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Original Article

Biochemistry Section

Association of Serum Hydrogen Sulphide Levels and Dyslipidaemia: A Cross-sectional Study

IPSITA BHATTACHARYA[1], ANKITA PRASAD[2], ANKITA BHATTACHARYA[3], PINAKI SAHA[4], NIRMALYA ROY[5], ARPITA SAHA[6], INDIRA BHASKAR BISWAS[7]

ABSTRACT

Introduction: Dyslipidaemia is an important risk factor for coronary artery disease. Oxidative damage to plasma lipoproteins ultimately results in the development of atherosclerosis. Since Hydrogen Sulphide (H_2S) is a cytoprotective molecule in oxidative stress, decreased H_2S levels may be a cause of dyslipidaemia.

Aim: To determine the relationship between the serum levels of H_2S with serum Triglycerides (TG), Total Cholesterol (TC), and High-density Lipoprotein Cholesterol (HDL-C) in cases of dyslipidaemia.

Materials and Methods: A cross-sectional study was conducted between December 2022 and June 2023 in the Departments of Biochemistry and Medicine at KPC Medical College, Jadavpur, Kolkata, West Bengal, India. Serum lipid profile {TC, TGs, HDL-C, and Low-density Lipoprotein Cholesterol (LDL-C) (calculated by Friedewald's equation)} and serum H_2S were measured in 70 cases of dyslipidaemia {according to National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) criteria} aged between 30 to 80 years and compared with an equal number of healthy volunteers as controls. The means of continuous variables were compared by independent t-tests and Mann-Whitney U tests. The Chi-square test was applied to compare gender distribution. Pearson's and Spearman's correlation coefficients were calculated for normal and non normal distributions, respectively.

Results: The mean age of dyslipidaemic patients (54.54 years±11.19) was significantly (p=0.0017) higher than that of the controls (47.02 years±16.18). Among the 70 cases of dyslipidaemia, 38 participants were male and 32 were female, while among the healthy controls (n=70), 42 participants were male and 28 were female. The serum H_2S levels in cases of dyslipidaemia (37.91±6.28 µmol/L) were significantly lower than in the healthy controls (58.52±12.92, p<0.01 µmol/L). A significant positive correlation was found between serum H_2S levels and HDL-cholesterol (r=0.81, p<0.001), whereas a negative correlation was found between serum H_2S and TG levels (r=-0.55, p<0.001).

Conclusion: In the present study, dyslipidaemia was associated with decreased levels of serum H_2S. Serum H_2S was positively correlated with serum HDL and negatively correlated with serum TG levels.

Keywords: Atherosclerosis, Body mass index, High-density lipoprotein cholesterol, Reactive oxygen species, Triglycerides

INTRODUCTION

Dyslipidaemia is defined as alterations of one or more lipoproteins and lipid parameters in the blood, such as increased TC, LDL-C, and/or TG levels, and decreased HDL-C levels [1,2]. It is a major risk factor for coronary artery disease [3] and is an important cause of cardiovascular disease with serious complications [4]. Increased levels of serum TC and LDL-C may contribute to higher mortality from cardiovascular diseases [5]. Apart from elevated plasma LDL-C levels leading to the development of atherosclerosis, other forms of dyslipidaemia, such as hypertriglyceridemia, are associated with other serious ailments like acute pancreatitis and Non Alcoholic Fatty Liver Disease (NAFLD) [6,7].

Dyslipidaemia has become widespread across the world over the past 30 years [8]. The most common form of dyslipidaemia throughout the world is hypercholesterolaemia [8]. Currently, both the urban and rural populations of India have a high prevalence of hypercholesterolaemia ranging between 25-30% and 15-20%, respectively [9]. However, the most common forms of dyslipidaemia in India are high LDL-C, low HDL-C, and hypertriglyceridemia [9]. There are multiple aetiologies of dyslipidaemia, including dietary factors, tobacco exposure, genetic causes, etc. [2]. Increased oxidative stress causes oxidative damage to plasma lipoproteins, ultimately leading to hyperlipidaemia and atherosclerosis [10,11].

