

## Evaluation of Serum Inosine, Hypoxanthine, and Carnitine as Ischemia Markers of Heart Disease

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### ABSTRACT

**Introduction:** Heart disease (HD) is a major global death cause, with adenosine triphosphate metabolites, carnitine, and other biomarkers aiding in timely detection and disease prediction. This study aims to investigate the changes in inosine, hypoxanthine, and carnitine levels in the serum of ischemic HD patients and to examine the relationship of these components with the extent of coronary artery blockage, as well as for the detection of coronary artery disease (CAD).

**Materials & Methods:** In this cross-sectional and case-control study, from patients who underwent angiographic assessment for suspected CAD, 40 control subjects, 40 stable angina patients, 40 unstable angina patients, and 40 myocardial infarction patients were selected. Serum samples were collected, and the amounts of inosine, hypoxanthine, and carnitine were measured using an HPLC-UV-visible method. The differences of the three substances in the patients and their clinical importances were analyzed by SPSS V.22 ( $P < 0.05$ ).

**Results:** The results of this study showed that the levels of inosine and hypoxanthine in patients were higher than those in the control group ( $P < 0.01$ ), while the level of carnitine was lower than that in the control group ( $P = 0.04$ ). Conversely, the concentration of carnitine in patients was lower than in the healthy control group, with respective concentrations of 31.84 (28.58-35.62), 34.01 (28.25-37.13), and 33.59 (28.58-38.87)  $\mu\text{g/ml}$ .

**Conclusion:** This study indicated that inosine and hypoxanthine could be effective and rapid discriminators, in comparison with other heart biomarkers, for the timely detection of HD.

**Keywords:** Heart disease, Inosine, Hypoxanthine, Carnitine, Cardiovascular diseases

### ➤ Cite this paper

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## Introduction

Cardiovascular disease is the leading cause of death and disability-adjusted life years (DALYs) worldwide, accounting for more than 17 million deaths annually (1). Atherosclerosis plaque, which often leads to coronary artery disease (CAD), has been associated with various risk factors such as genetics, diet, obesity, high blood pressure, smoking, high levels of low-density lipoprotein (LDL), age, and others (2). CAD is characterized by conditions such as stable CAD, unstable CAD, heart attack, and sudden death (3). Atherosclerosis plaque forms as a result of lipid accumulation within the endothelium. This accumulation triggers the migration of monocytes and macrophages to the lesion, where they release inflammatory cytokines such as interleukin 6 (IL-6) and IL-1 $\beta$ . The buildup of fat droplets within macrophages transforms them into foam cells, leading to the formation of cholesterol crystals (4).

Stable CAD, which is characterized by a 50% or more occlusion of the coronary artery, typically presents symptoms such as chest pain and discomfort in the neck and jaw (7). Unstable CAD is a condition that falls between stable CAD and a heart attack, increasing the risk of myocardial infarction and sudden death (6). Symptoms of unstable CAD appear during rest and mild activities, but no heart biomarkers are present (7). In unstable CAD, thrombosis and plaques block the coronary arteries, decreasing the blood flow rate (8, 9). A heart attack occurs when the blood supply to the heart tissue is interrupted, causing damage due to a lack of oxygen. This is typically caused by the blockage of coronary arteries and the rupture of fatty plaques (10, 11).

The most important markers for detecting damage to heart tissue are troponin I, troponin T, and creatine phosphokinase (CPK). These markers peak a few hours after ischemia. However, early stages of ischemia, such as stable and unstable angina, cannot be measured by these markers (12). It has been reported that ATP metabolites can be used for the diagnosis of ischemia (13). During ischemia, ATP is

converted into adenosine, inosine, hypoxanthine, and xanthine. These are then released into the bloodstream by equilibrative nucleoside transporters and concentrative nucleoside transporters (14). Carnitine is a non-standard amino acid that is synthesized from lysine and methionine in the liver and kidneys (15). The primary function of carnitine is to transport fatty acids into the mitochondria for  $\beta$ -oxidation. It has been suggested that carnitine can help reduce lipid profiles (16, 17). Carnitine also serves as an antioxidant, chelating iron ions and conjugating free radicals (18). This compound protects glutathione by enhancing the activity of superoxide dismutase, thereby preventing the accumulation of lipid peroxides. Additionally, carnitine can be utilized in inflammatory conditions to reduce inflammatory factors such as NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and TNF- $\alpha$  (tumor necrosis factor-alpha) (19-21).

Given the anti-inflammatory role of inosine, it may have beneficial effects in preventing oxidative stress, cancers, obesity, and cardiovascular diseases (22). Furthermore, the importance of plasma inosine and hypoxanthine levels as potential biomarkers for early detection of acute cardiac ischemia has been reported (14). Nevertheless, to clarify their exact diagnostic potential, additional clinical studies are mandatory (14). In this regard, the current study was conducted to evaluate the serum levels of inosine, hypoxanthine, and carnitine as useful identifiers of different types of CAD.

## Materials and methods

### Study Design

Participants of this cross-sectional and case-control study were selected from those patients who consecutively underwent angiographic assessment for their suspected CAD at the Fatemeh Zahra Heart Hospital of Sari in Iran, from January 2019 to March 2020.

