Original Research Article

Exploring the Interplay: Aspirin Therapy, Genetic Polymorphisms, and Homocysteine Levels in Cardiovascular Disease Patients

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ABSTRACT

Cardiovascular disease (CVD) is a significant worldwide health threat expected to cause 23.6 million deaths annually by 2030. Homocysteine is an amino acid that is generated during the breakdown of methionine. Elevated levels of homocysteine are recognised as a standalone risk factor for cardiovascular disease, such as coronary artery disease and stroke. Methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MS) play essential roles in regulating homocysteine levels and maintaining cardiovascular health. The C677T (rs1801133) mutation of the MTHFR gene is closely linked to coronary artery disease due to decreased enzyme activity while the A2756G mutation (rs1805087) in the MS gene disrupts the remethylation process and is linked to elevated homocysteine levels and a higher risk of cardiovascular disease. Aspirin is a key treatment for cardiovascular disease by preventing platelet activation and aggregation, reducing the likelihood of blood clot formation. In addition, aspirin usage seems to be connected to homocysteine levels in persons dealing with CVD. The precise interplay between aspirin therapy, genetic polymorphisms, and their collective impact on homocysteine levels in CVD patients remains unclear. Therefore, this investigation aims to explore the effects of genetic polymorphisms (MTHFR and MS genes) and aspirin therapy on homocysteine levels in CVD patients from two hospitals in Selangor, Malaysia. Blood samples from 52 patients were collected and analysed, with homocysteine levels quantified using LCMS-QQQ, aspirin abundance determined through LCMS-QTOF, and genetic polymorphisms of MTHFR and MS genes identified using RT-PCR. Interestingly, individuals with CVD exhibiting elevated homocysteine levels did not show the mutant genotype for neither MTHFR nor MS genes. Furthermore, the potential influence of aspirin therapy emerged as a plausible explanation for the observed lower homocysteine levels in these patients. Other variables than genetic predisposition may play a role in causing elevated homocysteine levels in people with CVD. Our findings suggest that aspirin therapy may have a potential impact on reducing homocysteine levels in these patients. However, more research is needed to understand the underlying mechanisms.

Keywords: Cardiovascular disease, Homocysteine, Aspirin, Methylenetetrahydrofolate reductase, Methionine synthase

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1.0 Introduction

CVD has remained the leading cause of death globally for decades due to the increasing prevalence of CVD risk factors and the aging population. CVD contributed to 422.7 million cases and 17.9 million fatalities in 2015 (1,2). According to studies, it is expected to cause 23.6 million deaths per year by 2030 (1–3). Over the past thirty years, the burden of CVD mortality and morbidity has increased in Malaysia. According to a report from the Malaysian Ministry of Health, CVD has remained the number-one cause of death since the 1980s(4). Age, smoking, genetics, hypertension, hyperlipidaemia, and diabetes mellitus are the main risk factors for heart disease that have been identified by the Framingham Heart Study (2). The prevalence of coronary artery disease and the onset of early coronary artery disease, however, cannot be entirely explained by this conventional variable alone. For instance, about 50% of patients with unstable angina or acute myocardial infarction do not have the traditional risk factors. New categories of atherosclerotic factors, like increased homocysteine, are currently being considered (2). Elevated levels of homocysteine in the bloodstream have been linked to a higher likelihood of developing atherosclerosis, coronary artery disease, and other cardiovascular incidents. The precise ways in which homocysteine contributes to cardio-vascular disease are currently being studied. It is believed to facilitate endothelial dysfunction, oxidative stress, inflammation, and thrombosis, all of which are significant factors in the development of cardiovascular disease. Elevated homocysteine levels are now being seen as a potential focus for intervention in preventing and managing CVD. Various approaches to reduce homocysteine levels, such as using B vitamins (including folate, vitamin B6, and vitamin B12) as supplements, have been investigated in clinical trials with varying outcomes. Additional study is required to enhance our comprehension of the correlation between homocysteine and cardiovascular disease (CVD) and to identify the most efficient methods for lowering homocysteine levels and reducing cardiovascular risk.

