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Cardiovascular manifestations of intermediate and major hyperhomocysteinemia due to vitamin B12 and folate deficiency and/or inherited disorders of one-carbon metabolism: a 3.5-year retrospective cross-sectional study of consecutive patients

Julien Levy,[1,2] *Rosa-Maria Rodriguez-Guéant,*[1,2,3] *Abderrahim Oussalah,*[1,2,3] *Elise Jeannesson,*[1,2] *Denis Wahl,*[4] *Stéphane Ziuly,*[4] *and Jean-Louis Guéant*[1,2,3]

[1]Department of Molecular Medicine, Division of Biochemistry, Molecular Biology, Nutrition, and Metabolism, University Hospital (CHRU) of Nancy, Nancy, France; [2]Reference Centre for Inborn Errors of Metabolism (ORPHA67872), University Hospital (CHRU) of Nancy, Nancy, France; [3]INSERM UMR_S 1256, Nutrition, Genetics, and Environmental Risk Exposure (NGERE), Faculty of Medicine of Nancy, University of Lorraine, INSERM, Nancy, France; and [4]INSERM UMR_S 1116 DCAC and CHRU-Nancy, Vascular Medicine Division and Regional Competence Center for Rare Auto-Immune Diseases, University of Lorraine, INSERM, University Hospital (CHRU) of Nancy, Nancy, France

ABSTRACT

Background: The association of moderate hyperhomocysteinemia (HHcy) (15–30 μmol/L) with cardiovascular diseases (CVD) has been challenged by the lack of benefit of vitamin supplementation to lowering homocysteine. Consequently, the results of interventional studies have confused the debate regarding the management of patients with intermediate/severe HHcy.

Objective: We sought to evaluate the association of intermediate (30–100 μmol/L) and severe (>100 μmol/L) HHcy related to vitamin deficiencies and/or inherited disorders with CVD outcomes.

Methods: We performed a retrospective cross-sectional study on consecutive patients who underwent a homocysteine assay in a French University Regional Hospital Center. Patients with CVD outcomes were assessed for vitamin B12, folate, Hcy, methylmalonic acid, and next-generation clinical exome sequencing.

Results: We evaluated 165 patients hospitalized for thromboembolic and other cardiovascular (CV) manifestations among 1006 patients consecutively recruited. Among them, 84% (138/165) had Hcy >30 μmol/L, 27% Hcy >50 μmol/L (44/165) and 3% Hcy >100 μmol/L (5/165). HHcy was related to vitamin B12 and/or folate deficiency in 55% (87/165), mutations in one or more genes of one-carbon and/or vitamin B12 metabolisms in 11% (19/165), and severe renal failure in 15% (21/141) of the studied patients. HHcy was the single vascular risk retrieved in almost 9% (15/165) of patients. Sixty % (101/165) of patients received a supplementation to treat HHcy, with a significant decrease in median Hcy from 41 to 17 μmol/L (IQR: 33.6–60.4 compared with 12.1–28). No recurrence of thromboembolic manifestations was observed after supplementation and antithrombotic treatment of patients who had HHcy as a single risk, after ∼4 y of follow-up.

Conclusion: The high frequency of intermediate/severe HHcy differs from the frequent moderate HHcy reported in previous observational studies of patients with pre-existing CVD. Our study points out the importance of diagnosing and treating nutritional deficiencies and inherited disorders to reverse intermediate/severe HHcy associated with CVD outcomes. *Am J Clin Nutr* 2021;113:1157–1167.

Keywords: homocysteine, vitamin B12, folate, cardiovascular disease risk, thromboembolic manifestations, inborn errors of metabolism, 1-carbon metabolism

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Supplemental Table 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

Address correspondence to J-LG (e-mail: jean-louis.gueant@univ-lorraine.fr) or R-MR-G (e-mail: rosa-maria.gueant-rodriguez@univ-lorraine.fr).

Abbreviations used: AAF, alternative allele frequency; ASAT, aspartate aminotransferase; ATB, antithrombin; CKD-EPI, chronic kidney disease-EPIdemiology formula; CV, cardiovascular; CVD, cardiovascular disease; GFR, glomerular filtration rate; Hcy, homocysteine; HHcy, hyperhomocysteinemia; KDIGO, kidney disease improving global outcomes; LA, lupus anticoagulant; MMA, methylmalonic acid; NGS, next-generation sequencing; RBC, red blood cell; UPLC, ultra performance liquid chromatography; VTE, venous thromboembolism; 1-CM, 1-carbon metabolism.