### Setting and Participants

The indices for determining patient and control groups were the coronary arteries (in each of the three main ones) with blockage  $\geq 50\%$  and  $< 50\%$ , respectively, in the angiography findings. The stable and unstable angina were determined through clinical symptoms (e.g., the quality and duration of chest pain and discomfort during rest or activity and the responsiveness to usual medications) and electrocardiographic findings by two independent cardiologists. Patients with myocardial infarction were distinguished based on the troponin I  $> 0.3$  ng/dl. The patients with other conditions (e.g., other cardiovascular diseases, chronic or acute inflammatory diseases, and malignancies) that probably may influence the amount of inosine, hypoxanthine, and carnitine were not entered in the study.

### **Sample Size**

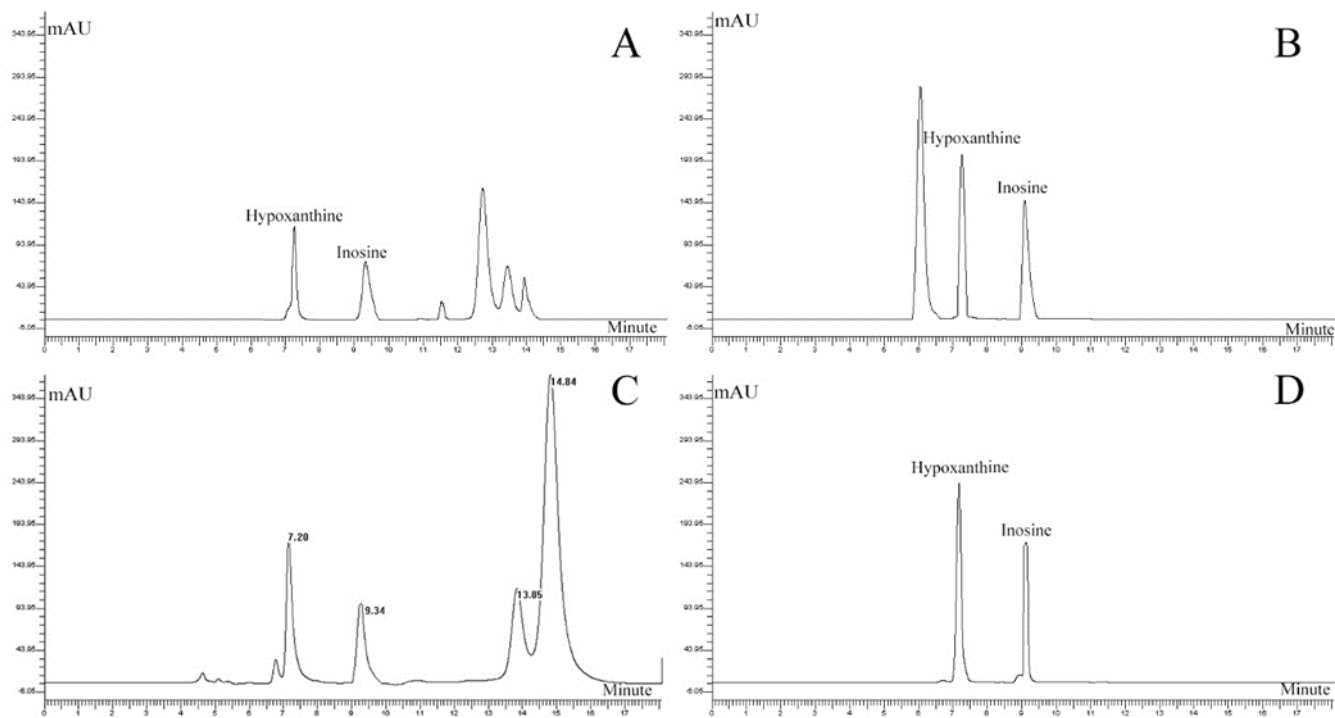
Based on Cochran's formula (23), 40 patients with stable angina, 40 patients with unstable angina, 40 patients with MI, and 40 control subjects (a total of 160 individuals) were included in the study.

