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Correlation between Lipid Peroxidation and Lipoprotein (a) Levels in Patients with Coronary Artery Disease

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ABSTRACT

Coronary Artery Disease (CAD) is the primary cause of mortality in developed and developing countries. Recently, oxidative stress has been reported to be associated with an increased incidence of atherosclerosis and cardiovascular disease. This study aims to investigate the levels of Vitamin C, Uric acid, bilirubin, and lipoprotein (a) (Lp (a)) in patients diagnosed with CAD and their relationship with Malondialdehyde (MDA) concentration. This study consists of 47 control subjects (28 women, 19 men) and 53 patients (15 women, 38 men) with CAD. Blood samples were collected after overnight fasting, and the sera were separated with low-speed centrifugation. MDA levels were determined through the TBARS method. Vitamin C and Lp (a) were determined through dinitrophenylhydrazine photometry and electro immunoassay (EID), respectively. Total bilirubin and Uric Acid (UA) were determined immediately by routine laboratory methods. The mean serum MDA and Lp (a) levels were higher in patients with CAD compared to the control group (MDA: 0.89±0.41 µmol vs. 0.66± 0.24 µmol, $p<0.05$), Lp (a): 35±20 mg/ml, vs. 26.2±14.6 mg/ml, $p=<0.05$). However, no significant correlation was observed between the patients with Lp (a) in their serum MDA and the control group. The mean total bilirubin level was higher in the control group compared to the CAD patients (1.030 vs. 0.830, $p<0.05$). A significant inverse relationship existed between the patient's bilirubin, vitamin C levels, and MDA. Other differences and relations were insignificant. Also, there was no significant difference between the frequency of APOA1 -75 genotypes (G/G, G/A, A/A) in the CAD patients versus the control group ($P>0.05$). Finally, the elevated serum levels of MDA and Lp (a) were known to be independent risk factors for coronary heart disease. Also, there was a significant correlation between serum MDA levels and age, bilirubin, and vitamin C in patients with CAD.

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Introduction

Since Coronary Artery Disease (CAD) is the primary cause of mortality in developed and developing countries, researchers worldwide

focus on it (Mohammadi Abgarmi *et al*., 2009). Atherosclerosis and coronary heart disease are gradual processes that develop over a lifetime. This process becomes dangerous when other risk factors accelerate (Efthimiadis *et al*., 2001). Numerous traditional risk factors of CAD, including genetic factors, age, sex, hypertension, diabetes, smoking, obesity, physical inactivity, and high blood cholesterol, can promote atherosclerosis and cardiovascular disease. Today, it is assumed that the traditional risk factors are only 50% involved in developing cardiovascular diseases and the new risk factors play a significant role in this process (Kalofoutis *et al*., 2007). Lipoprotein (a) (Lp (a)), homocysteine, and fibrinogen are considered the new risk factors for coronary heart disease (Kannel, 2002). Recently, oxidative stress has been reported to be associated with various events leading to increased atherosclerosis and cardiovascular disease (Weinbrenner *et al*., 2003). Over the last four decades, the role of oxidative stress in human disease has received much attention. The immune system produces superoxide anions and nitric oxide to defend the body against xenobiotics. Furthermore, it also poses an oxidative risk to its host (Mohammadi Abgarmi *et al*., 2017). Also, some exogenous substances and drugs cause oxidative stress during detoxification and excretion (Mohammadi Abgarmi *et al*., 2018; dos Santos Barbosa Ortega *et al*., 2019). This condition is caused by molecular damage, the hallmark of oxidative stress (Mohammadi Abgarmi *et al*., 2021). Poly-Unsaturated Fatty Acids (PUFAs) and their esters are the most sensitive molecules to oxidative damage in living tissues (Lagarde *et al*., 2018). Non-enzymatic peroxidation of the PUFAs, such as arachidonic acid, results in hydroperoxides, hydroxylated fatty acids, aldehydes, prostaglandin-like compounds, and short-chain alkanes (Street *et al*., 1994). In the absence of heme-containing proteins, iron, or copper ions, the carbon-carbon bond of lipid peroxides is broken (Fig. 1), resulting in malondialdehyde MDA and hydrodynamic (Ayala *et al*., 2014). Both compounds are highly reactive and damage various amino acids, proteins, nucleic acids, and enzymes. Furthermore, MDA is highly reactive, often indicating oxidative stress. The presence of an antioxidant is essential to prevent the harmful effects of these compounds. The antioxidant system consists of redox-sensitive antioxidants.