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Introduction

Cardiovascular diseases (CVDs) are a leading cause of death worldwide (1). The WHO defines CVDs as disorders affecting the heart and blood vessels, including coronary artery disease, cerebrovascular disease, peripheral arterial disease, congenital heart defects, deep vein thrombosis, and pulmonary embolism (1). Atherosclerosis is a prominent pathophysiological component of arterial CVDs (2) that is enhanced by cardiovascular (CV) risk factors, including smoking, high cholesterol, high blood pressure, diabetes, abdominal obesity, and psychosocial effects (3). These risk factors explain the major part but not the whole landscape of arterial CVDs. Major venous thromboembolism (VTE) risk factors include cancer, thrombophilia such as antiphospholipid syndrome, major surgery, and acute medical condition with prolonged immobilization. Other risk factors, such as an increase in homocysteine (Hcy) concentrations, produce other types of vascular injury and need additional evaluations. Hcy is an intermediate metabolite of 1-carbon metabolism (1-CM), which promotes endothelial dysfunction, oxidative stress, inflammation, cell proliferation, and thrombosis (4, 5). The increase in Hcy is usually classified into 3 categories, moderate (15–30 μmol/L), intermediate (30–100 μmol/L), or severe (>100 μmol/L) hyperhomocysteinemia (HHcy). The metabolism of Hcy relies on nutritional factors that include folate, vitamin B12, choline, riboflavin, and vitamin B6 (6–10). Deficiency in ≥1 of these vitamins produces HHcy, which can be reduced or even normalized by supplementation (10–14). Intermediate and severe HHcy may also be caused by renal failure and mutations in genes involved in 1-CM as well as those related to vitamin B12 and folate (15–17). The association between HHcy and CVD was first proposed >40 y ago by McCully, who reported vascular lesions in patients with intermediate and severe HHcy related to inherited disorders of 1-CM (18). Later, a vast majority of observational studies concluded that moderate HHcy is associated with CVD risk. These studies have shown that a moderate increase in Hcy is a risk factor for CV mortality, ischemic heart disease, peripheral arterial disease, and venous thrombosis (19–27). In contrast, most interventional studies with vitamin supplementation found no benefit of lowering Hcy in patients with or at risk of CVD (28–31). Several meta-analyses confirmed these conclusions and challenged the causality of the association between HHcy and CVD risk (28–31). Nonetheless, the influence of intermediate and severe HHcy on CVD outcomes in association with vitamin deficiencies and/or inherited metabolic disorders has not been studied explicitly in most observational and interventional studies (32), since a vast majority of these studies have included patients with baseline Hcy concentrations <30 μmol/L. The frequencies of genetic, metabolic, and nutritional causes and the effects on CVD outcomes of lowering intermediate/severe HHcy have earned a too limited interest, despite the previous study by McCully (18).

To address this gap in knowledge, we carried out a retrospective cross-sectional study that assessed consecutive patients in whom intermediate or severe HHcy was diagnosed during an assessment at the University Regional Hospital Center of Nancy for the occurrence of CVD outcomes. Some of these patients were subject to more in-depth metabolic and genetic evaluation and were treated at the Reference Centre for Inborn Errors of Metabolism (ORPHA67872).

Methods

Study design and population

We carried out a retrospective cross-sectional study on consecutive patients who had serum Hcy assessment during a consultation or hospitalization at the University Hospital of Nancy between January 2015 and August 2018.

Study aim

The primary aim was to evaluate the association with outcomes in patients with CVD of intermediate (30–100 μmol/L) and severe (>100 μmol/L) HHcy related to vitamin deficiencies and/or inherited disorders.

The secondary aim was to report HHcy evolution in patients after adapted supplementations and CVD manifestations during a 4-year follow-up. Reported outcomes included the decrease of HHcy and correction of B vitamin deficits and the occurrence of CVD manifestations related to coronary artery disease, cerebrovascular disease, peripheral arterial disease, deep vein thrombosis, VTE, and pulmonary embolism.

Patient selection criteria

Hcy was assayed in patients who were each assigned to 1 of 5 groups based on their disease, including CV/thromboembolic, neurological, cognitive, hematological, and digestive. Among these groups, we focused our study on patients affected by CV and/or VTE manifestations.

Data collected for the study

Clinical data were retrieved through electronic chart review using DxCare® software. We considered the following clinical data: sex; age; risk factors, including diabetes, smoking, arterial hypertension, and dyslipidemia; early CV family history; BMI (kg/m^2), obesity, chronic renal failure, hemodialysis, kidney transplant, bariatric surgery, and drug intake. Biological data, including those related to folate/vitamin B12 status, CV risk factors, and thrombophilia were extracted using the GLIMS laboratory information management system v9 (MIPS). Dyslipidemia and arterial hypertension were defined according to the guidelines of the European Society of Cardiology, with cutoffs of >1.60 g/L and <0.40 g/L for LDL cholesterol and HDL cholesterol, respectively, and cutoffs of >140 mmHg and >90 mmHg, for systolic and diastolic blood pressures, respectively. Biochemical data were retrieved from the Nancy Biochemical Database, which is a prospectively maintained database that collects biochemical results from consecutive patients hospitalized in 67 healthcare departments at the University Hospital of Nancy (33–36). The "Nancy Biochemical Database" is registered at the French National Commission on Informatics and Liberty, CNIL, under the record number 1763197v0.

Vitamin B12, folate, homocysteine, methylmalonic acid, and other biological assays

Vitamin B12 and folate serum concentrations were determined by the SNB SimulTRAC-box for radioimmunoassay with [57Co]-vitamin B12/[125]-folate as tracers (MP Biomedicals). Red blood cell (RBC) folate was determined using the same

radioimmunoassay method after total blood extract hemolysis in the presence of ascorbic acid. Plasma homocysteine and methylmalonic acid (MMA) concentrations were determined by ultra performance liquid chromatography (UPLC)-MS/MS, by using an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters Corporation). Patients were classified into the following 5 subgroups according to Hcy concentration: *1*) Hcy <15 μmol/L, *2*) Hcy: 15–30 μmol/L, *3*) Hcy: 30–50 μmol/L, *4*) Hcy: 50–100 μmol/L, and *5*) Hcy >100 μmol/L. We defined the vitamin B12 deficit by the association of a B12 concentration <150 pmol/L and an MMA concentration >0.35 μmol/L and/or Hcy >15 μmol/L. The deficit in folate was defined by a serum folate concentration <7 nmol/L and an Hcy concentration >15 μmol/L.