### **Measurements & Validity and Reliability**

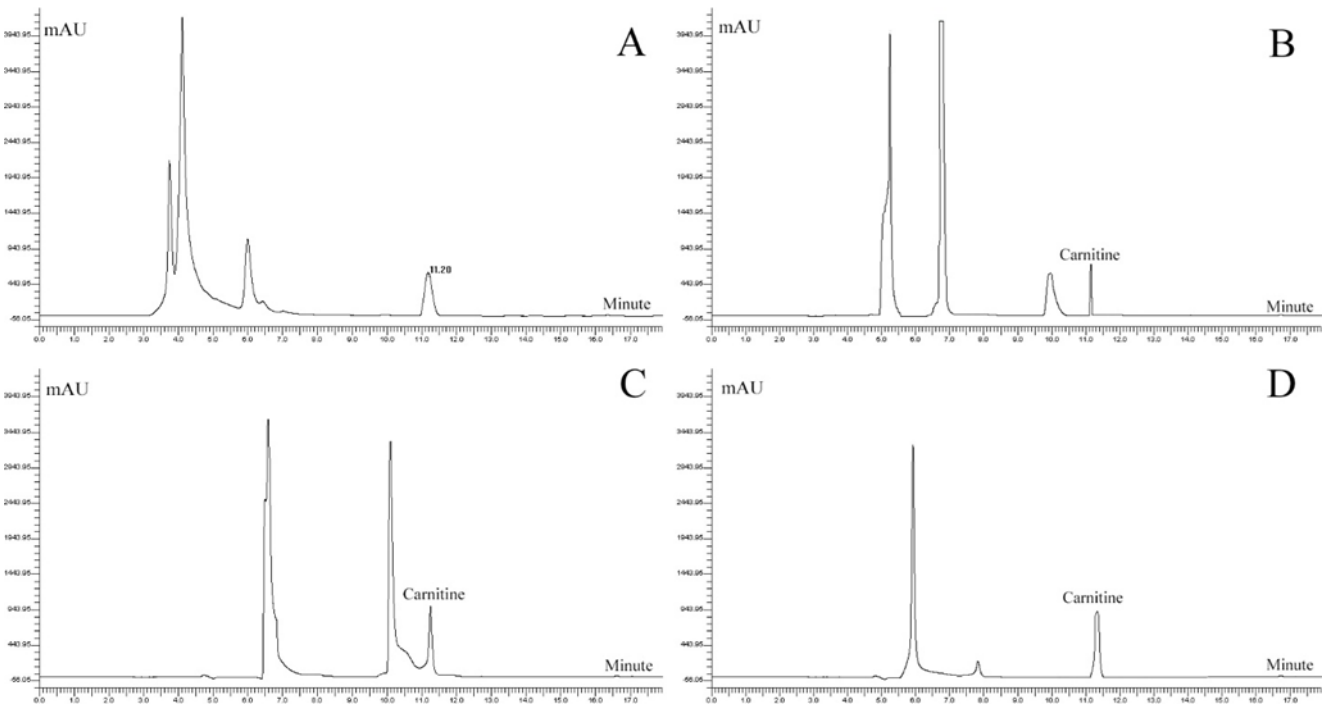
Inosine, hypoxanthine, carnitine, trifluoroacetic acid (TFA), acetonitrile, and citric acid were procured from Sigma Aldrich (Germany). Disodium hydrogen phosphate anhydrous, 2-propanol, and silver oxide were supplied by Merck (USA). HPLC-grade methanol and HPLC-grade water were obtained from Samchun (Korea), while tetrabutylammonium hydroxide, 2,4-dibromoacetophenone, and triethanolamine were prepared using products from Supelco (USA). Blood samples were collected from patients who had fasted for 12 hours, and to select patients who had MI or unstable angina, the sample was collected shortly after patients were referred to the emergency room, and the serum was immediately separated by centrifugation at 4000 rpm. The samples were then stored in a refrigerator at  $-80^{\circ}\text{C}$ . After that,

the frozen samples were kept under dry ice in a well-insulated container and transferred to Ilam University of Medical Sciences for biochemical analysis. Standards for inosine and hypoxanthine were prepared at concentrations of 250, 500, 1000, 3000, and 5000 ng/ml in HPLC-grade water. The standards for inosine and hypoxanthine were assessed using an HPLC-UV visible system at a wavelength of 254 nm with a C18-250 mm column. The mobile phase consisted of 90% 0.05% TFA and 10% methanol, with a flow rate of 0.5 ml/min (13). Carnitine was derivatized with 2,4-dibromoacetophenone in an anhydrous environment. The mobile phase consisted of acetonitrile and citric acid (0.018 mol/l) in a 90:10 ratio. To this, 250  $\mu\text{l}$  of triethanolamine was added, and the solution was prepared after degassing. 100  $\mu\text{l}$  of serum was mixed with 1 ml of acetonitrile and 1 ml of an ethanol solution in a 9:1 volume ratio. The sample was then processed twice using a mixture of anhydrous 400 mg  $\text{Na}_2\text{HPO}_4$  (9 parts) and  $\text{Ag}_2\text{O}$  and 400 mg  $\text{KH}_2\text{PO}_4$  (1 part). After centrifugation, 50  $\mu\text{l}$  of the derivative solution was mixed with 400 ml of supernatant and incubated at  $60^{\circ}\text{C}$  for 90 min. After cooling the sample, it was injected into the HPLC device, and the carnitine level was evaluated. The derivative solution was prepared by dissolving 40 mg of 2,4-dibromoacetophenone in 1 ml of acetonitrile, to which 100  $\mu\text{l}$  of tetrabutylammonium hydroxide was added. The flow rate was set at 1.2 ml/min, and the wavelength was 260 nm (24).

The serum proteins were precipitated using cold 2-propanol in a 1:3 ratio, mixed for 10 seconds, and then centrifuged at  $5^{\circ}\text{C}$  at 13500 rpm. The supernatant was then separated and filtered through a 0.2  $\mu\text{m}$  filter before being injected into an HPLC-UV visible detector. The chromatograms of inosine and hypoxanthine from the control group and patients, as well as the chromatogram related to the evaluation of carnitine in the patient's serum, are shown in Figure 1 and Figure 2.



**Figure 1.** Chromatograms of inosine and hypoxanthine in the serum of patients. A: Chromatogram related to the control group patient. B: Chromatogram related to the patient with myocardial infarction. C: Chromatogram related to the patient with stable coronary artery disease. D: Chromatogram related to the patient with unstable coronary artery disease.



**Figure 2.** Chromatogram of carnitine evaluation in serum of patients. A. Chromatogram related to the patient control group. B. Chromatogram related to myocardial infarction patient. C. chromatogram related to stable coronary artery patient. D. Chromatogram related to unstable coronary artery patient.

*Statistical and Data Analysis*

The obtained data was analyzed by SPSS V.22 based on parametric and non-parametric tests. Variable values were depicted as mean  $\pm$  SD, median (interquartile range), and number (percentage). To compare the values of non-parametric variables between patients and control individuals, the Kruskal-Wallis and Dunn's post hoc tests were utilized. For variables with parametric distribution one-way ANOVA and Bonferroni's post hoc examinations were accomplished. To compare dichotomous variables, the Chi-squared test was used. Receiver operating characteristics (ROC) curve analysis was applied to assign the exact cutoff amounts for inosine, hypoxanthine, and carnitine in

the study. The significance of all statistical analyses was based on the P value  $< 0.05$ .