The central point of the antioxidant system is Vitamin C (ascorbic acid). Ascorbate plays a crucial role in accumulating and inactivating free radicals generated in many phagocytes. Therefore, ascorbyl radicals are measured to indicate oxidative stress (Birben *et al*., 2012). Also, there was a relationship between the MDA levels and Lipid Per-Oxidation (LPO) in the missed tissue regeneration in earthworms that can be stimulated by plasma treatment (Gholami *et al*., 2022). The studies mention a relationship between the seminal MDA levels and sperm quality in fertile and infertile men. However, there is also a correlation between the sperm parameters and semen LPO total antioxidant levels in astheno- and oligoasthenoteratospermic men (Colagar *et al*., 2009; Colagar *et al*., 2013).

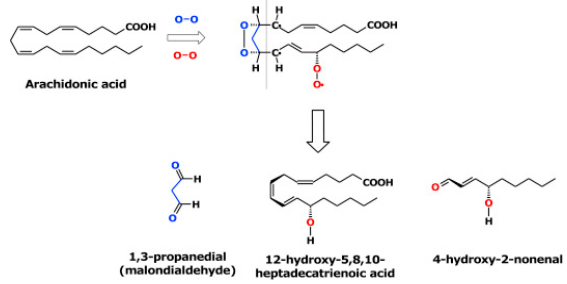

Fig. 1. Non-enzymatic arachidonic acid peroxidation: Reactive Oxygen Species (ROS) attack carbon-carbon double bonds of Poly-Unsaturated Fatty Acids (PUFAs), resulting in the production of MDA and 4-Hydroxy -2-Non-Eal (HNE).

Uric Acid (UA) is a poorly soluble substance derived from the metabolism of purines and acting as a scavenger of oxygen, peroxyl, and hydroxyl radicals. In humans, UA is excreted unchanged in the urine; however, in most mammals, it is oxidized to allantoin. Therefore, measurement of the oxidation product of UA, particularly allantois, may be an indicator of the oxidant production in vivo (Kushiyama *et al*., 2016). The impacts of bilirubin on inhibiting Low-Density Lipoprotein (LDL) oxidation have been studied. It was found that bilirubin, like endogenous UA, inhibits LDL oxidation, effectively reducing the risk of atherosclerosis and cardiovascular disease (Boon *et al*., 2012).

Lp (a) contains a glycoprotein called apolipoprotein-a (Apo (a)) and has a similar structure and lipid content to LDL. Apo (a), which determines the biological and immunological properties of Lp (a), has substantial structural similarity to plasminogen and consists of repeating domains called Kringle. The results of many epidemiological and mostly cross-sectional comparative studies show a direct relationship between the plasma Lp (a) concentration and the occurrence of CAD.

ApoA1 has four exons and is located on 11q23-q24. A common polymorphism of the ApoA1 promoter region consists of one substitution of G to A at the 75 bp upstream of the transcription start site. Many studies showed the association between the frequencies mentioned for polymorphism and CAD risk. Hence, the present study aimed to investigate vitamin C, UA, bilirubin, and Lp (a) in patients with CAD and their relationship to lipid peroxidation and conduct a genotype study of APOA1 -75G>A as a risk factor, which is strongly inversely associated with CAD (Schmidt *et al*., 2016).