The H_2S is one of the three important endogenous gaseous molecules with significant roles in cell signaling, other than nitric oxide and carbon monoxide [12]. Hydrogen sulfide (H_2S) plays several important physiological roles within the body, including protection against cardiovascular disease [13]. The enzymes known to synthesise H_2S in our body are cystathionine γ-lyase (CSE), Cystathionine β-Synthetase (CBS), and 3-Mercaptopyruvate Sulfurtransferase (3-(MST) [14,15]. The blood levels of serum H_2S are found to be lower in certain chronic disease states, such as diabetes, hypertension, and chronic kidney disease [16-18]. The imbalance of H_2S in our body may contribute to the development of vascular inflammation and atherosclerosis [19]. H_2S is also found to be cytoprotective in oxidative stress [20,21]. Hence, decreased levels of H_2S in the blood may be a causative agent of dyslipidaemia.

Jain SK et al., have observed a positive correlation between H_2S and HDL cholesterol levels in the blood [22]. H_2S has been found to reduce serum TG by activation of liver autophagy [23]. Researchers have also found that exogenous H_2S can reduce fatty liver blood TG and TC levels in obese mice [24]. It is a known fact that the enzyme Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) promotes hepatic Low-density Lipoprotein Receptor (LDLR) degradation and decreases the hepatic clearance of plasma LDL-C, thereby increasing LDL-C in the blood [25]. It has been reported that H_2S can inhibit the expression of PCSK9 [26].

With this background, the current study was planned to investigate the relationship between the serum levels of H_2S and the lipid parameters (serum TGs, TC, and HDL-cholesterol) in cases of

dyslipidaemia. The primary objective is to investigate the relationship between serum H_2S levels and serum TGs, TC, and HDL-C levels in cases of dyslipidaemia. The secondary objective is to investigate the relationship between serum H_2S levels and other associated anthropological parameters like Body Mass Index (BMI) and Waist-hip Ratio (WHR).

MATERIALS AND METHODS

This cross-sectional study was conducted in the Department of Biochemistry and Medicine at KPC Medical College, Jadavpur, Kolkata, West Bengal, India, between December 2022 and June 2023, after approval from the Institutional Ethics Committee (IEC No. KPCMCH/IEC/2022-23/51 dated 29/11/2022). Informed consent was taken from all the subjects enrolled in the study.

Inclusion criteria: The study was conducted including cases of dyslipidaemia aged between 30 to 80 years, including both males and females. An equal number of healthy volunteers were selected as controls. The cases were chosen from patients at the Medicine Outpatient Department (OPD) of the study Institute. Healthy controls were selected from the staff and faculty of KPC Medical College, as well as healthy relatives of the patients enrolled in the study who were willing to participate. Dyslipidaemia was diagnosed based on overnight fasting (9-12 hours) serum lipid profile estimation from the patients according to NCEP ATP III criteria, with serum TC>200 mg/dL, Serum TGs > 150 mg/dL, LDL-C (calculated) > 130 mg/dL, and/or HDL-C < 40 mg/dL [27]. Serum LDL-C was calculated using Friedewald's equation [28].

Exclusion criteria: Patients excluded from the study were those suffering from diabetes mellitus (except the prediabetic population), hypothyroidism, or other endocrine disorders; those receiving drugs like metformin, which may alter the level of H_2S; patients who were pregnant; those with cancer, collagen vascular diseases, or autoimmune diseases.

Sample size calculation: The formula used for the calculation of the sample size was n=Z2 P(1-P)/d2, where 'n' is the sample size, z=1.96 (95% confidence interval), p=47.3 (approximate prevalence of dyslipidaemia without diabetes mellitus according to Puri et al.,) [29], d=10% (Absolute precision). The sample size calculated was approximately 96. Among the 96 patients with dyslipidaemia without diabetes mellitus enrolled, 26 patients were excluded from the study due to dropout (9), the presence of hypothyroidism (12), a history of intake of Metformin (3), and a history suggesting a reproductive endocrine disorder with inadequate drug history (2).

