Journal of Population Therapeutics & Clinical Pharmacology

Original Article
DOI: 10.47750/jptcp.2023.1040

Haplotype analysis and linkage disequilibrium of MTHFR gene polymorphisms associated with recurrent thrombosis

Mustafa Ihssan Abbas Al-Mahroos,[1]* Mahmmod Riyadh Alhaleem,[2] Bahir Abdul Razzaq Mshimesh[3]

[1]College of Pharmacy, Alfarahidi University, Ministry of Higher Education and Scientific Research, Baghdad, Iraq

[2]Consultant Interventional Cardiologist, Ibn Al- Bitar Specialized Center for Cardiac Surgery, Ministry of Health, Baghdad, Iraq

[3]Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq

***Correspondence author:** Mustafa Ihssan Abbas Al-Mahroos, College of Pharmacy, Alfarahidi University, Ministry of Higher Education and Scientific Research, Baghdad, Iraq. Email: mustafamahroos1@gmail.com

Submitted: 08 November 2022; Accepted: 01 December 2022; Published: 10 January 2023

ABSTRACT

Haplotype analysis is the study of the origin of linked alleles set occurring on the same chromosome. Linkage disequilibrium (LD) is a population-based parameter that determines the inheritance or correlation between alleles of nearby genetic variants within a given population. The measurement of LD is important for biomedical research and is used in a wide range of applications. The study aimed to detect the true genetic effects of MTHFR polymorphisms that require a specific allele at several single nucleotide polymorphisms (SNPs) by using haplotype-based methods for analyzing all SNPs associated with recurrent thrombosis concurrently. This prospective case-control trial included 58 Iraqi patients (43 males and 15 females) aged between 20 and 64 years who suffered from different cardiovascular and thromboembolic disorders. The control group included 52 subjects composed of 41 males and 11 females without any thrombotic event history. The biochemical analysis of baseline homocysteine level, vitamin B12, and folic

acid level and the genetic polymorphism of MTHFR for two specific variants C677T and A1298C were performed. The haplotype block CA was more frequent among the control group than in the patient group (55.77% versus 35.34%) with a significant difference (OR [odds ratio] = 0.32, 95%CI [confidence intervals] = 0.17–0.61, $p < 0.001$). In contrast, the haplotype block CC was more common in the patient group (41.38%) than in the control group (20.19%) with a significant difference (OR = 3.11, 95%CI = 1.63–5.93, $p < 0.001$). The SNP C677T was in a strong LD (the measure D' was 0.83) with A1298C. Seven haplogenotypes were identified: M1M1, M1M2, M1M3, M1M4, M2M2, M3M3, and M2M4. The most common haplogenotype was M1M2 (CC/AC) representing 24.55% of the total participants and was considered as the wild genotype. The haplogenotype M1M1 (CC/AA) was more frequent in the control group than in the patient group (32.69% vs. 6.9%) with a significant difference (OR = 0.14, 95%CI = 0.89–27.6, $p = 0.004$). In contrast, the haplogenotype M2M2 (CC/CC) was more frequent among the patient group (10.34%) than in the control group (3.85%) with a significant difference (OR = 3.82, 95%CI = 1.26–14.5, $p = 0.018$).

The homozygous mutant genotype (CC) of the A1298C polymorphism was more common in patients with recurrent thrombosis than in those without recurrent thrombosis (33.33% vs. 12.9%) with a significant difference (OR = 3.8, 95%CI = 1.04–17.034). The most common recurrent thrombosis frequency for MTHFR combined genotypes was CC/CC and CT/AA (66.66%) from all cases, despite two-thirds of these cases being treated with aspirin or clopidogrel for secondary prevention of myocardial infarction and ischemic stroke. Other cases included were CC/AC (18.51%) and TT/AA (7.41%), as well as one case (3.71%) for each of CT/AC and CC/AA. The total percentage of CC/AC, TT/AA, CT/AC, and CC/AA for recurrent thrombosis cases was 33.34%, although 77.8% of these cases used aspirin or clopidogrel for secondary prevention of myocardial infarction and ischemic stroke. The median homocysteine in patients with recurrent thrombosis was higher than in those with no such disorder (75.5 μmol/L vs. 35.75 μmol/L), with a significant difference. The presence of C allele from the first SNP (CT) and A allele from the second SNP (AC) and the presence of CC/AA genotypes for two variants in individuals are considered as protective factors for exposure risk of ischemic heart disease, ischemic stroke, and venous thromboembolism. The individuals carrying C allele from the first SNP and C allele from the second SNP and those carrying CC/CC genotypes for two variants are at 3.11- and 3.82-fold risk of disease exposure respectively, compared to those carrying CC/AA genotypes. The SNP C677T was in a strong LD (the measure D' was 0.83) with A1298C. Individuals carrying CC homozygous genotype of A1298C are at a 3.8-fold risk of recurrent thrombosis than those with AA wild