Materials and Methods

Patients' selection

A case-control study was conducted under the supervision of the Ethics Committee of NIGEB with IR.NIGEB.EC.1397.9.2.G code Number. All patients were informed and signed the informed consent form. The study population consisted of 100 subjects, including 53 patients (38 men and 15 women) with CAD and 47 control subjects (19 men and 28 women) who had been referred for angiography to the Cardiovascular Research and Treatment Center of Afshar Hospital in Yazd. In the patient group, angiographic results showed coronary artery stenosis in at least one of the heart's main arteries of more than 50%. The control group was also a subject who had undergone angiography and had no stenosis in any of the three main arteries of the heart. Subjects who took antioxidant vitamins and had kidney, liver, diabetes, or smoking were excluded from the study. The acceptable age range for both groups was 40 to 70 years, and the control group and patients were matched entirely in age and sex. A questionnaire was prepared for each subject, including age, sex, family history, taken medications, occupation, and subject weight. The questionnaire was completed at the time of blood sample collection. The average sample size and the patients' selection were determined according to similar studies, and control limitations were applied to estimate the total sample size.

Sampling

10 mL of fasting blood was collected from all subjects between 7-9 am. Serum samples were separated from the whole blood after clotting (60 minutes) by centrifugation (10 minutes at 1500 rpm). UA and bilirubin were measured on the sampling day. The remaining serum was divided into three parts and stored in the freezer at -70 °C for three months to determine the MDA, Lp (a), and Vitamin C levels. Also, the WBC of all samples was used for DNA extraction through the salting-out method (Miller *et al*., 1988) to determine the quality of the DNAs.

MDA measurement

The MDA concentration was determined by Thio-Barbituric Acid (TBA) method. Serum (250µl) was mixed with 1 mL of TCA for deproteination. After verification, 700 ml of supernatant was mixed with 500µl TBA (0.4%), then kept in the 90_95 °C water bath for 50 minutes. After cooling, the mixtures were centrifuged at 4,000 rpm at 4 °C for 5 minutes. Then, the spectrophotometer was used to determine the absorbance at 532 nm.

Vitamin C measurement

Plasma ascorbic acid was oxidized by copper (CU) II and converted to dehydroascorbic acid. The compound reacted with an acidic 2, 4-Di-Nitro-Phenyl-Hydrazine (DNPH) solution to form the red compound bis-hydrazone with an absorption maximum of 520 nm. A DTCS solution was first prepared according to the appropriate instructions to measure Vit C through photometry. To each sample tube, 250 µL of serum sample and 1 ml of 5% TCA solution were added and, after mixing, centrifuged at 3000 rpm for 10 minutes to precipitate serum proteins. Depending on the number of samples, standards, and controls, a small tube was selected, 0.5 ml of the TCA

solution was added to the control tube, 0.5 ml of the corresponding standard solution was added to each of the standard tubes, and 0.5 ml of the filtered solution was added to each test tube. 200 µl of the DTCS mixture was added to all tubes. After sealing the tubes and mixing them, they were placed in a 37°C water bath for three hours. All tubes were then placed in a mixture of ice and water for 10 minutes, and 1 ml of a 12 M sulfuric acid solution was added to each tube. The solution was kept at room temperature for at least 30 minutes and then read at 520 nm. The absorbance and concentration of the standards were used to draw the standard curve, and the concentration of the samples was calculated from the standard curve.

Uric acid and total bilirubin measurement

UA and total bilirubin levels of serum were measured by the enzymatic and DCA methods, respectively, and according to the instructions of the Pars Azmun kit (Cat. No.: 1021036) and (Cat. No.: 2181023) using the RA -1000-TECHNICON Autoanalyzer (USA).

Lp (a) measurement

Lp (a) was measured by electro immunodiffusion method. Briefly, we first cleaned the electrophoresis plates (7cm × 21cm) with alcohol cotton and placed them on the surface. 0.22 g of agarose was weighed and dissolved in tris-borate buffer (3 g of Tris base with 0.7 g of EDTA disodium salt and 8.2 g of boric acid, and dissolved in 200 CC of water, adjusting the pH to 8.5). The solution was then cooled, and when the temperature reached 56 °C, about 80 µL of Lp (a) antiserum was added. After getting well-mixed, it was immediately spread on the glass. After 15-20 minutes, the gel was placed horizontally in a humidified, closed environment in the refrigerator for one hour to achieve the required firmness. The wells were done at a distance of 1 cm from the edge of the glass; then, the glass was placed in an electrophoresis tank.