Study Procedure

Four millilitres of overnight fasting blood samples were collected in ordinary red-capped clot vials without any additives from the patients for the study of serum lipid profile. Informed consent was obtained from both cases and controls. Blood was drawn aseptically in sterile vials from all consenting individuals. The collected whole blood was centrifuged for five minutes at 3000 rpm, and the serum was stored in a -20°C refrigerator. Serum TGs, TC, and HDL-cholesterol were estimated by enzymatic kit methods. The details of the instrument, methodology, and cut-off values are mentioned in [Table/Fig-1].

Variables	Methodology	Cut-off value	Analyser
TG	Enzymatic (GPO) kit method	>150 mg/dL	Fully automated biochemistry analyser SYS 400.
TC	Enzymatic (CHOD-PAP) kit method	>200 mg/dL	
HDL-C	Immuno FS homogenous method.	<40 mg/dL	
LDL-C	Friedewald's equation {LDL=Total Cholesterol-(HDL+ Triglycerides/5)}	>130 mg/dL	
H_2S	Spectrophotometric manual method	<50.47 µM/L	

[Table/Fig-1]: Parameters of serum lipid profile and H_2S level with methods of estimation and reference ranges.
TC: Total cholesterol; HDL-C: High-density cholesterol; TG: Triglycerides; LDL-C: Low-density cholesterol; CHOD-PAP: Cholesterol oxidase peroxidase single reagent; GPO: Glycerol phosphate oxidase; FS: Fluid stable

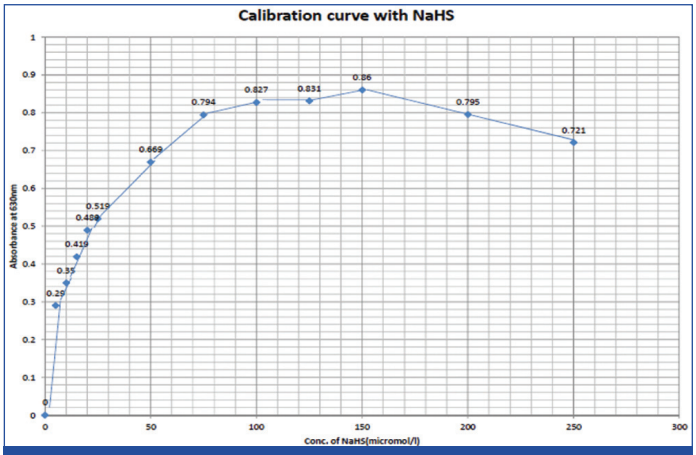
LDL-C levels were calculated by Friedewald's equation: LDL=TC-(HDL+TGs/5) [28].

The serum H_2S levels were estimated using a spectrophotometric method that had been standardised and described earlier by one of the authors [30] and modified in the Biochemistry research laboratory at KPC Medical College. This modified method has also been published previously [31]. The method involves the reaction of sulfide with N, N-dimethyl p-phenylene diamine sulfate in the presence of Fe^{3+} in hydrochloric acid as the oxidising agent. Methylene blue is produced in the reaction, which is then read at a wavelength of 670 nm.

To perform the test, 75 µL of serum was taken in capped glass test tubes. To this, 425 µL of Phosphate Buffered Saline (PBS) and 250 µL of 10% trichloroacetic acid were added. The tube was capped, properly covering the mouth, and then centrifuged at 3000 rpm for 30 minutes. The supernatant obtained after centrifugation was transferred to another capped glass test tube.

In the next step, 250 µL of 1% zinc acetate, 133 µL of 20 mM N, N-dimethyl-p-phenylene diamine sulfate in 7.2 mM HCl, 133 µL of 30 millimolar $FeCl_3$ in 1.2 mM of HCl, and 60 µL of 10% NaOH were added to the supernatant. The test tubes were capped and incubated for 10 minutes at room temperature.