genotype of the same SNPs due to high levels of homocysteine. This highlighted the role of homocysteine in atherogenic process, indicating that moderate-to-severe level of homocysteine is a potential risk factor for hypertension, cardiovascular disease, and stroke.

Keywords: *Haplotype, linkage disequilibrium, haplogenotype, recurrent thrombosis*

INTRODUCTION

A haplotype is the combination of alleles for different polymorphisms that occur on the same chromosome and for any given extent of chromosomal DNA in individuals. Haplotype analysis is the study of the origin of linked alleles set occurring on the same chromosome.[1]

Linkage disequilibrium (LD) is a population-based parameter that determines the inheritance or correlation between alleles of nearby genetic variants within a given population. The measurement of LD is important for biomedical research and is used in a wide range of applications.[2]

Methods based on single nucleotide polymorphisms (SNPs) have low power for detecting a true genetic effect that requires a specific allele at several SNPs. This can be detected by using haplotype-based methods for analyzing all SNPs concurrently.

OBJECTIVE

The study aimed to detect the true genetic effects of MTHFR polymorphisms that require a specific allele at several SNPs by using haplotype-based methods for analyzing all SNPs associated with recurrent thrombosis concurrently.

SUBJECTS AND METHOD

Study design

This prospective case-control trial conducted from September 2020 to September 2021 included 58 Iraqi patients (43 males and 15 females, forming the patient group) aged between 20 and 64 years with different cardiovascular and thromboembolic disorders at different wards in Ibn Al-Bitar Specialized Center for Cardiac Surgery, Al-Imamein Al-Kadhimaein Medical City, and Dr. Saad Al-Watri Hospital for Neurosciences. The control group included 52 subjects (41 males and 11 females) without any thrombotic event history.

The biochemical analysis of baseline homocysteine level, vitamin B12, and folic acid level and the genetic polymorphism of MTHFR for two specific variants C677T and A1298C were performed in the pioneer molecular pathology laboratory. This study obtained approval from the scientific committee in the College of Pharmacy at the University of Mustansiriyah and the scientific and administrative committee in the Ministry of Health. All participants submitted written consent.

Inclusion criteria

The patients were screened and selected based on the following inclusion criteria: (1) patients with ischemic heart disease and myocardial infarction, who were diagnosed under the supervision of cardiologist subspecialties; (2) transient ischemic attack or ischemic stroke patients under the supervision of neurologist subspecialties; and (3) patients with venous thromboembolism (deep vein thrombosis and pulmonary embolism) under the supervision of cardiologist subspecialties.

Exclusion criteria

The patients with any condition that may interfere with the study protocol were excluded,

including: (1) patients with venous thromboembolisms having a history of risk factors of previous surgical procedure, bone fracture, and malignancy; (2) patients with chronic renal failure; and (3) patients of Alzheimer or Parkinson disease.

MATERIALS

Table 1 lists the kits used in this study.

METHOD

EDTA anti-coagulated blood (2-mL sample) samples were obtained from participants and the whole blood sample was used for the analysis. The allele-specific real-time PCR technique (quantitative real-time PCR) was used for detecting MTHFR genetic polymorphism (wild, heterozygous, homozygous) in two specific variants (C677T and A1298C) as SNP to assess allele frequency or genotype distribution (CC,TT,CT and AA,CC,AC respectively).