## Results

The conditions of the patients were compared based on common cardiovascular biomarkers, as well as levels of inosine, hypoxanthine, and carnitine. When comparing cardiovascular risk factors such as triglycerides, total cholesterol, LDL, HDL (high-density lipoprotein), and BMI between patients and controls, no significant difference was observed. However, a significant difference was found in hs-CRP, blood pressure, inosine, hypoxanthine, and carnitine levels in the control group, as shown in Table 1.

**Table 1.** The clinical characteristics of the patients under study.

	Control subjects (40)	Stable CAD patients (40)	Unstable CAD patients (40)	MI patients (40)	P-value
Age	64.80 $\pm$ 10.42	61.71 $\pm$ 8.23	61.80 $\pm$ 10.09	61.01 $\pm$ 9.87	0.61
male(female)	20(17)	20(18)	20(20)	20(20)	0.45
BMI (kg/m <sup>2</sup> )	25.81 $\pm$ 0.97	26.01 $\pm$ 0.62	25.90 $\pm$ 0.51	26.12 $\pm$ 0.71	0.71
Total Cholesterol (mg/dl)	182.22 $\pm$ 34.0	187.54 $\pm$ 66.0	186.20 $\pm$ 37.0	185.00 $\pm$ 64.01	0.27
Triglycerides (mg/dl)	151.80(118.71-190.52)	152.04(121.31-201.46)	154.11(119.81-200.73)	153.22(117.98-202.68)	0.09
LDL-C (mg/dl)	99.49 $\pm$ 23.55	111.30 $\pm$ 20.71	115.01 $\pm$ 30.83	112.54 $\pm$ 27.920	0.08
HDL-C (mg/dl)	48.9.77 $\pm$ 9.77	44.1 $\pm$ 8.17	44.41 $\pm$ 7.28	43.05 $\pm$ 10.01	0.041
hsCRP (mg/l)	1.63(1.12-2.67)	3.28(2.81 $\pm$ 4.08)	4.06(2.55-5.71)	4.00	0.03*
Blood pressure	6	12	16	18	0.0001*
Diabetes Mellitus	12	10	13	9	0.08
Family history	6	6	5	10	0.06
Smoking	3	7	6	8	0.07
Lipid drug using	10	36	28	34	0.001*, a
Hypoxanthine ( $\mu$ g/ml)	0.61(0.43-0.71)	5.88(4.86-6.45)	6.63(5.96-7.02)	6.65(5.73-6.99)	0.0001*
Inosine ( $\mu$ g/ml)	0.46(0.35-0.56)	3.25(2.25-3.68)	3.38(2.51-4.06)	3.41(2.26-4.11)	0.0001*
Carnitine ( $\mu$ g/ml)	36.89.00(30.72-42.26)	33.59(28.58-38.87)	34.11(25.52-37.13)	31.84(28.58-35.62)	0.041*

Data are displayed as the mean  $\pm$  SD, median (25th-75th interquartile range), and count. A comparison was made between the control subjects and the other patients groups using one-way ANOVA /Kruskal-Wallis and their related post hoc tests.

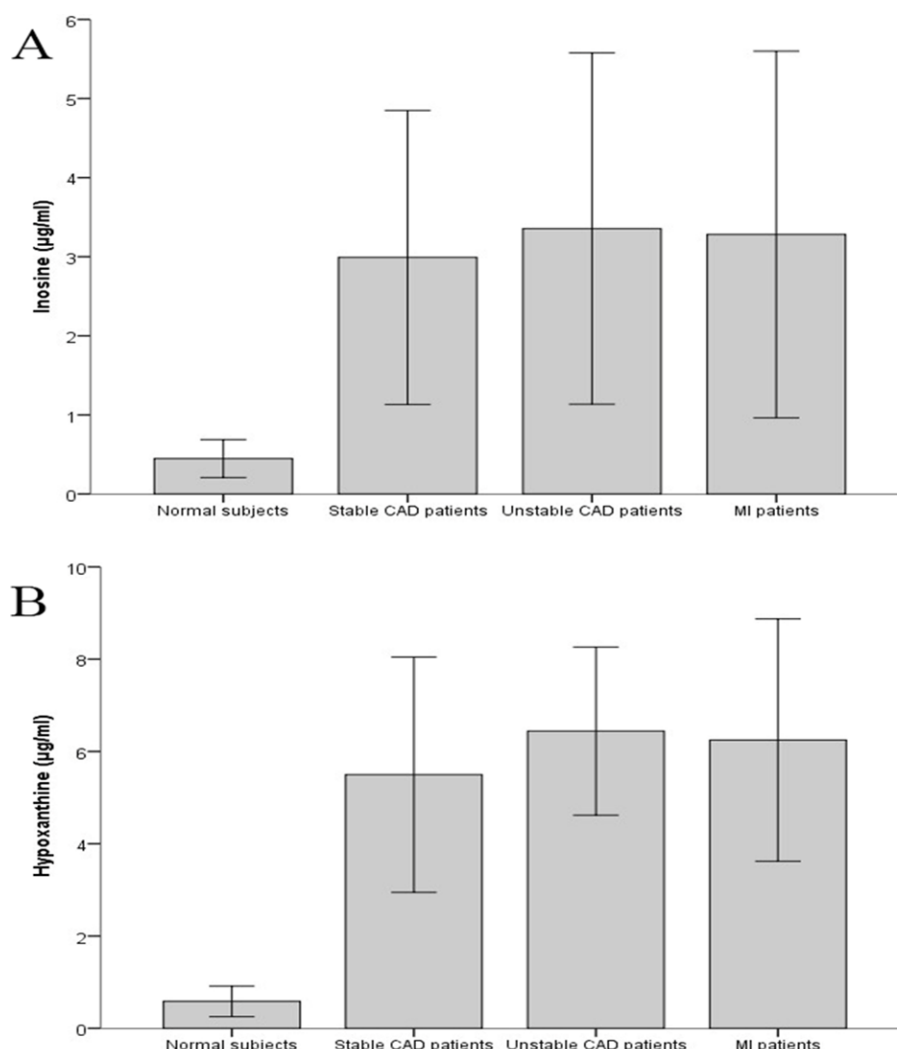
\* Kruskal-Wallis and Dunn's post hoc test; Control subjects vs Stable CAD, Unstable CAD and MI patients.