Approximately, 3 µL of the appropriate serum and standards were added to the wells. The apparatus chamber was closed, and the samples were electrophoresed at 80 volts for at least 18 hours. The plates were then placed in sodium chloride 8.8gr/l for 3 hours to wash the unprecipitated proteins with the antibodies. The gel was placed in distilled water overnight and then dried at 50 °C. The dried gel was immersed in the blue dye solution of Coomassie blue for 20 minutes and then washed 2-3 times. The surface area of the plate's blue rockets represented the Lp (a) concentration in the serum samples or standard solutions.

Electro Immuno-Diffusion (EID) is a simple, rapid method for quantitating immunoglobulins in dilute biological fluids. In the Immunodiffusion method, agarose gel with 1% to 2% concentrations of assay proteins in biological solutions was used as a semi-solid culture where molecules could be easily dispersed. The protein content was the basis for separating the components of proteins in this method. A calibration curve can analyze the existence or absence of Lp (a) on the agarose gel.

APOA1 genotyping

Approximately 100-500 ng of DNAs were amplified via thermocycler (PEC lab Co., France) using AMPLIQON PCR Kit (AMPLIQON Co., Denmark) in a 12.5 µL reaction mixture containing 10 picomoles of forward and reverse specific primer for APO A1-75 G to A polymorphism with annealing temperature 57°C for 45 seconds. Then, about 9 µL of the PCR product was digested at 37°C overnight with 10 units of *Msp*I restriction enzyme (New England Biolabs Inc., USA), based on Moradi *et al*. (2022) methods. The digested PCR product was resolved on an 8% polyacrylamide gel and visualized by silver staining methods (Chhabra *et al*., 2005). G to A at -75 bp polymorphism resulted in the loss of the MspI site, and the existence of 183 bp represented the 'A' allele. The genotypes were GG, GA, and AA (Chhabra *et al*., 2005).

Statistical analysis

Statistical analysis was performed using SPSS Software (version 16). An independent Student t-test and Mann-Whitney test were performed to compare the case and control groups in normal and non-normal distribution. The results were expressed as mean ± standard deviation. $P < 0.05$ was considered statistically significant. The Pearson correlation test was used to determine

the correlation between the data with normal and non-normal distributions, respectively.

Results

Case and control demography

The present study was performed on 100 serum samples (53 patients and 47 controls). The mean age of the study population was 54.25± 8.61 years, with a minimum of 40 and a maximum of 69 years. The age distribution of the study population was normal. The patients' mean age was 55.24 ± 7.53 years, and that of the control group was 53.10 ± 9.62 years.

Serum MDA analysis

The serum MDA level was 0.89±0.41 µmol in the studied patients. At the same time, this value was 0.66± 0.24 µmol in the control group. A significant difference was observed between the mean values of the case and control groups (P <0.05) (Table 1).

According to Table 1, the serum MDA concentrations measured by the photometric thiobarbituric acid method had a normal distribution. Therefore, the "t-student test" was used to compare the mean concentration of the serum MDA in the CAD patients and controls. The serum MDA was 0.89±0.41 µM and 0.66±0.24 µM in patients and controls, respectively. A comparison of the mean serum in the two groups shows that the serum MDA concentrations in the patients with CAD are significantly higher than the controls (P <0.05).

Comparison of Vit C, UA, Bilirubin, and Lp (a) serum levels

There was no significant difference between the VIT C and UA concentrations in the control and patient groups (P> 0.05). In contrast, the bilirubin concentration was higher in the control group than in the patient group (P <0.05). Also, the Lp (a) concentration was higher in the patient group (P< 0.05).

VIT C and total bilirubin concentrations in the study population were not normally distributed. Hence, the Mann-Whitney test was used to compare the mean concentrations of these compounds in the control and patient groups. The other variables listed in Table 1 had a normal distribution, resulting in the use of the student t-test to compare the means of the variables in the control and patient groups. The concentration of VIT C and UA in control and patient groups were not significantly different (P> 0.05), while the concentration of bilirubin in the controls was higher than the patients (P <0.05). Also, the mean concentration of Lp (a) in the patient group was significantly higher than the control group (P <0.05).