Detection of SNPs

DNA purification was required to remove the blood constituents such as hemoglobin that could interfere with the acquisition of the fluorescence signals. SNPs were detected in untreated whole blood and serum by using a real-time PCR assay applied to MTHFR C677T and MTHFR A1298C polymorphisms.[3–5] Table 2 shows the primers and probes for genotyping of SNPs to MTHFR.[5]

Statistical analysis

Statistical analyses were performed using SPSS software version 25.0 (SPSS, Chicago). Data with non-normal distribution were presented as median and range and analyzed using the Kruskal–Wallis test (for three groups' comparison). Categorical variables were expressed as number and percentages, and analyzed with Chi-square test.

TABLE 1. Kits used in the study.

Kits	Manufacturer	Origin
Bosphore thrombophilia detection kit	Anatolia Genework	Turkey
Homocysteine colorimetric assay kit	Elabscience	China
Human folic acid ELISA kit	Bioassay Technology	China
Human vitamin B12 ELISA kit	Bioassay Technology	China

Bosphore thrombophilia detection kit used in this study consisted of real-time polymerase chain reaction (PCR) reagents.

TABLE 2. Primers and probes for genotyping of SNPs to MTHFR.

Gene and genotype	Primer/Probe	Reporter	Sequences
MTHFR	Primer forward Primer reverse		TGACCTGAAGCACTTGAAGGAGAA GGAAGAATGTGTCAGCCTCAAAGA
C677T	Probe C Probe T	FAM TET	ATGAAATCGGCTCCCG ATGAAATCGACTCCCG
MTHFR	Primer forward Primer reverse		GGAGGAGCTGCTGAAGATGTG TCTCCCGAGAGGTAAAGAACAAA
A1298C	Probe A	FAM	AAAGACACTTTCTTCACTG
	Probe C	TET	AGACACTTGCTTCACTG

FAM is 6-carboxyfluorescein whereas TET is 6-carboxy-4,7,29,79 tetra-chloro-fluorescein. The variant base in underlined. The sequence is 5' to 3'.
SNP, single nucleotide polymorphism.

The Hardy-Weinberg Equilibrium (HWE) was used for calculating the genetic variation of a population at equilibrium. The deviation of different genotypes from HWE was calculated online (https://www.easycalculation.com/health/hardy-weinberg-equilibrium-calculator.php). If there is no deviation between observed genotype frequencies in a population and the frequencies predicted by the equation, the genotype frequencies in a population are in good accordance with HWE.[6]

Haplotype analysis was performed utilizing structural and functional genomic information for improving the accuracy of genomic prediction. LD analysis was done by calculating Lewontin's D′ for the association of genetic polymorphisms using SHEsis software platform.[7]

The association of different genotypes and alleles frequency with the diseases was evaluated using the odds ratio (OR), for determining the risks of genetic mutation, and binary logistic regression for calculating the corresponding 95% confidence intervals (CI). A p-value less than 0.05 indicated a statistically significant difference.

RESULTS AND DISCUSSION

Haplotype analysis of MTHFR

As the two polymorphisms are located on the same gene, haplotype blocks were formed by combining each allele in the first SNP (C677T) with another allele from the second SNP (A1298C).

These haplotype blocks were constructed using SHEsis software. Table 3 shows the most frequent haplotypes in the patient and control groups. The haplotype block CA was more frequent among the control group than in the patient group (55.77% vs. 35.34%) with a significant difference (OR = 0.32, 95%CI = 0.17–0.61, p < 0.001). In contrast, the haplotype block CC was more common in the patient group (41.38%) than in the control group (20.19%) with a significant difference (OR = 3.11, 95%CI = 1.63–5.93, p < 0.001). Although the haplotype block TA was more common among the control group than in the patient group (27.88% vs. 18.1%), the difference was not statistically significant.

The presence of C allele from the first SNP (CT) and A allele from the second SNP (AC) in individuals is considered a protective factor against exposure risk of ischemic heart disease, ischemic stroke, and venous thromboembolism. The individuals carrying C allele from the first SNP and C allele from the second SNP are at a 3.11-fold risk of disease exposure than those carrying CC/AA genotypes.