A calibration curve was prepared using concentrations of 5-250 mM/L of Sodium Hydrogen Sulfide (NaHS) as shown in [Table/Fig-2] according to Bhattacharya A et al., and the serum H_2S levels were calculated based on this curve [31].



[Table/Fig-2]: Calibration curve of serum H_2S assay.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Microsoft excel 2007 Analysis ToolPak for descriptive statistics, independent t-tests, and scatterplots. The online free social statistics calculator was used for normality tests, Mann-Whitney U tests, Chi-square tests, and the calculation of correlation coefficients. The continuous data were checked for normality using the Kolmogorov-Smirnov Test. Data comparison was conducted using an independent t-test (2-tailed) for normal distribution and Mann-Whitney U Tests for non normal distribution. The comparison of categorical data (gender distribution) was done by the Chi-square test. Pearson's and Spearman's correlation coefficients were calculated to determine the relationship between variables with normal and non normal distributions, respectively. A p-value of <0.05 was considered statistically significant.

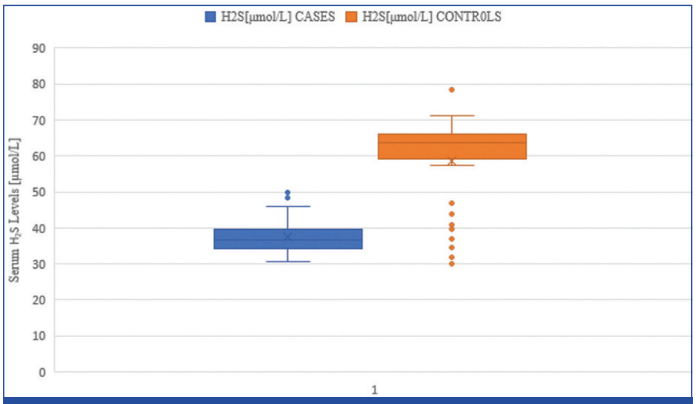
RESULTS

In the present study, the mean age of dyslipidaemic patients (54.54 years±11.19) was significantly higher (p=0.0017) than that of their healthy counterparts (47.02 years±16.18). Among the 70 cases of dyslipidaemia, 38 participants were male and 32 were female, whereas among the healthy controls, 42 participants were male and 28 were female. The mean BMI, WHR, fasting, and Postprandial

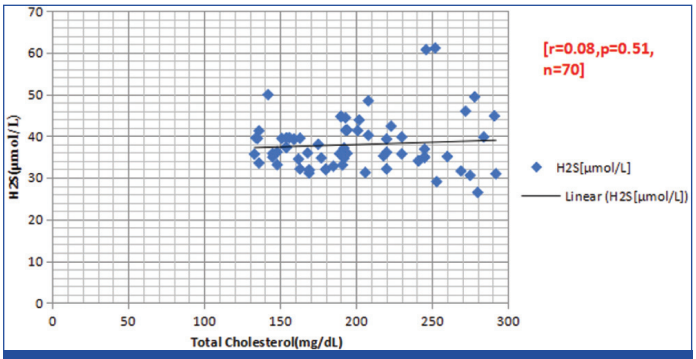
Variables	Cases (n=70) Mean±SD, p-value(K-S)	Controls (n=70) Mean±SD, p-value (K-S)	p-value
Age (in years)	54.54±11.19, 0.68	47.02±16.18, 0.33	0.0017
Gender (M/F)	38/32	42/28	0.49
BMI (kg/m^2)	24.31±2.75, 0.93	23.73±5.06, 0.50	0.40
WHR	0.94±0.16, 0.01	0.91±0.15, 0.15	0.25
FBS (mg/dL)	92.48±11.57, 0.17	91.65±11.51, 0.108	0.67
PPBS (mg/dL) [2 hours]	130.95±16.23,0.12	131.68±17.71, 0.022	0.79
Serum TC (mg/dL)	198.14±44.97, 0.22	139.05±30.81, 0.38	<0.001
Serum HDL-C (mg/dL)	37.42±5.97, 0.01	50.77±7.63, 0.007	<0.001
Serum TG (mg/dL)	188±97.54, 0.009	106.78±27.39, 0.08	<0.0001
Serum LDL-C [calculated] (mg/dL)	123.08±41.45,0.6	64.27±27.86,0.4	<0.001
Serum H_2S (µmol/L)	37.91±6.28, 0.41	58.52±12.92, <0.01	<0.0001