a Kruskal-Wallis and Dunn's post hoc test; Unstable CAD patients vs Stable CAD and MI patients, P = 0.03

An evaluation of inosine and hypoxanthine levels in patients and the control group revealed that the concentrations of these compounds were significantly higher in patients compared to the

control group ( $P < 0.05$ ). Specifically, the levels of inosine and hypoxanthine were found to be 9 times higher than those in the control group. Furthermore, our data indicated that the concentrations of these two compounds were higher than those in the stable

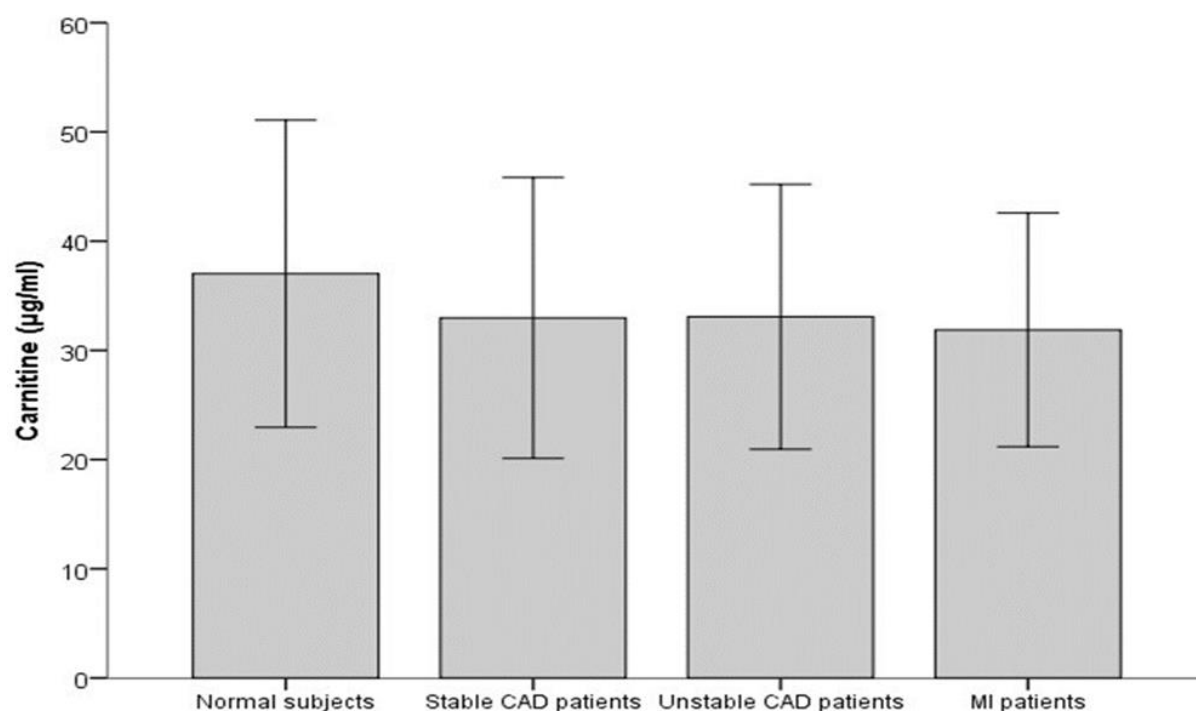
coronary artery group ( $P < 0.05$ ). However, there was no significant difference in the levels of these two compounds between patients with unstable coronary arteries and myocardial infarction ( $P = 0.09$ ), Figure 3.



**Figure 3.** Comparison of inosine and hypoxanthine levels between patients and controls. A. The content of inosine was higher in patients than in the control group (Kruskal-Wallis and Dunn's post hoc test,  $P = 0.0001$ ). B. The content of hypoxanthine was higher in the three groups of patients than in the control group (Kruskal-Wallis and Dunn's post hoc test,  $P = 0.0001$ ). The concentrations of inosine and hypoxanthine were higher in the unstable CAD and MI patients compared to the stable CAD patients (Kruskal-Wallis and Dunn's post hoc test,  $P < 0.05$ )

The levels of carnitine in patients indicated that they were higher in the control group compared to the patient groups ( $P = 0.04$ ). However, there was no

significant difference in carnitine levels among the different patient groups, as shown in Figure 4 and Table 1 ( $P = 0.17$ ).