Homocysteine can contribute to the development of cardiovascular disease through various pathways, including its negative impact on vascular endothelium and smooth muscle cells, leading to changes in preclinical artery structure and function. Increased levels of homocysteine are associated with endothelial dysfunction, oxidative stress, inflammation, and a prothrombotic state. These factors can lead to the development and advancement of atherosclerosis, thrombotic events, and ultimately, cardiovascular events such as heart attacks and strokes. High levels of homocysteine are closely linked to various harmful processes in the cardiovascular system. Homocysteine rise is closely associated with endothelial dysfunction, which is marked by reduced vasodilation and heightened vascular inflammation. Endothelial dysfunction is a crucial factor in the development and advancement of atherosclerosis, a primary pathogenic mechanism responsible for most cardiovascular incidents. Elevated homo-cysteine levels contribute to oxidative stress by disrupting the balance between reactive oxygen species generation and the body's antioxidant defenses. Oxidative stress worsens endothelial dysfunction and encourages vascular inflammation, leading to the formation of atherosclerotic plaques. Homocysteine is involved in promoting a prothrombotic condition, which means it stimulates the production of blood clots. This setting that promotes blood clotting raises the likelihood of clot-related events like heart attacks and

strokes. Thus, focusing on homocysteine levels shows potential as a therapeutic strategy for treating cardiovascular disease. Lowering homocysteine levels by lifestyle changes and pharmacological treatments like folic acid supplementation can help alleviate endothelial dysfunction, oxidative stress, inflammation, and the prothrombotic state. This comprehensive strategy can stop the advancement of atherosclerosis and lower the chances of cardiovascular events such as heart attacks and strokes, ultimately leading to better patient results (5).

Methylenetetrahydrofolate reductase (*MTHFR*) is an enzyme that converts homocysteine to methionine, which is an important step in the methylation cycle. The methylation cycle is a biological process that is essential for several physiological activities, such as DNA synthesis, repair, and neurotransmitter production. *MTHFR* converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is necessary for the remethylation of homocysteine to methionine. Methionine, in turn, acts as a precursor for S-adenosylmethionine (SAM), a methyl donor involved in many methylation events in the body. Reduced enzymatic activity may result from polymorphisms (mutations) in the *MTHFR* gene, namely the C677T and A1298C variants. Blood homocysteine levels may rise because of this decline in *MTHFR* activity. Because high homocysteine levels can cause inflammation and blood vessel damage, they have been linked to an increased risk of CVD. Sustaining homocysteine levels at acceptable levels is critical for cardiovascular health. It is crucial to monitor and control homocysteine levels with a healthy diet that includes enough B vitamins (such as folate, vitamin B6, and vitamin B12). Additionally, tailored strategies to control homocysteine levels and lower related health risks may be advantageous for people with particular *MTHFR* gene variations. *MTHFR* enzymatic activity is decreased in people having Val residues, with a mean enzymatic activity of 30% in the (TT) homozygous (Val/Val) state and 65% in the (CT) heterozygous (Ala/Val) condition (6). This decreased *MTHFR* activity can lead to an increase in plasma homocysteine levels, especially when folate consumption is low (6,7). Increased homocysteine levels are associated with an increased risk of CVD. Whereas methionine synthases transform the amino acid homocysteine to methionine that is required for protein synthesis and other metabolic functions. Methionine synthase (*MS*) regulates DNA transcription, epigenetic expression, and gene regulation requires cobalamin and folate as cofactors. In humans, the *MS* gene encodes methionine synthase and mutations in this gene can influence enzyme performance as well as blood levels of homocysteine and folate (8).