Linkage disequilibrium

Figure 1 displays the results of LD analysis. LD plot was constructed using combined genotype data from all individuals (patient and control group). The SNP C677T was in a strong LD (the measure D' was 0.83) with A1298C.

Haplogenotypes for two variants of MTHFR

Different haplogenotypes are formed by combining each allele in the first SNP (C677T) with another allele from the second SNP (A1298C).

TABLE 3. Most frequent haplotype blocks.

Haplotype blocks	Patient group (n = 116)	Control group (n = 104)	P	OR (95%CI)
CA	41 (35.34%)	58 (55.77%)	<0.001*	0.32 (0.17–0.61)
CC	48 (41.38%)	21 (20.19%)	<0.001*	3.11 (1.63–5.93)
TA	21 (18.1%)	29 (27.88%)	0.860	0.57 (0.30–1.08)

Data presented as number of patients (n) and percentage (%) for categorical variables.
Binary logistic regression for categorical variables.
*P ≥ 0.05 is considered nonsignificant, *P < 0.05 is considered significant.*

Accordingly, CA was represented and proposed as M1 and CC was represented and proposed as M2, whereas TA and TC were elucidated as M3 and M4, respectively. This study identified seven haplogenotypes, namely M1M1, M1M2, M1M3, M1M4, M2M2, M3M3, and M2M4. The most common haplogenotype was M1M2 (CC/AC) representing 24.55% of the total participants and was considered as the wild genotype. Table 4 presents the haplogenotypes distribution.

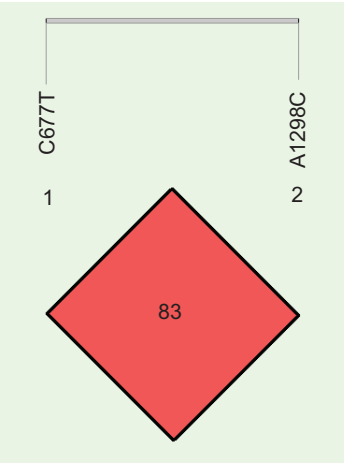

FIGURE 1. Linkage disequilibrium pattern in the *MTHFR* gene. The red area indicates strong linkage disequilibrium. The maximum D' value is 1.

The haplogenotype M1M1 (CC/AA) was more frequent in the control group than the patient group (32.69% vs. 6.9%) with a significant difference (OR = 0.14, 95%CI = 0.89–27.6, p = 0.004). In contrast, the haplogenotype M2M2 (CC/CC) was more frequent among the patient group (10.34%) than the control group (3.85%) with a significant difference (OR = 3.82, 95%CI = 1.26–14.5, p = 0.018).

The presence of CC/AA genotypes for two variants in individuals is considered a protective factor against exposure risk of ischemic heart disease, ischemic stroke, and venous thromboembolism. The individuals carrying CC/CC genotypes for two variants are at a 3.82-fold risk of disease exposure than those carrying CC/AA genotypes.

Association of gene polymorphisms with recurrent thrombosis

This study investigated two SNPs of the MTHFR gene (C677T and A1298C) for their association with recurrent thrombosis incidence for different diseases.

For the C677T variant, the distribution of different genotypes and alleles frequencies was compared between patients with and without recurrent thrombosis. As Table 5 shows, there are no significant difference.

However, the homozygous mutant genotype (CC) of the A1298C polymorphism was more common in

TABLE 4. The frequency of MTHFR haplogenotypes in the patient and control groups.