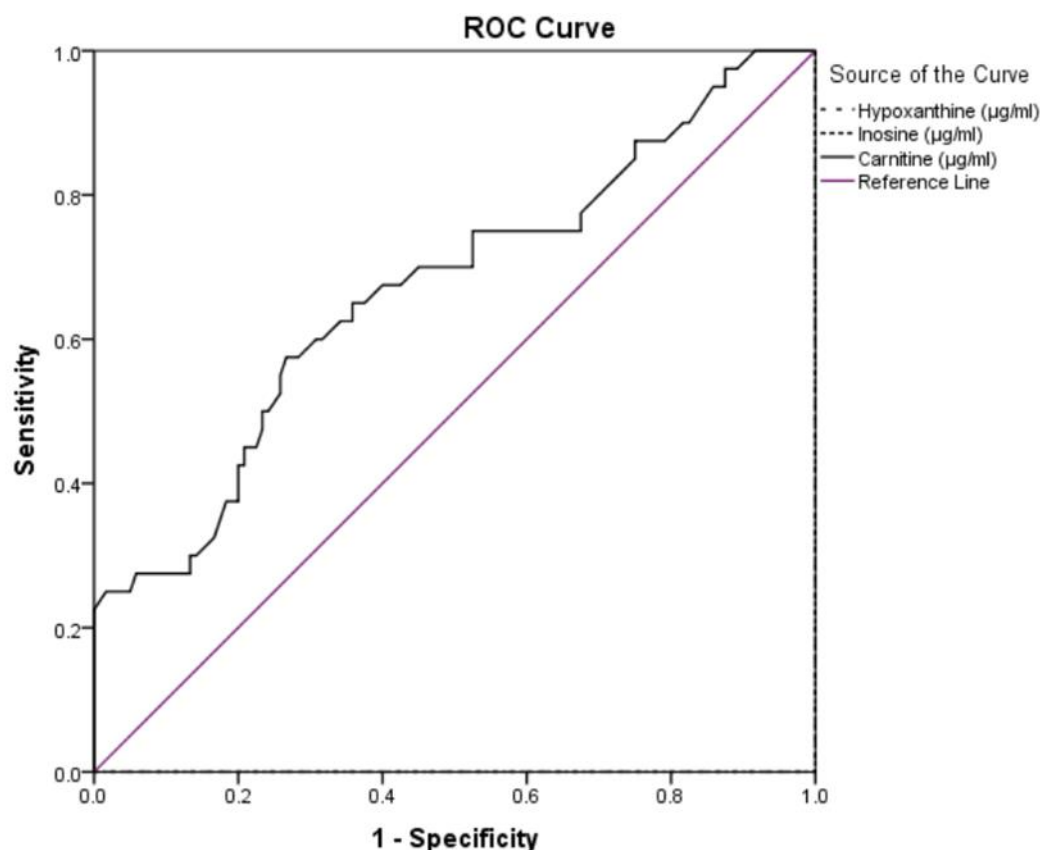


**Figure 4.** Comparison of carnitine levels in patients and control group. Carnitine level was decreased in patients compared to the control group ( $P = 0.04$ )

#### ***Differentiation power of inosine, hypoxanthine, and carnitine in patients and control group***

To determine whether the levels of hypoxanthine, inosine, and carnitine in the serum of the study participants could differentiate and distinguish between the individuals, a Receiver Operating Characteristic (ROC) curve analysis was performed. The evaluation of inosine, hypoxanthine, and carnitine revealed that these three compounds could be detected at minimum values of 0.33 g/ml, 0.41 µg/ml, and 7.35 µg/ml for inosine, hypoxanthine, and carnitine, respectively. The results indicated that inosine and hypoxanthine had a combined area under