Aspirin is commonly used to prevent and treat CVD by inhibiting platelet aggregation. Nevertheless, a notable problem occurs with aspirin resistance (AR) despite its widespread usage. AR occurs when platelet aggregation is not effectively prevented in patients who are receiving regular or high doses of aspirin. Resistance to antiplatelet medication can be observed in 5% to 40% of instances, affecting its efficacy. Platelet aggregation is crucial in the development of cardio-vascular disease. Aspirin resistance may impact the effectiveness of aspirin in preventing cardiovascular events. It is essential to tackle this resistance to achieve the best treatment results and lower the chances of CVD related issues (9).

The exact relationship between aspirin therapy, genetic polymorphisms, and their combined effect on homocysteine levels in patients with CVD remains uncertain.

Therefore, the study aims to evaluate the impact of genetic polymorphisms (*MTHFR* and *MS* genes) and aspirin therapy on homocysteine levels in cardiovascular patients. Specifically, we explore the influence of *MTHFR* and *MS* gene polymorphisms, along with aspirin therapy, on homocysteine concentrations in cardiovascular patients. This research addresses the implications of these factors, enhancing our understanding of aspirin's role in managing homocysteine levels among cardiovascular patients.

2.0 Materials and Methods

2.1 Experimental design

Ethical approval was obtained from the Research Ethics Committee (REC/03/2021 (FB/14). This study is a randomised study involved 52 male patients seeking emergency care presenting CVD symptoms at the Hospital Al-Sultan Abdullah, Puncak Alam, Selangor, Malaysia, and the Clinical Training Centre Sungai Buloh, Selangor, Malaysia. The inclusion criteria for this study were male patients who were admitted for cardiovascular events, age 40 and above. Meanwhile, the exclusion criteria for this study were patients with severe dehydration and renal disease. A total of 3 ml of blood samples was collected from patients who agreed to participate in this study. The blood samples were aliquoted for genotyping (1.5 ml), quantitation of homocysteine, and measurement of abundance of aspirin (1.5 ml). Patients were grouped according to their homocysteine level, patients with homocysteine more than 15 µmol/L were grouped in hyperhomocysteinemia. Moreover, all the patients are already on aspirin medication.

2.2 Genotyping of MTHFR (rs1801133) and MS (rs1805087)

DNA was extracted from the whole blood using a DNA purification kit. The extraction was done according to the protocol that was given by the manufacturer. Followed by the DNA integrity test by measuring the DNA obtained using a Nanodrop 2000 Spectro-photometer (Thermo Fisher Scientific, Waltham, MA). The DNA yield is considered a good DNA yield if the concentration of DNA is above 50 ng/µl, 260/280 ratio of reading ~1.8, and ~2.0 for 269/230 ratio. The in-house primer polymerase chain reaction (PCR) method was developed to determine the *MTHFR* and *MS* polymorphisms. The in-house designed primers of *MTHFR* and *MS* are listed in the Table 1. HRM analysis was performed on (PCRmax Eco 48 qPCR system). The steps in this PCR consisted of the steps consisted of an initial denaturing step at 95°C for 5 minutes, followed by 45 cycles of 95°C for 31 seconds (DNA denaturation), 95°C for 15 seconds (Primer annealing), 65°C for 15 seconds (Extension) and a melting curve (gradual temperature changes up to 95°C, over 15 seconds).

2.3 Quantification of homocysteine

Blood homocysteine level was quantitated from plasma. The plasma samples were stored at -20 ºC and thawed at least 30 minutes before the extraction process. Quantification was conducted using multiple reaction monitoring (MRM) modes based on the parents and product ion transition for L-Homocysteine (136 > 90.1, 56.2) and d8-DL-Homocysteine (140 > 94.1, 59.3) as internal standard. The approach was validated for linearity, sensitivity, accuracy, precision, recovery, and stability.