Other biochemical parameters, including fasting plasma concentrations of glucose, cholesterol, triglycerides, HDL cholesterol, aspartate aminotransferase (ASAT), alanine aminotransferase, C-reactive protein, and albumin were determined by colo-turbidimetry on an AU 2700 Olympus Chemistry Analyzer (Beckman Coulter). LDL-cholesterol values were calculated using the Friedwald formula. Plasma creatinine was used to calculate the glomerular filtration rate (GFR) using the Chronic Kidney Disease–EPIdemiology formula (CKD-EPI) (33). Hematological parameters, including hemoglobin and mean corpuscular volume, were determined by fluorescence flow cytometry on a Sysmex® XN-9000 analyzer (Sysmex Corporation). Activated partial thromboplastin time, prothrombin time, and fibrinogen were determined by a viscosity-based detection system on a STAR MAX2 analyzer (Stago). Antithrombin (ATB), protein C, and protein S assays were determined by photo-optical clot detection on an ACLTop 700 (Werfen). Antiphospholipid antibodies, which included lupus anticoagulant, IgG and IgM anticardiolipin antibodies (aCL), and IgG and IgM anti-β_2-glycoprotein I antibodies were performed according to established guidelines as previously described (37). The genotyping of coagulation factors II (*F2*, NM_000506.4: c.97G > A, rs1799963) and V (*F5*, NM_000130.4: c.1601G > A, rs6025) variants was performed using loop-mediated isothermal amplification (LACAR kit) on a LightCycler LC480 instrument (Roche). The genotyping of the *MTHFR* (NM_005957.5: c.665C > T), (NM_005957.4: c.1286A > C), *MTRR* (NM_002454.3: c.66A > G), AU: (NM_002454.3: c.524C > T), and *MTR* (NM_000254.2: c.2756A > G) variants was performed using fluorescence resonance energy transfer on a LightCycler LC480 instrument.

Next-generation clinical exome sequencing

Patients were assessed at the Department of Personalized Medicine at the University Hospital of Nancy, Reference Center of Inborn Errors of Metabolism, to look for genetic defects that could elucidate their clinical presentation. We performed a molecular diagnosis approach based on next-generation sequencing of 4813 genes using the TruSight One sequencing panel. All of the details related to the panel, including the technical note and the gene list, are available at https://emea.illumina.com/products/by-type/clinical-research-products/trusight-one.html. We have developed a standardized and systematic approach for analyzing and prioritizing the genetic variants in the setting of our molecular diagnosis approach, as previously described (38, 39). The strategy used for filtering and prioritizing the genetic variants had the following steps: *1*) import all retrieved genetic variants; *2*) retain only low-frequency and rare variants by excluding variants with a reported alternative allele frequency (AAF) >5% in the following databases: the Exome Aggregation Consortium (ExAC) and the Genome Aggregation Database (gnomAD Exomes). The aim of step *2*) is to retain rare (AAF <1%) and low-frequency (AAF <5%) variants which exhibit relatively large effects on disease risk, such as those involved in severe monogenic diseases; *3*) exclude intronic or synonymous variants; and *4*) prioritize genetic variants for their association with the studied phenotype using the PhoRank gene ranking based on the Phevor algorithm. The Phorank score was calculated according to the RefSeq Genes 105v2, NCBI gene source, using the following ontologies: Human Phenotype Ontology, Gene Ontology, and OMIM Phenotype Ontology. Functional variant annotation was performed using the following tools: MutationTaster (40), Functional Analysis through Hidden Markov Models (FATHMM) (41), MetaSVM/MetaLR scores (42), Sorting Tolerant From Intolerant (SIFT) (43), Protein Variation Effect Analyzer (Provean) (44), and deleterious annotation of genetic variants using neural networks (DANN) (45). Variant pathogenicity was reported according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) variant guidelines (46). All bioinformatic analyses were performed using the SNP & Variation Suite (v8.8.3; Golden Helix, Inc.).

Statistical analyses

All quantitative variables are shown as the median IQR (25th–75th percentile) and qualitative variables as percentages and 95% CI. We used the Mann-Whitney *U* test or the Kruskal-Wallis test to compare the distribution of biological biomarkers according to the different subgroups, as appropriate. We compared Hcy concentrations before and after treatment using the Wilcoxon paired test. Statistical analyses were performed using Stata SE, version 12.1, based on a 2-sided type I error with an α level of 0.05.

Results

Clinical and biological characteristics of patients

During the study period, 1400 patients had a plasma Hcy assay. Among these, 394 were excluded for missing data on extraction. Of the 1006 remaining patients, a total of 165 patients had a plasma Hcy determination in relation to a diagnosis of CV and/or thromboembolic disease (**Figure 1**), including 152 patients admitted to the department of cardiology for arterial thrombosis, VTE, vasculitis, vascular-placental pathology, and arterial + venous thrombosis, and 13 patients admitted in other departments for hematological or digestive disease associated with CVD outcomes. Details of the clinical and biological characteristics of the 165 patients are reported in **Table 1**. The median age was 46 y (IQR: 35–59 y) and the proportion of males was 57% (94/165). Most of the patients had arterial (79/165, 47.8%) or venous thrombosis (65/165, 39.3%), and 80.6% (133/165) of patients had ≥1 risk factor for CVD other

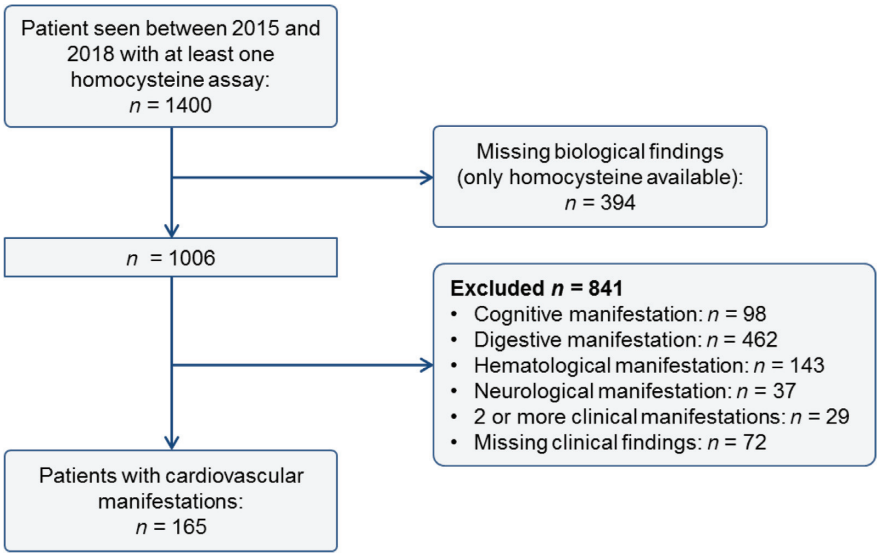

FIGURE 1 Flow chart reporting patient selection in the study.