Haplogenotypes	Patients group (n = 58)	Control group (n = 52)	P	OR (95%CI)
M1M2 (CC/AC)	17 (29.31%)	10 (19.23%)	**0.014***	1.0
M1M1 (CC/AA)	4 (6.9%)	17 (32.69%)	**0.004***	**0.14 (0.89–27.6)**
M1M3 (CT/AA)	13 (22.41%)	12 (30.06%)	0.425	0.64 (0.52–4.74)
M1M4 (CT/AC)	6 (10.34%)	6 (11.54%)	0.449	0.59 (0.43–6.72)
M2M2 (CC/CC)	13 (22.41%)	2 (3.85%)	**0.018***	**3.82** (1.26–14.5)
M3M3 (TT/AA)	5 (8.62%)	2 (3.8%)	0.865	1.31 (0.13–5.51)
M2M4 (CT/CC)	0 (0%)	3 (5.77%)	0.182	0.2 (0.46–55.8)

Data presented as number of patients (n) and percentage (%) for categorical variables.
Binary logistic regression for categorical variables.
*P v≥ 0.05 is considered non-significant, *P < 0.05 is considered significant.*

TABLE 5. The frequency of different genotypes and alleles of C677T polymorphism in patients with and without recurrent thrombosis.

C677T	No recurrent thrombosis (n = 31)	Recurrent thrombosis (n = 27)	P	OR (95%CI)
Genotypes				
CC	19 (61.29%)	15 (55.56%)	0.800	1.0
CT	9 (29.03%)	10 (37.04%)	0.862	1.18 (0.17–8.02)
TT	3 (9.68%)	2 (7.4%)	0.617	1.67 (0.23–12.35)
Dominant model				
CC+CT	28 (90.32%)	25 (92.59%)	0.759	1.0
TT	3 (9.68%)	2 (7.4%)	0.568	0.75 (0.12–4.84)
Recessive model				
CC	19 (61.29%)	15 (55.56%)		1.0
CT+TT	12 (38.71%)	12 (44.44%)		1.27 (0.44–3.61)
Alleles				
C	47 (75.81%)	40 (74.07%)	0.830	1.0
T	15 (24.19%)	14 (25.93%)		1.1 (0.47–2.54)

Data presented as number of patients (n) and percentage (%) for categorical variables.
Binary logistic regression for categorical variables.
*$P \geq 0.05$ is considered nonsignificant, *$P < 0.05$ is considered significant.*

patients with recurrent thrombosis than in those without recurrent thrombosis (33.33% vs. 12.9%) with a significant difference (OR = 3.8, 95%CI = 1.04–17.034). Neither the dominant nor the recessive model showed significant association indicating a co-dominant inheritance as shown in Table 6.

The individuals carrying CC homozygous genotype of A1298C are at a 3.8-fold risk of recurrent thrombosis than those carrying AA wild genotype of the same SNPs. A recent study discussed modifiable risk factors and combination of genetic factors for recurrent ischemic stroke, concluding that cumulative effect of both risk factors and genetic factors were associated with recurrent ischemic stroke.[8] Another study revealed that patients with genetic predisposition had a significantly increased risk of recurrence of thrombotic events.[9] A case report by Alexandru Bostan described a 29-year-old female patient, who developed thrombophlebitis during the 17th week of her second pregnancy, which later complicated to right deep vein thrombosis in her 24th week of pregnancy and was treated with enoxaparin. After delivery, the patient suffered from cerebral vein thrombosis after third day postpartum. Upon investigation, the patient carried heterozygous mutation of MTHFR A1298C genotype.[10]

Observation of combined genotypes with recurrent thrombosis and history of anti-platelets

Of the participants, 27 cases from the patient group recorded for recurrent thrombosis with different diseases. Table 7 lists these cases as combined genotypes.

The most common recurrent thrombosis frequency for MTHFR combined genotypes was CC/CC and CT/AA (66.66%) from all cases, despite two-thirds of these cases being treated with aspirin or clopidogrel for secondary prevention of myocardial infarction and ischemic stroke. Other cases included were CC/AC (18.51%) and TT/AA (7.41%), as well as one case (3.71%) for each of CT/AC and CC/AA. The total percentage of CC/AC, TT/AA, CT/AC, and CC/AA for recurrent thrombosis cases was 33.34%, although 77.8% of these cases used aspirin or clopidogrel for secondary prevention of myocardial infarction and ischemic stroke. A recent study

TABLE 6. The frequency of different genotypes and alleles of A1298 polymorphism in patients with and without recurrent thrombosis.

