM, Wehr CM, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci USA. 1997;94(7):3290-3295.
12. Malatak JJ, Morran MM, Moughan B. Isolated congenital malabsorption of folic acid in a male infant: insights into treatment and mechanism of defect. Pediatrics. 1999;104(5 Pt 1):1133-1137.
13. Shin DS, Mahadeo K, Min SH, et al. Identification of novel mutations in the proton-coupled folate transporter (PCFT-SLC46A1) associated with hereditary folate malabsorption. *Mol Genet Metab.* 2011;103(1):33-37.

14. Borutzky A, Crompton B, Bergmann AK, et al. Reversible severe combined immunodeficiency phenotype secondary to a mutation of the proton-coupled folate transporter. *Clin Immunol.* 2009;133(3):287-294.

15. Keller MD, Ganesh J, Heltzer M, et al. Severe combined immunodeficiency resulting from mutations in MTHFD1. *Pediatrics.* 2013;131(2):e629-e634.