than HHcy, including 39.8% (53/165) with 1 risk factor, 38.3% (51/165) with 2 risk factors, 17.2% (23/165) with 3 risk factors, and 4.5% with 4 or 5 risk factors (6/165). The most frequently observed risk factors were smoking (78/165, 47%), hypertension (64/165, 39%), and dyslipidemia (56/165, 34%). Few patients had a family history of CVD (9/165, 5%). Around a quarter of patients (44/165, 26%) had ≥1 abnormal biological marker of thrombophilia.

The median Hcy concentration was 38.9 μmol/L (IQR: 31.5–51.5). The majority of patients (94/165, 57%) had an intermediate HHcy (30–100 μmol/L), and 27% (44/165) had an Hcy >50 μmol/L. Only 6% (10/165) of patients had a normal Hcy concentration and 10.3% (17/165) had a moderate HHcy (15–30 μmol/L). There were no significant differences in Hcy concentrations between the different groups of CV/VTE events (**Figure 2**).

Vitamin B12 was assayed in 87 cases (55%) and folate in 50 of these cases (57%). A deficit in vitamin B12 was reported in 36 cases (41%), a deficit in folate in 18 cases (36%), and a combined deficit in both vitamins in 6 cases (7%). The median concentrations of Hcy and MAA in B12-deficient patients were 36.3 μmol/L (IQR: 27.7–46.5) and 0.58 μmol/L (IQR: 0.37–0.79), respectively (**Supplemental Table 1**). The median concentrations of Hcy and MMA in folate-deficient patients were 42 μmol/L (IQR: 38.8–59.2) and 0.33 μmol/L (IQR: 0.17–0.62), respectively (Supplemental Table 1). In this subgroup, 2 patients had renal failure, with abnormal GFR indicative of renal failure (CKD-EPI at 10 and 32 mL/min/1.73 m^2, respectively). The B12 deficiency was mainly due to malabsorption and/or mutations in genes of B12 absorption and/or iatrogenic effects of proton pump inhibitors. HHcy was also related to moderate and severe renal failure in 44 other cases (27%), including 23 cases (52%) with kidney disease improving global outcomes (KDIGO) stage 3, 10 with KDIGO stage 4 (23%), and 11 with end-stage renal failure (KDIGO stage 5) (25%) (Supplemental Table 1).

Patients with HHcy as the single risk factor of CV diseases

A subgroup of 15 cases (9%) had HHcy with no other identified CVD risk. The median age of this subgroup was 33 y (IQR: 27–47), and the proportion of males was 53% (8/15). The median concentrations of Hcy and MMA were 50.4 μmol/L (IQR: 31.3–50.4) and 0.225 μmol/L (IQR: 0.11–0.335), respectively. Forty-seven percent of cases (7/15) had an Hcy concentration between 30 and 50 μmol/L and 53% (8/15) had Hcy >50 μmol/L. Only 2 patients had a deficit in folate. The median concentrations of vitamin B12 and serum folates were 191 pmol/L (IQR: 130–292) and 9.9 nmol/L (IQR: 8.3–18.5), respectively. Among the 15 patients, 7 had venous thrombosis, 5 had arterial thrombosis, and 1 had both arterial and venous thrombosis. Most of the patients (10/15, 66.7%) were assessed at the National Reference Center of Inborn Errors of Metabolism, 5 (33.3%) were diagnosed with a genetic abnormality related to 1-CM, and 10 (66.7%) received a supplementation therapy.

Patients treated in the National Reference Center of Inborn Errors of Metabolism

Forty-six patients were referred to the National Reference Center of Inborn Errors of Metabolism, 95.6% (44/46) adults and 4.4% (2/46) children. Among these patients, 45.6% (21/46) had arterial thrombosis, 39% (18/46) venous thrombosis, 8.7% (4/46) had both arterial and venous thrombosis, and 4.3% (2/46) had vascular placental pathology. Next-generation sequencing (NGS) exome analysis was performed in 30 patients (65.2%). We reported variants with minor allele frequency <0.05 in genes of vitamin B12 metabolism and 1-CM in 14 patients (73%, 14/19), including in *CUBN*, *AMN*, and *GIF* genes related to B12 absorption, *CD320*, *ABCC1*, and *MMAB* genes related to blood transport and intracellular metabolism of vitamin B12 cases, and *MTR* and *CBS* genes encoding enzymes of the 1-CM (**Table 2**).

TABLE 1 Characteristics of the 165 patients included in the study[1]