[Table/Fig-3]: Description of clinical and biochemical parameters of cases and controls (N=140).
BMI: Body mass index; WHR: Waist-hip ratio; FBS: Fasting blood sugar; PPBS: Postprandial blood sugar; TC: Total cholesterol; HDL-C: High-density cholesterol; TG: Triglycerides; LDL-C: Low-density cholesterol; K-S: Kolmogorov Smirnov Test for normality (p>0.05 signifies normal distribution); p-value <0.05 is considered statistically significant

Blood Sugar (PPBS) levels did not differ significantly between cases and controls [Table/Fig-3]. The serum H_2S levels in dyslipidaemia cases (37.91±6.28 µmol/L) were significantly lower than in healthy controls (58.52±12.92, p<0.01 µmol/L) [Table/Fig-3,4]. In the Mean±2SD analysis of cases from the current study, the cut-off value of serum H_2S for dyslipidaemia was 50.47 µmol/L [32].

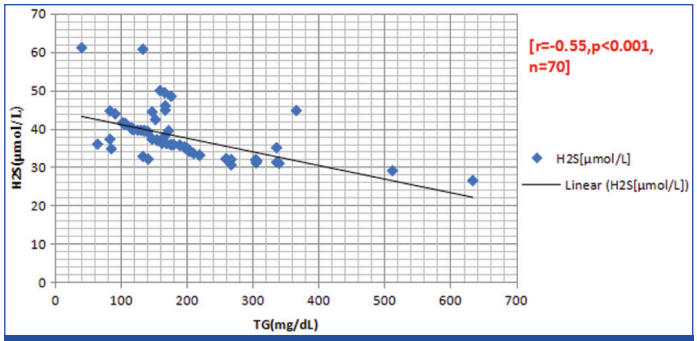


[Table/Fig-4]: Box Whisker plot showing serum H_2S levels in cases and controls (N=140).

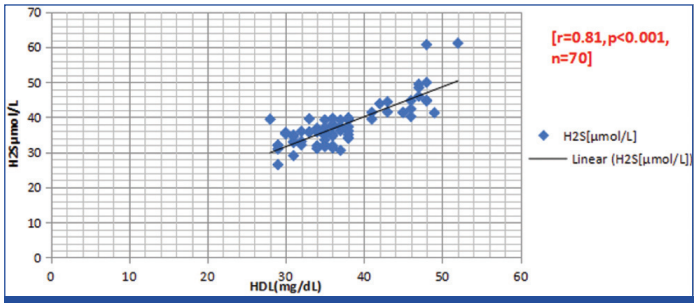


[Table/Fig-5]: Scatterplot showing correlation between serum H_2S and Total Cholesterol (TC) levels.

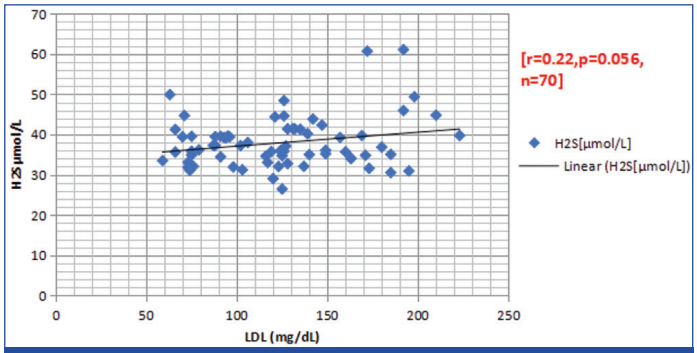
No significant correlation was found between serum TC and H_2S levels (r=0.08, p=0.51, N=70) [Table/Fig-5]. There was a negative correlation (r=-0.55, p<0.001, n=70) between serum H_2S levels and serum TG levels [Table/Fig-6]. In contrast, a positive correlation (r=0.81, p<0.001, n=70) was found between serum H_2S and HDL-C [Table/Fig-7] in cases of dyslipidaemia. No significant correlation between serum H_2S and LDL-C levels (calculated) was found in the current study (r=0.22, p=0.056, n=70) [Table/Fig-8]. A negative correlation (r=-0.44, p<0.05, n=70) was also found between



[Table/Fig-6]: Scatterplot showing correlation between serum H_2S and Triglyceride (TG) levels.