Table 1. List of primers

List Of Primes	Primer Sequence
MTHFR Forward Primer	GCCCAGCCACTCACTGTTTTAGTTCAGG
MTHFR Reverse Primer	GGTGGAGTGCTGAGTTCGCTGAGTTCTT
MS Forward Primer	TCAGTGTTCCCAGCTGTTAGA
MS Reverse Primer	GGAGTGTAAAAGGCTTTGGATCA

Linearity was observed between 250 – 3000 ng/mL with a correlation coefficient ($r^2 = 0.98$). Before analysis was done, QC samples at three different concentrations which are low, medium, and high (250, 1250, and 2500 ng/ml) were prepared and analysed to assure the LC/MS/MS was stable. Then, the result obtained was compared with the calibration curve. The QC sampled were accepted if the result is between ±20% accuracy. The data was processed by Agilent MassHunter Qualitative Analysis software (B.09.00.).

2.4 Measurement of aspirin abundance

Upon collection, the plasma blood samples were stored at -20 °C to preserve the aspirin within the plasma. Before the extraction process, the samples were thawed for at least 30 minutes. Subsequently, the plasma blood samples underwent liquid chromatography and full-scan mass spectrometry to collect data on all metabolites potentially present in the sample. Notably, this analysis focused on aspirin components, including salicylate, 2,3-hydrobenzoate, gentisate, salicyl acyl, and salicylurate. The measurement of metabolomics profiles was done using LC/MS-QTOF. The global metabolomics analysis method which was established and optimized in-house was used. A 1200 Rapid Resolution system (Agilent Technologies, USA) complete with a binary pump and degasser, well-plate auto-sampler with thermostat, thermostatic column compartment, and an Agilent 6520 Q-TOF mass spectrometry equipped with ESI source was used to analyse the samples. Column Zorbax Eclipse Plus C-18 (1.8 µm particle size, 2.1 x 100 mm column dimensions) was used for chromatographic separation of sample extracts and maintained at 40ºC during the run. Samples were in both positive modes. To ensure the integrity of the data, tuning of the machine to adjust the TOF and quadrupole parameters to achieve the desired signal intensity and resolution is done regularly. The data was processed by Agilent MassHunter Qualitative Analysis Software B.09.00.

2.5 Statistical analysis

The data were imported into IBM Statistical Package for Social Sciences (SPSS) Statistics (Version 15). Before data entry into SPSS, the questionnaire underwent coding. Frequencies and descriptive statistics were examined to assess normality and identify any outliers or erroneous data. In cases where the data did not exhibit a normal distribution, square root transformation was applied (sqrt). The collected data underwent analysis using the Paired Sample t-test and one-way ANOVA.

3.0 Results

3.1 Genetic Polymorphisms of MTHFR (rs1801133) and MS (rs1805087)

Most of the patients were Malay males (40), and the others were Chinese males (7) and Indian males (5) with a mean age of 60 ± 8.88 years old. Tables 2 and 3 display homocysteine levels in patients with MTHFR (rs1801133) and MS (rs1805087) genetic polymorphisms, respectively. The ANOVA test performed

revealed no significant difference between the groups for the MTHFR (rs1801133) and MS (rs1805087) polymorphisms (p=0.994787 and p=0.973223), respectively. This suggests that there was no statistically significant difference in the levels of homocysteine based on the MS (rs1805087) and MTHFR (rs1801133) genotype.

Table 2. Homocysteine levels in patients with *MTHFR* (rs1801133) genetic polymorphisms

Genotype	*Count*	*Average (µmol/l)*	*SD*
GG	15	10.85625	±5.650054
GA	30	10.53567	±4.982
AA	7	10.44571	±1.627184

Table 3. Homocysteine levels in patients with *MS* (rs1805087) genetic polymorphisms

Genotype	*Count*	*Average (µmol/l)*	*SD*
AA	19	10.78368	±5.129228
AG	33	10.63788	±4.769704
GG	0		