Demographic data	*n*	Whole population
Age, median (IQR), y	165	46 (35–59)
Male sex, % (95% CI)	94/165	57.0 (49.3–64.6)
BMI, kg/m^2	104	24.5 (20.8–29.8)
Patients' medical history and risk factors		
Tobacco consumption	78/165	47.3 (39.6–55)
Obesity	27/165	16.4 (10.7–22.1)
Hypertension	64/165	38.8 (31.3–46.3)
Dyslipidemia	56/165	33.9 (26.6–41.2)
Diabetes	13/165	7.9 (3.72–12)
Cardiovascular disease	133/165	80.6 (74.5–86.7)
Kidney transplantation	9/165	5.4 (1.95–8.96)
Severe renal failure	21/141	14.9 (9.02–20.78)
Hemodialysis	17/165	10.3 (5.62–15)
Family history of early cardiovascular event	9/165	5.45 (1.95–8.96)
Laboratory findings, median (IQR)		
Homocysteine, μmol/L	165/165	38.9 (31.5–51.5)
Methylmalonic acid, μmol/L	65/165	0.61 (0.26–0.82)
Vitamin B12, pmol/L	87/165	191 (130–292)
Folates, serum, nmol/L	50/165	9.9 (5.9–18.5)
Folates, erythrocyte, nmol/L	29/165	769 (537–1149)
Hemoglobin, g/dL	153/165	13.6 (12.1–15.1)
MCV, fL	153/165	93 (89–97)
Glomerular filtration rate, mL/min	141/165	72 (54–94)
Total cholesterol, g/L	113/165	5.02 (3.99–6.01)
HDL cholesterol, g/L	97/165	1.09 (0.95–1.33)
LDL cholesterol, g/L	92/165	3.11 (2.36–4.16)
Triglycerides, g/L	112/165	1.75 (1.06–2.65)
ASAT, U/L	120/165	23 (18–30)
ALAT, U/L	120/165	20 (14–32)
Albumin, g/L	18/165	37.2 (33.6–41.9)
Iron, μmol/L	99/165	14.0 (9.3–19.0)
C-reactive protein, mg/L	110/165	4.7 (1.5–14.4)
Patient subgroups according to Hcy concentration stratum		
Stratum 1 (<15 μmol/L)	10/165	6.1(2.4–9.7)
Stratum 2 (15–30 μmol/L)	17/165	10.3 (5.6–15)
Stratum 3 (30–50 μmol/L)	94/165	57 (49.3–64.6)
Stratum 4 (50–100 μmol/L)	39/165	23.6 (17.1–30.2)
Stratum 5 (>100 μmol/L)	5/165	3.03 (0.4–5.7)
Patient subgroups according to the KDIGO classification		
KDIGO stage 1	43/141	30.5 (22.8–38.2)
KDIGO stage 2	54/141	38.3 (30.2–46.4)
KDIGO stage 3	23/141	16.3 (10.1–22.5)
KDIGO stage 4	10/141	7.09 (2.8–11.4)
KDIGO stage 5	11/141	7.8 (3.3–12.3)
Thrombophilia investigations, median (IQR)		0 (—)
TP (%)	102/165	89 (82–96)
aPTT ratio	128/165	1.07 (0.94–1.24)
Fibrinogen (g/L)	131/165	3.50 (2.93–4.30)
Testing for LA, aCL, and aB2GP1	133/165	80.6 (74.5–86.7)
Positive result for LA, aCL, and/or aB2GP1	28/133	21.1 (14–28.1)
Positive result for LA (class IIa)	14/28	50 (30.3–69.7)
Positive result for aCL (class IIb)	9/28	32.1 (13.7–50.6)
Positive result for aB2GP1, (class IIc)	0/28	0 (—)
Positive result	23/28	82.1 (67–97.3)
Double positive result	4/28	14.3 (0.5–28.1)
Triple positive result	1/28	3.57 (0–10.9)
Antithrombin deficiency, % (95% CI)	6/75	8.0 (1.7–14.3)
Protein C deficiency, % (95% CI)	10/62	16.1 (6.7–25.5)
Protein S deficiency, % (95% CI)	8/57	14.0 (4.74–23.3)
Factor II (prothrombin, G20210A), median (IQR)		
Wild type, homozygous	60/67	89.6 (82.0–97.1)
Heterozygous	7/67	10.4 (2.93–18.0)
Variant, homozygous	0/67	0 (—)

(*Continued*)

TABLE 1 (*Continued*)

Demographic data	*n*	Whole population
Factor V Leiden, median (IQR)		
Wild type, homozygous	16/26	61.5 (41.5–81.6)
Heterozygous	10/26	38.5 (18.4–58.5)
Variant, homozygous	0/26	0 (—)

[1] Values are *n*/total number of subjects, median (IQR), or % (95% CI). aB2GP1, anti-β_2-glycoprotein I antibodies; aCl, anticardiolipin antibodies; ALAT, alanine aminotransferase; aPTT, activated partial thromboplastin time; ASAT, aspartate aminotransferase; Hcy, homocysteine; LA, lupus anticoagulant; KDIGO, kidney disease improving global outcomes; MCV, mean corpuscular volume.

In addition, we reported variants with allele frequency >0.05 in *MTHFR* and *MTRR* genes of 6 other cases. The influence of these gene variants on HHcys was uncertain as these subjects also had low plasma B12 concentrations.

Additionally, the rs1801133 polymorphism of *MTHFR* was assessed in 55 patients (33.3%), including 21 patients referred to the National Reference Center. Among them, 28 patients (50.9%) were homozygous for the minor allele of *MTHFR*, a frequency that was 5-fold higher than that reported in the French population (47). The median (IQR) concentration of Hcy was 48 μmol/L (IQR: 38.2–58.7), 36.2 μmol/L (IQR: 32.5–42.7), and 34.6 μmol/L (IQR: 24.6–41.7) in patients exhibiting a homozygous genotype for the minor allele (*TT*), heterozygous genotype (*CT*), and homozygous genotype for the major allele (*CC*), respectively. HHcy concentrations were was significantly higher in patients with the *TT* genotype, compared with those with either the *CT* or *CC* genotype ($P < 0.01$).