According to the results presented in Table 2, there was a positive and significant relationship between the concentration of MDA in the patient group and age (P <0.05). In the control group, this relationship was insignificant. There was a significant inverse correlation between VIT C and MDA in the control and patient groups (P <0.05). In contrast, there was no correlation between UA and Lp (a) in both the control and patient groups (P>0.05). Bilirubin was inversely related to serum MDA in the patient group (P <0.05), whereas no such relationship was observed in the control group (P >0.05).

Since the serum MDA concentrations measured by the TBARS method were generally distributed in the study population, the Pearson Correlation Coefficient Test was used to examine the correlation between the MDA concentration and other factors. Table 2 shows a positive and significant relationship between the MDA concentration in the patient group and age (P= 0.017). In contrast, this relationship was insignificant in the control group. There was a significant negative correlation between VIT C, bilirubin, and the MDA in the patient group. Also, a positive and significant correlation was observed between the serum UA and MDA in the patient group. There was no significant relation between UA and Lp (a) and MDA in the two groups.

APOA1-75G/A genotyping

The results showed that although the frequency of APOA1 -75 AA genotype was lower in the CAD patients compared to the controls, this difference was insignificant (P>0.05) (table3). Compared to the control group, this insignificant difference was also observed in the APOA1 -75 A allele in the CAD patients.

Table1. Mean serum concentration of MDA, VIT C, UA, bilirubin, and Lp (a) in control and patient groups.

Variable	CAD Patients Mean ± SD	Controls Mean ± SD	P-value
VIT C(mg/dL)	0.614± 1.10	0.546± 0.044	Not significant
UA (mg/dL)	6.52±1.5	6.6±1.8	Not significant
Bilirubin (mg/dL)	0.83±0.39	1.03±0.30	< 0.05
Lp (a) (mg/dL)	35.15±20.07	26.21±14.57	< 0.05
MDA (µmol)	0.89±0.41	0.66± 0.24	< 0.05

Table 2. Correlation between serum MDA concentration and age, Vit C, UA, total bilirubin, and Lp (a) in control and patient groups.

CAD Patients			Controls	
Variable	**Correlation coefficient**	**P-value**	**Correlation coefficient**	**P-value**
Age(years)	0.455	0.017	0.236	0.333
Lp(a) (mg/dL) Total	0.72	0.638	0.339	0.084
Bilirubin(mg/dL)	- 0.449	0.008	0.146	0.320
Vit C (mg/dL)	-0.420	0.036	0.017	0.910
UA (mg/dL)	0.394	0.049	0.044	0.756

Table 3. Genotype and allele frequencies of the APOAI gene polymorphism (-75 G/A) among CAD cases and controls

Genotype/ Allele		Cases (n=53)	Controls (n=47)	OR (95%CI)	P-value
Genotype	GG	31 (58.5%)	32 (68%)	1.00 (Reference)	P>0.05
	GA	19 (35.8%)	11 (23.5%)	0.96 (0.68,1.36)	
	AA	3 (5.7%)	4 (8.5%)	0.50 (0.28,0.88)	
Allele	G (95% CI)	81 (76.4%)	75(79.8%)	1.00 (Reference)	P>0.05
	A (95% CI)	25 (23.6%)	19(20.2%)	0.76(0.59,0.98)	

Discussion

Atherosclerosis is the leading cause of death in various countries. Numerous studies have shown that LDL oxidation has a significant role in atherogenesis, and detection of susceptibility of lipids to oxidation and quantification of its byproducts determine the oxidation rate. Walter *et al*. (2004) measured MDA in the serum of 634 patients with CAD by HPLC and spectrophotometry. Their studies showed that serum MDA levels were the best predictor of cardiovascular events in patients with stable angina. They showed that the serum MDA is an independent risk factor for CAD and is associated with other established risk variables and inflammatory indicators (Walter *et al*., 2004). *Dincer et al*. (1999), Kavocas *et al*. (1997), and Kostner *et al*. (1997) demonstrated an increase in the MDA levels in patients with coronary artery stenosis. Based on previous studies and current findings, it can be concluded that a high concentration of serum MDA is a risk factor for coronary artery disease. In our study, the serum MDA concentration was significantly higher in the patients with CAD than in the controls; MDA could also play an essential role in developing atherosclerosis in our study population.