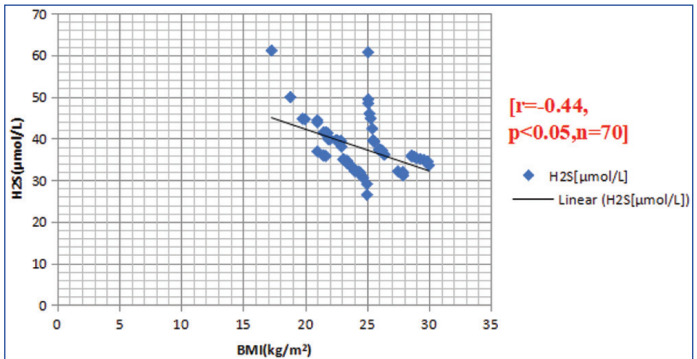


[Table/Fig-7]: Scatterplot showing correlation between serum H_2S and HDL-cholesterol levels.



[Table/Fig-8]: Scatterplot showing a correlation between serum H_2S and LDL cholesterol levels (calculated)

serum H_2S and BMI [Table/Fig-9], whereas there was no significant relationship between serum H_2S and WHR, FBS, or PPBS.



[Table/Fig-9]: Scatterplot showing correlation between serum H2S levels and BMI.

DISCUSSION

The current research findings show significantly lower serum H_2S levels (37.91±6.28 µmol/L) in cases of dyslipidaemia compared to the healthy controls (58.52±12.92 µmol/L, p<0.01), indicating an association between dyslipidaemia and low serum H_2S levels (p<0.0001). It is indicative of the fact that low H_2S levels can contribute to the development of dyslipidaemia. Since dyslipidaemia leads to atherosclerosis, these findings corroborate with the previous research indicating that H_2S has anti-atherosclerotic functions. Furthermore, experimental evidence suggests that H_2S supplementation is beneficial in preventing atherosclerosis [4].

The role of H_2S in the preservation of endothelium, antioxidant action, prevention of inflammatory responses, relaxation of blood vessels, ion channel regulation, etc., contributes to the mechanisms that lead to preventing the atherosclerotic processes [33]. Oxidative stress is an important underlying mechanism in hyperlipidaemia [10]. It has been found that H_2S decreases the development of atherosclerosis by reducing lipid peroxidation in the heart following ischaemic injury induced by isoproterenol by acting as scavengers of Reactive Oxygen Species (ROS) molecules e.g., H_2O_2 and O_2- [34]. Moreover, research has shown that H_2S decreases the development of atherosclerosis by reducing the stimulation of nuclear factor kappa B (NF-κB), decreasing cell adhesion cytokine expressions, ROS generations, and the induction of apoptosis [35,36]. The anti-atherosclerotic property of H_2S is corroborated by the finding of the positive correlation between serum HDL and H_2S in the present study (r=0.81, p<0.001, n=70).