3.2 Quantification of Homocysteine level and Aspirin abundance

All patients received aspirin promptly upon admission to the emergency departments of both hospitals. Following admission, patients were stratified based on their homocysteine levels, with individuals presenting less than 15 µmol/L categorized as the normal group and those with levels exceeding 15 µmol/L designated as the hyperhomocysteinemia group. The mean homocysteine levels for each group were determined as 8.9 ± 2.71 µmol/L for 44 patients in the normal group and 19.42 ± 4.00 µmol/L for 8 patients in the hyperhomocysteinemia group. Subsequently, these results were scrutinized with aspirin abundance as shown in Table 4. The data is presented in Table 5, showcasing the distribution of aspirin abundance across varying homocysteine levels in both groups. The statistical analysis, as indicated by the t-test result (p=0.000359), demonstrates a notable and statistically significant difference in aspirin abundance between patients with normal homocysteine levels and those with hyperhomocysteinemia. This significant finding holds potential implications for elucidating the intricate relationship between homocysteine levels and the efficacy or metabolism of aspirin in these distinct patient groups. Each sample was run once using LCMS-QTOF and the mean and standard deviation (SD) values were calculated for each group (normal homocysteine level and hyper-homocysteinemia) as shown in Table 5.

4.0 Discussion

The study aims to evaluate the impact of genetic polymorphisms (*MTHFR* and *MS* genes) and aspirin therapy on homocysteine levels in CVD patients from two different hospitals in Selangor, Malaysia. Blood samples from 52 patients were analysed using advanced techniques to identify genetic variations and measure homocysteine levels and aspirin abundance. Patients with elevated homocysteine levels did not have mutated genotypes for the *MTHFR* or *MS* genes, which contradicts conventional knowledge. The findings indicate that aspirin therapy could help control homocysteine levels in these patients, emphasising wider benefits beyond antiplatelet actions and supporting tailored approaches to cardiovascular disease management.

The metabolic pathway of homocysteine plays a crucial role in "one-carbon metabolism." The presence of functional polymorphisms in key genes

Table 4: Five metabolites of Aspirin were found in the hyperhomocysteinemia group in different Retention Time (RT)

Metabolites	Aspirin	Salicylic Acid	Gentistic	Salicylurate	Salicyl Phenolic Glucoronide
Retention time (RT)	3.424	2.40	2.059	8.136	1.568

Table 5: Distribution of aspirin abundance across normal homocysteine and hyperhomocysteinemia groups

Groups	*N*	*Abundance (%)*	*SD*
Normal Homocysteine *(<15 µmol/l)*	44	4.05	±0.49
Hyperhomocysteinemia *(>15 µmol/l))*	8	0.86	±3.49

related to one-carbon metabolism, such as *MTHFR* and *MS*, can significantly impact folate metabolism. Consequently, these genetic variations can exert a profound influence on intracellular methylation reactions. An increase in homocysteine, referred to as hyperhomocysteinemia, signals a decline in methylation ability. This condition can contribute to several health issues, including the development of atherosclerosis (10,11). Recent studies have stated that the *MTHFR* and *MS* gene genotypes and hyperhomocysteinemia might be changed by variations in genes coding for other homocysteine metabolizing enzymes (10). *MTHFR* serves as an enzyme facilitating the conversion of homocysteine to methionine, a crucial process reliant on folate as a cofactor. Variations in the *MTHFR* gene, such as C677T and A1298C, have the potential to diminish enzymatic activity, resulting in elevated homocysteine levels in the bloodstream. Elevated homocysteine levels are correlated with an augmented susceptibility to CVD and hypertension (12).

Our investigation uncovered interesting findings about the genetic makeup of hyperhomocysteinemia in cardiovascular patients the two hospital sites. The occurrence of mutant genotypes for the *MTHFR* and *MS* genes was lower than expected, indicating possible complications in the genetic pathways responsible for hyperhomocysteinemia in this community. The study revealed that the mutant genotype frequency for *MTHFR* was 12.7%, with the heterozygous genotype being the most common at 54.5%, followed by the homozygous genotype at 27.3%. The observed frequencies for the *MS* gene genotypes were lower than expected. The limited variety in genotypes among the patient groups made it difficult to use typical genetic models to evaluate the impact of genotype combinations on hyperhomocysteinemia. Although limited, this study offers important initial observations on the genetic factors influencing hyperhomocysteinemia in cardiovascular patients. Our study found no evidence supporting an increased risk associated with the combination of *MTHFR* and *MS* gene variations in hyperhomocysteinemia. This contradicts the hypothesis suggesting a synergistic interaction between these genes. These findings underscore the need for further research to clarify the complex genetic basis of hyperhomocysteinemia in cardiovascular patients.