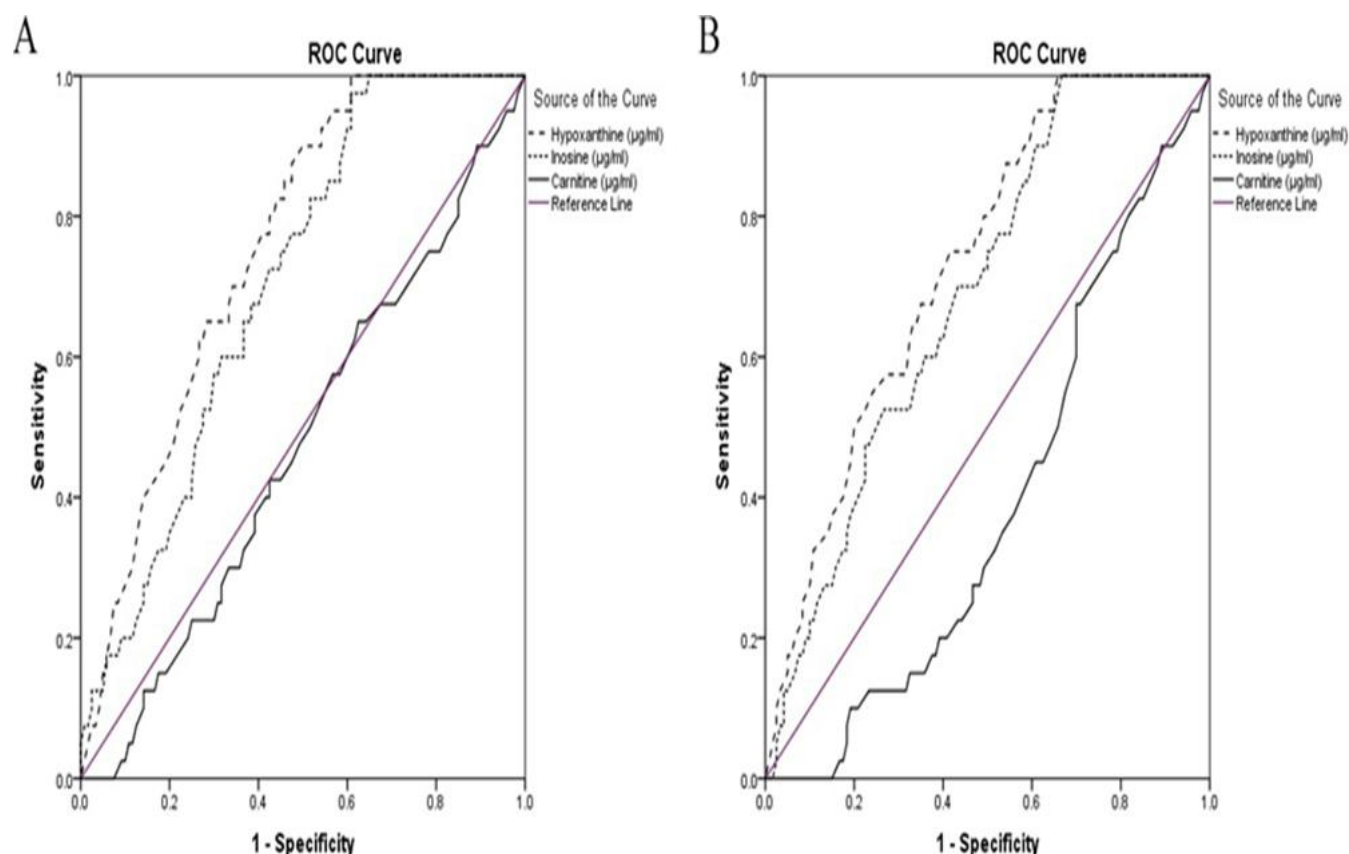
the curve (AUC) of 100% [1.00 (0.99-1.00);  $P = 0.0001$ ], demonstrating excellent diagnostic capability. Using inosine and hypoxanthine, patients could be distinguished from the control group with a sensitivity and specificity of 90% and 85%, respectively. In contrast, carnitine showed a lower AUC of 0.67 (0.57-0.77;  $P = 0.0001$ ), indicating its limited effectiveness in differentiating between patients and controls (Figure 5). Carnitine also could differentiate between stable, unstable, and myocardial infarction patients from the control group, with a sensitivity and specificity of 65% and 75%, respectively (Figure 5).



**Figure 5.** Receiver operating characteristic (ROC) curve analyses to assigned the cut off values of hypoxanthine, inosine, and carnitine to differentiate between the patients (stable, unstable CAD and MI) and control groups. Due to the strong differences in inosine and hypoxanthine levels between control subjects and patients the area under the curve for these two (in combination) was 100% [1.00(0.99-1.00);  $P = 0.0001$ ], However, this number for carnitine was [0.67(0.57-0.77);  $P = 0.0001$ ].

On the other hand, the results indicated that inosine, hypoxanthine, and carnitine were not able to differentiate stable coronary artery patients from other patients (Figure 8,  $P > 0.05$ ). However, upon evaluation, it was found that inosine and hypoxanthine, with minimum values of 2.74  $\mu\text{g/ml}$  and 6.05  $\mu\text{g/ml}$ , respectively, could differentiate unstable coronary artery patients from stable coronary artery patients (Figure 8,  $P = 0.0001$ ). The sensitivity and specificity for inosine were 72% and 68%, respectively, while for hypoxanthine, they were 70% and 76%, respectively. Carnitine, however, was unable to differentiate unstable coronary artery

patients from stable coronary artery patients (Figure 8,  $P = 0.6$ ). The minimum values for inosine and hypoxanthine were 2.79  $\mu\text{g/ml}$  and 5.98  $\mu\text{g/ml}$ , respectively. Inosine had a sensitivity and specificity of 65% and 60%, respectively. For hypoxanthine, these values were 70% and 72%, respectively. These compounds can differentiate myocardial infarction patients from stable coronary artery patients (Figure 8,  $P = 0.0001$ ). However, carnitine was unable to differentiate myocardial infarction patients from those with stable coronary artery disease (Figure 6,  $P = 0.48$ ).



**Figure 6.** Differentiation power of inosine, hypoxanthine, and carnitine for stable and unstable coronary artery disease (CAD) and myocardial infarction (MI) patients. A. Inosine, with a sensitivity of 72% and specificity of 68%, and hypoxanthine, with a sensitivity of 70% and specificity of 76%, were capable of differentiating unstable CAD patients from stable CAD patients ( $P = 0.0001$ ). However, carnitine was not able to differentiate unstable CAD patients from other patients ( $P = 0.6$ ). B. Inosine with sensitivity and specificity of 65% and 60% and hypoxanthine with sensitivity and specificity of 70% and 72% differentiated MI patients from stable CAD patients ( $P = 0.0001$ ), while carnitine cannot differentiate MI patients from stable CAD patients ( $P = 0.48$ ).