A1298C	No recurrent thrombosis (n = 31)	Recurrent thrombosis (n = 27)	P	OR (95%CI)
Genotypes				
AA	10 (32.26%)	12 (44.44%)	**0.036***	**1.0**
AC	17 (54.84%)	6 (22.22%)	0.394	0.32 (0.12–1.18)
CC	4 (12.9%)	9 (33.33%)	**0.016***	**3.8 (1.04–17.34)**
Dominant model				
AA+AC	27 (8.71%)	18 (66.67%)	0.071	1.0
CC	4 (12.9%)	9 (33.33%)		3.37 (0.9–12.64)
Recessive model			0.342	
AA	10 (32.26%)	12 (44.44%)		1.0
AC+CC	21 (67.74%)	15 (55.56%)		0.59 (0.20–1.73)
Alleles				
A	37 (59.68%)	30 (55.56%)	0.654	1.0
C	25 (40.32%)	24 (44.44%)		1.18 (0.57–2.49)

Data presented as number of patients (n) and percentage (%) for categorical variables.
Binary logistic regression for categorical variables.
*P ≥ 0.05 is considered non-significant, *P < 0.05 is considered significant.*

TABLE 7. Recurrent thrombosis cases with a combination of MTHFR genotypes and percentage of history of aspirin or clopidogrel.

Combination of genotypes C677T/A1298C	Recurrent thrombosis (n = 27)	History of (aspirin or clopidogrel) in cases with recurrent thrombosis for each combination	Without recurrent thrombosis (n = 31)
CC/CC	9 (33.33%)	5 (55.56%)	4 (12.9%)
CT/AA	9 (33.33%)	7 (77.78%)	4 (12.9%)
CC/AC	5 (18.51%)	3 (60%)	12 (38.7%)
TT/AA	2 (7.41%)	2 (100%)	3 (9.7%)
CT/AC	1 (3.71%)	1 (100%)	5 (16.1%)
CC/AA	1 (3.71%)	1 (100%)	3 (9.7%)

Data presented as number of patients (n) and percentage (%) for categorical variables.

concluded that despite aspirin usage in patients with ischemic heart disease, there were recurrent episodes of heart attack, which was related to the presence of cardiovascular risk factors.[11]

Association of serum levels of homocysteine, vitamin B12, and folic acid with recurrent thrombosis

The median homocysteine, vitamin B12, and folic acid were compared between two groups of recurrent and nonrecurrent thrombosis with no significant differences. However, the median homocysteine in patients with recurrent thrombosis was much higher than in those with no such disorder (75.5 µmol/L versus 35.75 µmol/L) with a significant difference statistically as shown in Table 8.

The high levels of homocysteine in patients with recurrent thrombosis demonstrated the role of homocysteine in atherogenic process, indicating that moderate-to-severe level of homocysteine is a

TABLE 8. Association of serum levels of homocysteine, vitamin B12, and folic acid with recurrent thrombosis.

Variable	Homocysteine (µmol/L)	B12 (pmol/L)	Folic acid (nmol/L)
Recurrent thrombosis			
No	35.75 (13.75–74.5)	175 (106–843)	3.5 (1.2–33.7)
Yes	75.5 (14.5–101)	168 (35–415)	4.55 (1.05–7.4)
P	**0.041**	0.354	0.239

Data presented as median and range for numerical variables.
Kruskal–Wallis test (for three groups' comparison) for numerical and non-normally distributed variables.
*$P \geq 0.05$ is considered non-significant, *$P < 0.05$ is considered significant.*

potential risk factor for hypertension, cardiovascular disease, and stroke.[12–14]

CONCLUSIONS

The presence of C allele from the first SNP (CT) and A allele from the second SNP (AC) and the presence of CC/AA genotypes for two variants in individuals are considered as protective factors for exposure risk of ischemic heart disease, ischemic stroke, and venous thromboembolism. The individuals carrying C allele from the first SNP and C allele from the second SNP and those carrying CC/CC genotypes for two variants are at 3.11- and 3.82-fold risk of disease exposure respectively, compared to those carrying CC/AA genotypes.