Cystathionine β-synthase knockout mice have been reported to exhibit decreased HDL-C levels in plasma compared to normal mice due to decreased synthesis of endogenous H_2S [35]. The significant negative correlation between serum TG levels and H_2S (r=-0.55, p<0.001, n=70) again shows the possible causative role of decreased serum H_2S in the development of increasing TG levels in the blood. This can be explained by the research finding of preventing hypertriglyceridemia by stimulating liver autophagy through the AMP-activated Protein Kinase (AMPK) and Target-of-rapamycin (TOR) pathway [23]. The present study shows no significant correlation between serum H_2S levels with serum TC and LDL-C levels (calculated). Previous experiments with CSE knockout mice have shown increased plasma levels of cholesterol and LDL-C [37]. Jain SK et al., have shown no statistically significant relationship between serum TGs and H_2S, but a significant negative correlation is found with the LDL/HDL cholesterol ratio in only 36 healthy volunteers with no history of chronic illness [22]. The current study has found no relationship between serum H_2S levels and LDL-C as well as TC levels. The role of garlic and onion in the diet in increasing levels of H_2S and HDL in the blood and decreasing LDL-C levels cannot be ruled out in the current study as well as previous research findings [38]. Further evaluation of serum H_2S in independent cases of hypertriglyceridemia and hypercholesterolaemia, with adequate dietary history and measurement of serum LDL-C directly by standard and validated enzymatic methods, is necessary for finding out the exact relationship with serum H_2S levels.

The relationship between blood H_2S levels and lipid parameters, obesity, and blood glucose levels is indeed complex. A negative correlation has been found between serum H_2S and BMI in the present study, but no relationship has been found with WHR. The current study also shows no relationship between serum H_2S levels and FBS and PPBS levels. Studies in diabetic patients have shown significantly lower levels of H_2S in the blood [39,40]. A positive correlation between FBS and plasma H_2S is observed by Saha P et al., [30]. Reduced serum H_2S levels may be associated with impaired glucose tolerance, as found by Bahadoran Z et al., [41]. The role of antidiabetic drugs in H_2S metabolism needs to be explored to determine the relationship between blood glucose levels and serum H_2S. Diabetes cases have been excluded from the study, but prediabetes cases were not excluded. Whiteman M et al., have found waist circumference to be an independent predictor of plasma H_2S levels [40]. Plasma H_2S levels were found to be inversely correlated with visceral fat area, total body fat, waist circumference, BMI, TC, and LDL-C levels (p<0.05) by Fan D et al., [42]. Serum sulfide levels were found to be increased in subjects with morbid obesity in proportion to the fat mass, and the percentage change in serum sulfide concentration is positively correlated with the percentage change in BMI [43]. The underlying pathology of alterations in blood H_2S levels in obesity is still unknown.

Limitation(s)

A separate study is necessary to consider the relationship of individual types of dyslipidaemia with serum H_2S with an increased sample size. LDL-C has been measured by the Friedewald equation in this study due to limited resource availability. Estimating direct LDL-C would be a better way to assess the relationship with serum H_2S.

CONCLUSION(S)

The current study concludes that a positive correlation exists between serum H_2S and serum HDL. Additionally, a negative correlation between serum H_2S and serum triglycerides was also found. Furthermore, a significant negative correlation has been found between BMI and serum H_2S levels. Comparative studies may be further designed to assess the relationship between serum H_2S levels and dyslipidaemia in cases with or without diabetes mellitus, metabolic syndrome, obesity, hypothyroidism, and other endocrinological disorders. Prospective cohort studies with larger sample sizes are needed to investigate the role of blood H_2S levels as an independent causative factor in dyslipidaemia.

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PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Biochemistry, KPC Medical College, Kolkata, West Bengal, India.
2. Assistant Professor, Department of Biochemistry, KPC Medical College, Kolkata, West Bengal, India.
3. Assistant Professor, Department of Biochemistry, KPC Medical College, Kolkata, West Bengal, India.
4. Associate Professor, Department of Biochemistry, KPC Medical College, Kolkata, West Bengal, India.
5. Professor, Department of Medicine, KPC Medical College, Kolkata, West Bengal, India.
6. Research Assistant, Department of Biochemistry, KPC Medical College, Kolkata, West Bengal, India.
7. Professor and Head, Department of Biochemistry, KPC Medical College, Kolkata, West Bengal, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Pinaki Saha,
Associate Professor, Department of Biochemistry, KPC Medical College, 1F, Raja Subodh Mallick Road, Jadavpur, Kolkata-700032, West Bengal, India.
E-mail: spinaki1973@gmail.com

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