Volpi and Tarugi (1998) found no significant association between age and increased MDA in adult men. In addition to the influence of sex on lipid peroxidation, Ide *et al*. (2002) also examined the influence of age on this factor. Their results showed no significant relationship between age and serum MDA levels. Given that the patients have several specific conditions and characteristics, it is impossible to correctly assume the significance of the relationship between age and MDA and interpret it accurately. In the control group, the results have a higher degree of confidence. Therefore, according to the previous studies, we reject the relationship between age and MDA.

In this study, the serum Lp (a) levels were higher in the patients with coronary artery stenosis than in the control group. Although the Lp (a) levels vary in different populations depending on their race, various studies suggest that elevated Lp (a) levels, both in men and women, are a risk factor for atherosclerosis (Emerging Risk Factors Collaboration, 2009).

In a study conducted by Kostner *et al*. (1997) on patients with angina, the Lp (a) levels of 100 healthy subjects were compared to 100 patients. The Lp (a) levels were significantly higher in the patients. In another study, Burman confirmed the increased Lp (a) levels in patients with coronary artery disease. Also, our study found no significant relationship between the Lp (a) levels and MDA. Burman et al. showed no significant association between the Lp (a) level and lipids, lipoproteins, and other risk factors. Therefore, he introduced Lp (a) as an independent risk factor for coronary heart disease (Burman *et al*., 2004).

The present study also evaluated Vit C in both control and patient groups. The results conveyed that the levels of Vit C were insignificantly different between the control and patient groups. In a case-control study performed by Delport *et al*. (1998) on 41 patients with CAD and 41 controls, the Vit C levels were lower. Nevertheless, there was no correlation between the level of this vitamin and the risk of atherosclerosis (Delport *et al*., 1998). In another study by Bokalova et al., plasma lipophilic antioxidants such as vitamin E and carotenoids were lower in patients with CAD than the healthy subjects. However, VIT C was not significantly different between the control and patient groups (Bakalova *et al*., 1995). Dincer *et al*.'s (1999) findings also confirmed a decrease in Vit C levels in patients with atherosclerosis. The conflicting reports confirm no difference between the control and patient groups' Vit C. However, in patients, its inverse correlation with MDA indicates the effect of this vitamin in preventing lipid peroxidation.

In a study by Levinson (1997) on 254 CAD patients, a negative correlation was found between the serum bilirubin levels and the percentage of vascular occlusions in the CAD patients; however, there was no relationship with other risk factors, such as triglycerides and HDL cholesterol. HDL cholesterol remains a standard marker of Cardio-Vascular (CV) risk. Thus, its reduction is a major factor in raising the CV risk (Bhargava *et al*., 2022; Sirtori *et al*., 2022). In our study, the bilirubin concentration in the controls was higher than the patients, and there was a negative correlation between the total bilirubin and serum MDA in the patient group. Furthermore, the patient group obtained a significant inverse correlation between the MDA concentration and serum bilirubin. Therefore, we also confirm the role of bilirubin as an endogenous antioxidant in preventing lipid peroxidation.

The UA concentrations were also compared in the control and patient groups, indicating that the difference between the two groups was insignificant. Also, there was no relationship between the UA and serum MDA levels in the control and patient groups. Bickel et al. examined the effect of the serum UA concentrations on death in patients with coronary artery stenosis and compared the patients with UA concentrations of 5.1 mg/dL to those with UA higher than 7.1 mg/dL. It was reported that the mortality rate increased from 3.4 to 17.1. The difference in the UA levels between the two groups can explain the normal distribution around the mean and the small sample size.

Conclusion

Elevated MDA and Lp (a) levels are critical risk factors for CAD and require further attention. A significant association between serum MDA levels and antioxidants such as UA, bilirubin, and VIT C in patients with CAD was observed within this study. Given the small size of the study population, we suggest further large-scale studies to confirm the current findings.

Conflicts of interest

The authors declared no conflicts of interest.

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