Individuals with CC homozygous genotype of A1298C are at a 3.8-fold risk of recurrent thrombosis than those with AA wild genotype of the same SNPs due to high levels of homocysteine. This highlighted the role of homocysteine in atherogenic process, indicating that moderate-to-severe level of homocysteine is a potential risk factor for hypertension, cardiovascular disease, and stroke.

ACKNOWLEDGMENT

The authors would like to thank Alfarahidi University and Mustansiryiah University (www.uomustansiryiah.edu.iq), Baghdad-Iraq, for their support in this work.

REFERENCES

1. D'Amelio AM, Jr., Monroy C, El-Zein R, et al. Using haplotype analysis to elucidate significant associations between genes and Hodgkin Lymphoma. Leuk Res. 2012; 36(11): 1359–1364. https://doi.org/10.1016/j.leukres.2012.07.014
2. Myers TA, Chanock SJ, and Machiela MJ. LD link R: an R package for rapidly calculating linkage disequilibrium statistics in diverse populations. Front Genet. 2020; 11(157): 1–5. https://doi.org/10.3389/fgene.2020.00157
3. Livak KJ. Allelic discrimination using fluorogenic probes and the 59 nuclease assay. Genet Anal. 1999; 14: 143–149. https://doi.org/10.1016/S1050-3862(98)00019-9
4. Mutlak QM, Abdulridha MK, and Al-Huseini LA. Study the distribution of rotavirus genotypes in vaccinated and non-vaccinated children in Babylon Province. Iraqi J Pharm Sci. 2021; 30(2): 167–176. https://doi.org/10.31351/vol30iss2pp167-176
5. Ulvik A, and Ueland P. Single nucleotide polymorphism (SNP) genotyping in unprocessed whole blood and serum by real-time PCR application to SNPs affecting homocysteine and folate metabolism. Clin Chem. 2001; 47(11): 2050–2053. https://doi.org/10.1093/clinchem/47.11.2050
6. Lewis CM. Genetic association studies: design, analysis and interpretation. Brief Bioinform. 2002; 3(2): 146–153. https://doi.org/10.1093/bib/3.2.146
7. Yong Yong SHI, and Lin HE. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic

association at polymorphism loci. Cell Res. 2005; 15(2): 97–98. https://doi.org/10.1038/sj.cr.7290272

8. M'barek L, Sakka S, Megdiche F, et al. Traditional risk factors and combined genetic markers of recurrent ischemic stroke in adults. J Thromb Haemost. 2021; 19(10): 2596–2604. https://doi.org/10.1111/jth.15448
9. Elassal G, Hamed H, and Elgamal R. Study of some genetic predisposition in pulmonary embolism. Egypt J Chest Dis Tubercul. 2014; 63: 1039–1046. https://doi.org/10.1016/j.ejcdt.2014.05.007
10. Bostan A, Ţăpoi LC, Barcan MN, et al. Cerebral vein thrombosis associated with MTHFR A1289C mutation gene in a young postpartum woman. Arch Clin Cases. 2019; 6(1): 26–30. https://doi.org/10.22551/2019.22.0601.10150
11. Alsharidah AS, Alsuhaibani DS, Alsuhaibani HA, et al. Aspirin resistance among patients with new and recurrent ischemic heart disease episodes in Qassim region, Saudi Arabia. Eur Rev Med Pharmacol Sci. 2022; 26: 2106–2116.
12. Wang Y, Chen S, Yao T, et al. Homocysteine as a risk factor for hypertension: a 2-year follow-up study. PLoS One. 2014; 9(10): e108223. https://doi.org/10.1371/journal.pone.0108223
13. Chrysant SG, and Chrysant GS. The current status of homocysteine as a risk factor for cardiovascular disease: a mini review. Exp Rev Cardiovasc Ther. 2018; 16(8): 559–565. https://doi.org/10.1080/14779072.2018.1497974
14. Piazzolla G, Candigliota M, Fanelli M, et al. Hyperhomocysteinemia is an independent risk factor of atherosclerosis in patients with metabolic syndrome. Diabetol Metab Syndr. 2019; 11: 87. https://doi.org/10.1186/s13098-019-0484-0