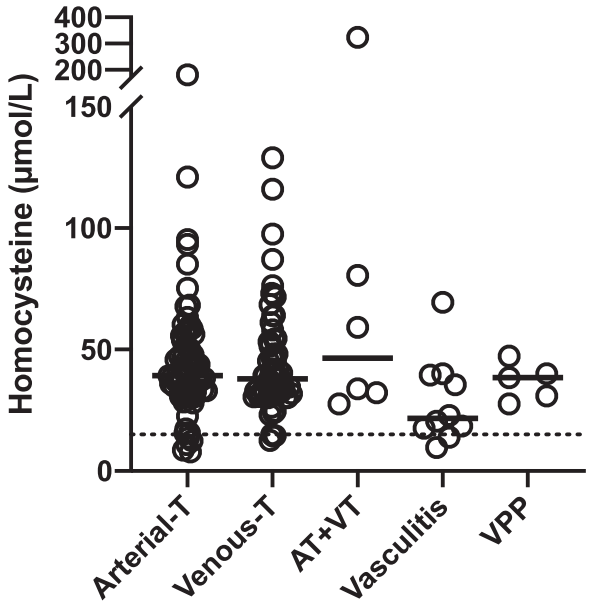

FIGURE 2 Concentrations of homocysteine according to CV manifestations in the 165 cases (arterial thrombosis 79/165; venous thrombosis 65/165; arterial thrombosis + venous thrombosis 6/165; vasculitis 10/165; vascular placental pathology 5/165). The cutoff of homocysteine (15 μmol/L) is indicated by dotted lines. Bars represent medians. No difference was reported among groups, Mann-Whitney U-test. AT, arterial thrombosis; CV, cardiovascular; VPP, vascular placental pathology; VT, venous thrombosis.

Supplementation of patients

Of the 165 studied patients, 101 (61.2%) received a supplementation therapy with vitamin B12 and/or folate and/or betaine and/or vitamin B6 and/or riboflavin in relation to either a folate or vitamin B12 deficiency or a genetic cause of HHcy. Of the 101 treated patients, 44 (43.5%) were treated and followed at the National Reference Center of Inborn Errors of Metabolism, according to the international guidelines of inherited disorders of the 1-CM. After a median follow-up of 44 months, the median Hcy concentration was lowered from a baseline median Hcy value of 41.5 µmol/L (IQR: 33.6–60.4) to a median posttreatment Hcy value of 17.6 (IQR: 12.1–28) µmol/L (difference 23.9 µmol/L; $P < 0.001$) (**Figure 3**). The supplementation was less efficient in patients treated outside the National Reference Center of Inborn Errors of Metabolism than in those followed in the center, with decreases in Hcy of 19.1 µmol/L and 39.3 µmol/L, respectively (Figure 3B and C). We observed no recurrence of CVD outcomes in all treated patients who had no other CVD risk factors than HHcy after nearly 4 y of follow-up. These patients were treated with antithrombotics and adapted supplementation for vitamin deficiency or genetic disorders.

Discussion

Our study highlights the high frequency of intermediate or severe HHcy (84%, 138/165) in 165 patients who had undergone an assessment of their plasma Hcy concentration in the setting of thromboembolic and other CV manifestations, among 1006 patients consecutively recruited during 3.5 y at a University Regional Hospital Center in France. Among these patients, 84% had HHcy >30 μmol/L, which was related to vitamin B12 and/or folate deficiency, a genetic cause, or renal failure.

The search for a deficiency in vitamin B12 and/or folate was carried out in only 53% of the reported patients, including 44 patients studied in the reference center for metabolic diseases. The US and European Societies of Cardiology do not recommend including HHcy as a risk factor of CVD, and as a consequence, there is a lack of consideration and/or knowledge among cardiologists to diagnose and treat patients with intermediate and severe HHcy. The vitamin B12 deficiency was established according to evocative clinical symptoms, low concentration of B12, and increased Hcy and/or MMA. Nearly 62% of tested patients had a deficit in vitamin B12 or folate, with the same CV outcomes as the other cases. The B12 deficiency was related to malabsorption and/or mutations in the *CUBN*, *AMN,* or *GIF* genes involved in B12 absorption and/or iatrogenic effects of

TABLE 2 Results of NGS exome analysis in the study[1]

Patient	Age (y)	Sex	Cardiovascular manifestation	Homocysteine (μmol/L)	MMA (μmol/L)	B12 (pmol/L)	Folate (nmol/L)	Folate, red cell (nmol/L)	Gene	Variant (HGVS, c.)	Variant (HGVS, p.)	Alternative allele frequency
Vitamin B12 absorption gene												
P1	26	M	VT, AT (Others)	80.5	0.3	121	45	NA	*AMN*	c.773T > C	p.Leu258Ser	4.31×10^{-3}
P2	40	M	VT (DVT), PE	87.1	0.31	648	14.9	NA	*GIF*	c.910C > T	p.Pro304Ser	9×10^{-4}
P3	31	M	AT (MI)	51.9	0.34	116	45	NA	*CUBN*	c.5840C > A	p.Ser1947Tyr	2.63×10^{-4}
P4	27	M	VT (DVT), PE	51.4	NA	144.9	25	NA	*CUBNH*	c.2461T > G	p.Leu821Val	6×10^{-5}
									CUBN	c.8635C > A	p.Leu2879Ile	2.64×10^{-2}
P5	31	F	AT (Others)	60.4	0.21	62.58	NA	1806	*CUBN*	c.6469A > G	p.Asn2157Asp	5.62×10^{-3}
P6	39	M	PE	48.2	NA	NA	NA	1852	*CUBN*	c.7365T > A	p.Asp2455Glu	5.62×10^{-3}
									AMN	c.829A > G	p.Thr277Ala	4.96×10^{-3}
P7	31	F	VT (DVT)	71.7	NA	175	NA	564	*CUBN*	c.6469A > G	p.Asn2157Asp	5.62×10^{-3}
Vitamin B12 intracellular transport gene												
P9	35	F	VT (DVT), PE	116	0.21	222	NA	501	*ABBC1*	c.1299G > T	p.Arg433Ser	1.36×10^{-2}
P10	58	M	AT (MI)	57	NA	90	NA	2111	*CD320*	c.11G > A	p.Gly4Asp	2.29×10^{-3}
P13	35	M	AT (MI)	58.6	0.63	306	32	385	*MMAB*	c.556C > T	p.Arg186Trp	1.4×10^{-4}
P14	38	F	VT (SVT)	45.9	NA	73.9	NA	224.53	*MMAB*	c.∗1742_∗1752 delAAAAAAA AAAA	Intronic	Not reported*
I-CM enzyme genes												
P11	57	M	AT (MI)	31.9	0.87	406	45	NA	*MTR*	c.2044-2A > T	p.Gly999Glu	1.27×10^{-2}
P12	28	M	VT (DVT), PE	73.2	0.12	NA	12.2	NA	*MTR*	c.2044-2A > T	p.Gly999Glu	1.27×10^{-2}
P8	30	M	AT (Others)	40.5	NA	324.1	8.8	769.1	*MTRR*	c.1549A > G	p.Thr517Ala	4.48×10^{-3}
P19	3	M	VT (Others)	64.3	NA	1311	45	1311	*CBS*	c.526 G > A	p.Glu176 Lys	Not reported