Figure 6. Differentiation power of inosine, hypoxanthine, and carnitine for stable and unstable coronary artery disease (CAD) and myocardial infarction (MI) patients. A. Inosine, with a sensitivity of 72% and specificity of 68%, and hypoxanthine, with a sensitivity of 70% and specificity of 76%, were capable of differentiating unstable CAD patients from stable CAD patients ( $P = 0.0001$ ). However, carnitine was not able to differentiate unstable CAD patients from other patients ( $P = 0.6$ ). B. Inosine with sensitivity and specificity of 65% and 60% and hypoxanthine with sensitivity and specificity of 70% and 72% differentiated MI patients from stable CAD patients ( $P = 0.0001$ ), while carnitine cannot differentiate MI patients from stable CAD patients ( $P = 0.48$ )

## Discussion

Diagnosing during ischemia, particularly in the case of myocardial infarction, is a critical process. Typically, heart markers rise in the blood several hours after a myocardial infarction, which can lead to irreversible damage (25, 26). Two studies conducted by Farthing in 2006 (13) and 2015 (18), as well as a study by Al-Shamiri in 2009 (27), have demonstrated that ATP metabolites, particularly inosine and hypoxanthine, increase during angina, which is in line with the results of our study. The studies further demonstrated that inosine and hypoxanthine are compounds released from the heart during ischemia and can be detected in the blood. Measurement of these compounds has diagnostic value. The elevated levels of hypoxanthine observed in all the groups

studied suggest a short half-life of inosine in the bloodstream, as it is rapidly metabolized by blood enzymes (26). This suggests that hypoxanthine, due to its longer presence in the bloodstream, may serve as a more suitable marker than inosine. In the current study, both inosine and hypoxanthine concentrations were used to differentiate patients with unstable CAD and MI from those with CAD. However, they were unable to distinguish between patients with unstable angina and MI. The primary reason for this observation is not entirely clear, but it could be attributed to the similarities in the pathology of unstable angina and MI during clinical symptoms (28-30). Adenosine and inosine function as vasodilatory compounds that are released during ischemia and aid in the reestablishment of blood flow (31-32). Diadenosine polyphosphates are recognized as alerting compounds. Studies conducted by Garcia et al. (33), Stavrou et al. (34), and Luo et al. (35) have demonstrated that diadenosine polyphosphates, which are released from the heart during ischemia, exert a protective effect on the heart and are converted into inosine and hypoxanthine in the bloodstream. In this regard, the elevation of inosine and hypoxanthine in the bloodstream, as such in our study, may be a compensatory mechanism against ischemia injuries in the heart. However, the exact reason for this phenomenon deserves further research.

The findings of this study also revealed that carnitine levels were lower compared to the control group, although there was no significant difference in carnitine levels among the various patient groups. The formation of atherosclerotic plaques is a complex process involving inflammatory reactions and immune cells, characterized by high lipid profiles and oxidation in the early stages. The decrease in carnitine levels suggests its protective role in atherosclerosis. However, this study does not definitively establish whether carnitine's protective role is due to increased  $\beta$ -oxidation or its antioxidant activity. It's also worth noting that carnitine could potentially have a negative impact through its

metabolite, trimethylamine N-oxide (TMAO) (34). Wu et al. (36) and Claus et al. (38) have demonstrated that the gastrointestinal microbiomes or the liver can metabolize carnitine into TMAO. This has led to the observation of TMAO's role in the development of atherosclerosis. Zhao et al. revealed that gastrointestinal microbiomes can decrease carnitine levels while increasing TMAO levels. Furthermore, they discovered a correlation between gastrointestinal microbiomes and the development of atherosclerosis (39). But does the activity of gastrointestinal microbes influence the reduction of carnitine? To answer this question necessitates a study that explores the relationship between carnitine levels, the rate of coronary artery blockage, and the activity of gastrointestinal microbes. It's important to note that the majority of the body's carnitine needs are met through dietary intake (40). It would indeed be beneficial to further examine the nutritional impact of carnitine. Given these interpretations, it's clear that the effects of carnitine are more complex than what was addressed in this study. The levels of these compounds were assessed in relation to the degree of vascular congestion. However, no significant correlation was found between the levels of inosine and hypoxanthine and vascular congestion. Interestingly, higher levels of inosine and hypoxanthine were observed in patients with lower rates of clogging. This could be attributed to the increase in these compounds during acute ischemia. On the other hand, carnitine levels in patients were found to be lower than those in the control group. Whether the lipid-lowering (16, 17) or antioxidant (18) effects of carnitine that may be related to its cardioprotective capacity or the decreased levels of carnitine concentration in CAD patients in the current study, additionally, demonstrate the involvement of carnitine in CAD.

It should be noted the limitations of the study included the control group, who were persons who underwent coronary angiography for their suspected CAD and likely may not be a typical sample of a normal population. However, the subjects with other

conditions that probably may affect the levels of inosine, hypoxanthine, and carnitine were not included in the study.

### Conclusion

The findings of this study indicate that hypoxanthine, inosine, and carnitine concentrations in serum may serve as effective markers for differentiation and identification of patients with stable and unstable angina and MI patients from normal subjects. Despite the observed reduced level of carnitine in the patients compared to the control subjects, the complex function of carnitine in CAD requires further research to fully comprehend its role in the pathogenesis of CAD.

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### Ethical Considerations

The present study received ethical approval from the Ethics Committee of Ilam University of Medical Sciences (IR.MEDILAM.REC.1398.009). Written informed consent captured from the participant in accordance with the Declaration of Helsinki guidelines, and following principles for conducting interventions involving human subjects were considered.

### Financial Disclosure

This research was supported by the Ilam University of Medical Sciences, Iran.

### Competing Interests' Disclosure

There are no conflicts of interest for the study.

### Authors' contributions

Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Software, Data Curation, Writing— Original Draft Preparation, Writing— Review & Editing, Visualization, Supervision, Project Administration: HN, ASN, SG, GB.

### Writing Disclosure

This work was written and prepared by the authors independently, without the use of any outside writing agency. All ideas, words, and other material are all the writers.

### Data Availability Statement

The corresponding author may be contacted at any reasonable time for the data that back up the study's conclusions.

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