The mechanism of action for aspirin involves inhibiting platelet activation and aggregation through the permanent blockage of the COX-1 enzyme, which produces thromboxane A2, a substance inducing platelet aggregation. This action prevents excessive platelet clumping, reducing the risk of harmful blood clot

formation (13,14). Despite the effect of aspirin that can lower the homocysteine level in patients, some studies suggest that some cardiovascular patients might have aspirin resistance. Aspirin resistance can be categorized into two types: laboratory resistance and clinical resistance. Laboratory aspirin resistance is characterized by aspirin's inability to hinder platelet thromboxane A2 production or to inhibit platelet function tests (such as platelet aggregation) that rely on thromboxane production. On the other hand, clinical aspirin resistance is described as aspirin's failure to prevent atherothromboembolic ischemic events in patients prescribed the medication. However, this issue is more precisely termed aspirin treatment failure (14,15).

In our study, the mean aspirin abundance for hyperhomocysteinemia patients is higher than those with normal homocysteine level. It is worth mentioning, however, that the first line of therapy for these individuals was aspirin, which has been linked to lower plasma homocysteine levels. The patients with hyperhomocysteinemia are suspected to have aspirin resistance which may may lead to inability of the aspirin to protect individuals from thrombotic events, prolong bleeding times, decrease the generation of TXA2, or have the expected effects on platelet function *in vitro* studies. According to several research, the percentage of the population with cardiovascular and cerebrovascular disorders that are aspirin resistance ranges from 5% to 60%. Due to differences in aspirin resistance classifications, testing and measuring method employed across research, limited sample sizes and different populations studied, it is challenging to determine the precise prevalence of aspirin resistance from this investigation of platelet response to aspirin today involves a variety of laboratory procedures, including measurement of thromboxane production, platelet aggregation and platelet activation as well as bleeding time. Increased platelet turnover, genetic variations in COX-1 and other thromboxane biosynthesis genes, overexpression of nonplatelet sources of thromboxane production and medication interactions are a few potential aspirin pathways (14,16,17).

5.0 Conclusion

In summary, this research delved into the intricate interplay between homocysteine levels, genetic polymorphisms—specifically those related to the *MTHFR* and *MS* genes—and the effects of aspirin therapy on homocysteine levels in cardiovascular patients. Contrary to conventional assumptions, the study revealed that individuals with CVD and elevated homocysteine levels do not always exhibit the mutant genotype for *MTHFR* or *MS* genes. Furthermore, the potential impact of aspirin therapy on reducing homocysteine levels introduces an additional layer of complexity to our understanding of these connections. This investigation underscores the importance of nuanced exploration, considering multifaceted factors that influence homocysteine levels in CVD patients, and it paves the way for further research into the role of aspirin and genetic polymorphisms in shaping cardiovascular outcomes.

Authorship contribution statement

SSMAS: Carried out the experiments, perform analysis and drafted the manuscript**; SSS**: Participated in the design and served as principal investigator throughout its execution and helped to draft the manuscript; **MIKM & MAMM:** Participated in coordination of the study and helped to draft the manuscript**; MSR:** Participated in the data analysis and

helped to draft the manuscript**; TLK & MZS:** Participated in the design and data analysis of the study**; RJJ**: Participated in the design and coordination of the study and helped to draft the manuscript**.**

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Conflict of Interest

The authors declared that they have no conflict of interest to disclose.

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