[1]AT, arterial thrombosis; DVT, deep venous thrombosis; MI, myocardial infarction; NA, not available; NGS, next-generation sequencing; PE, pulmonary embolism; SVT, superficial venous thrombosis; VT, venous thrombosis. *Not reported in genome aggregation database (GenomAD v2.1.1).

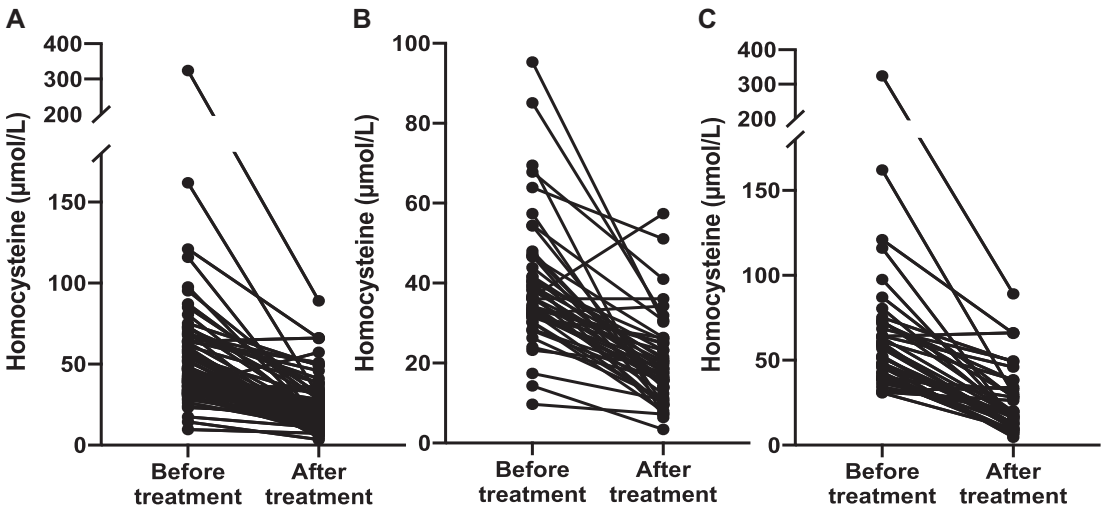

FIGURE 3 Pairwise comparison of Hcy values in patients before and after diagnosis-oriented therapy which included ≥1 of the following treatments: cobalamin, folic acid, pyridoxine, and betaine. All CV patients (A): After a median follow-up of 44 mo, the median Hcy concentration was lowered from a baseline median Hcy value of 41.5 µmol/L (IQR: 33.6–60.4) to a median posttreatment Hcy value of 17.6 µmol/L (IQR: 12.1–28), difference 23.9 µmol/L; $P < 0.001$. Patients outside the National Reference Center of Inborn Errors of Metabolism ($n = 57$) (B): After a median follow-up of 44 mo, the median Hcy concentration was lowered from a baseline median Hcy value of 36.25 µmol/L (IQR 31.9–43.9) to a median posttreatment Hcy value of 17.15 µmol/L (IQR: 12.4–24.9, difference 9.1 µmol/L; $P < 0.001$. Patients followed at the National Reference Center of Inborn Errors of Metabolism ($n = 44$) (C): After a median follow-up of 44 mo, the median Hcy concentration was lowered from a baseline median Hcy value of 57 µmol/L (IQR: 42.5–72.7) to a median post-treatment Hcy value of 17.7 µmol/L (IQR: 12.1–32.7) (difference 39.3 µmol/L; $P < 0.001$. Hcy concentrations before and after treatment were compared using the Wilcoxon paired test. CV, cardiovascular; Hcy, homocysteine.

proton pump inhibitors. Four patients had malabsorption related to ileitis in the context of Crohn's disease. The increased risk of thromboembolic outcomes associated with HHcy has already been reported in Crohn's disease, where it is secondary to vitamin B 12 deficiency caused by ileitis (48).

NGS-based molecular diagnosis to search for rare genetic variants related to inherited disorders of vitamin B12 and HHcy had never been performed in a CVD adult population, to our knowledge. All patients recruited in the reference center who had a familial history of HHcy were screened by clinical exome sequencing. In total, we reported variants of 1-CM genes in 19 cases (11% of the reported population). The identified genes were related to the absorption and metabolism of vitamin B12 and key enzymes of the 1-CM. Our data show, therefore, that the presence of 1-CM genetic variants is dramatically enriched in patients with intermediate to severe HHcy, compared with reference populations. The identification of genetic variants made it possible to provide personalized therapeutic approaches and follow-up adapted to the inherited disorders, using vitamin B12 and/or folate and/or vitamin B6 and/or betaine.

Severe chronic kidney disease was observed in 15% (21/141) of patients with HHcy in the absence of any other causal factor. The association of HHcy with CVD outcomes can be secondary to chronic kidney disease (49). Conversely, vascular disease may impair renal function, which in turn could lead to HHcy (50). Renal failure produces HHcy through reduced filtration and decreases the transsulfuration of Hcy, leading to increased Hcy plasma concentrations (51). Of note, renal failure contributed to HHcy in 9 cases with B12 and folate deficiency. Besides the well-known influence of renal failure in HHcy, it has been shown that renal failure may increase the risk of vitamin B12 deficit (52) and impairs the cellular uptake of B12 (53).

To our knowledge, most of the previous retrospective cross-sectional and interventional studies have reported the rate of CVD manifestations in patients consecutively recruited with median Hcy <15 µmol/L. Most interventional studies have evaluated the occurrence of CVD outcomes in subjects with Hcy median concentrations ranging from 9.8 to 14.6 µmol/L and normal serum folate and B12 median/mean concentrations (**Supplementary Table 2**). In particular, the SU.FOL.OM3 interventional study of CVD patients recruited in France reported a mean plasma Hcy at 12.8 µmol/L and serum folate at 15.2 nmol/L at baseline, indicative of a low prevalence of folate deficit, despite the absence of folate fortification in this country (Supplemental Table 2). In contrast, the median Hcy of our case series was as high as 38.9 µmol/L, and only 6% (10/165) of the patients had Hcy <15 µmol/L. Another limitation of most of the observational studies is that Hcy has been assayed a long time after the onset of the vascular event. Therefore, it is impossible to distinguish the influence of Hcy on the development of atherosclerosis and arterial thrombosis from the influence of CVD and related treatments on secondary Hcy (54). A strong relation has been identified between Hcy and carotid stenosis in prospectively recruited patients (55). The percentage of subjects with carotid stenosis ≥25% increased linearly, from 25% for Hcy at 8 µmol/L to 50% when Hcy was >18 µmol/L. These differences remained highly significant after adjusting for age, sex, HDL cholesterol, systolic blood pressure, and smoking. Therefore, we assumed that the risk of vascular lesions was much higher in our series than in the previous observational studies in regard to the association between the magnitude of HHcy and the risk of CVD (55). Several observational studies have shown an increased plasma Hcy in patients with arterial thrombosis (54, 56–64), with median Hcy concentrations even lower than the

cutoff of 15 μmol/L. In most of these studies, patients presented CVD outcomes before the age of 55. In our study, we reported arterial thrombosis and VT as prominent outcomes at a median age of 46 y (IQR: 35–59).

Up to three-fourths of CVD can be attributed to what is considered as classical risk factors, highlighting the search for other second-line risk factors (65). We reported HHcy as the single risk factor of CVD in 9% (15/165) of cases. These patients were younger and had higher Hcy concentrations and a higher rate of genetic substratum for HHcy compared with patients with other risk factors (Supplemental Table 1). These results point out HHcy as a biomarker of CVD risk, as previously suggested (66, 67). Indeed, the addition of Hcy concentration to the Framingham Risk Score significantly improved the risk prediction in post hoc analyses of the MESA (Multi-Ethnic Study of Atherosclerosis) and NHANES III studies (66, 67).

Despite the association between plasma Hcy and CVD events reported in most observational studies, the randomized controlled trials and meta-analyses have shown that Hcy-lowering interventions by vitamin B12, folate, and/or vitamin B6 did not reduce the occurrence of most CVD, even if debate still exists on a possible benefit in stroke patients (68, 69). Our study may help increase understanding of and decrease the discrepancy between observational studies and randomized clinical trials. In the trials which did not include patients with renal failure or heart transplantation, the Hcy concentration at baseline ranged from 9.7 to 14.3 μmol/L, a range of concentrations close to the Hcy of control populations and dramatically lower than those reported in our study. Consequently, these trials lowered Hcy by <5 units, while in our study, the specific treatment of deficiencies and inherited disorders produced a decrease of 24.5 units of median Hcy concentrations, with no recurrence of CVD outcomes after several months of follow-up in patients without other risk factors. Furthermore, the studies reported in the literature did not evaluate the effect of the treatment on CVD outcomes in patients with B12 and/or folate deficiency and/or genetic causes of HHcy at baseline. They did not consider the influence of B vitamin supplementation in the subgroup of patients with Hcy >30 μmol/L, despite the initial evidence provided by the Hcy vascular hypothesis published in 1969 by McCully (18). This first report on Hcy association with CVD observed early athero-thrombosis in children linked to a dramatic increase in plasma Hcy due to mutations in key genes of 1-CM. Subsequent studies reported that treatment of severe HHcy with folic acid and vitamin B6 significantly reduced the risk of CV events and venous thrombosis in a series of 32 patients with congenital homocystinuria (70). Supplementation with riboflavin may also be effective to reduce the CVD risk related to hypertension in patients with intermediate or severe HHcy (71). Despite this evidence, large-scale epidemiological studies and interventional studies have only focused on the vascular risk generated by moderate HHcy.

In conclusion, our study shows a high frequency of vitamin B12 and/or folate deficiency and/or genetic causes related to intermediate/severe HHcy in patients consecutively recruited for CVD occurrence at a University Regional Hospital Center. The high frequency of intermediate/severe HHcy differs from that of moderate HHcy reported in observational studies of patients with pre-existing CV disease. For many practitioners, the failure of intervention studies to prevent the occurrence or recurrence of CVD outcomes has ruled out the need to assess and treat HHcy. The results of interventional studies have jeopardized the debate for the proper management of patients with intermediate to severe HHcy. Our study points out the importance of diagnosing and treating nutritional deficiencies and inherited disorders of 1-CM in order to reverse intermediate/severe HHcy and their potential impact on CVD outcomes. On the other hand, there is no benefit of B vitamin supplements in patients who already present a CV disease and for whom there is a modest elevation of Hcy. This message should be translated into recommendations by medical societies.

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Data Availability

Data described in the manuscript will be made available upon request.

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