Is elevated serum ceruloplasmin level associated with increased risk of coronary artery disease?

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AY Göçmen, E Şahin, E Semiz, S Gümüşlü. Is elevated serum ceruloplasmin level associated with increased risk of coronary artery disease? Can J Cardiol 2008;24(3):209-212.

BACKGROUND: An imbalance between the lipid peroxidation process and antioxidative protection is associated with the pathophysiology of coronary artery disease (CAD). The authors aimed to determine the relationship between the contributors of antioxidant protection, such as paraoxonase-1 (PON1) activity, albumin, vitamin C and ceruloplasmin (CP) levels, and lipid peroxidation indicators.
METHODS: In the present study, the activity of PON1 was measured, together with serum concentrations of a variety of lipid constituents, albumin, vitamin C and CP levels, and lipid peroxidation indicators (conjugated dienes [CDs] and thiobarbituric acid-reactive substances [TBARS]). Data were gathered from 26 nondiabetic, angiographically proven, Turkish CAD patients and 26 healthy controls living in the Antalya region (Turkey).
RESULTS: CAD patients had significantly lower PON1 activity, high-density lipoprotein cholesterol, vitamin C and albumin concentrations, and higher CP, CD and TBARS concentrations than the controls. In the entire study population (n=52), serum CP levels were positively correlated with TBARS and CD levels, and negatively correlated with albumin and vitamin C levels, as well as with PON1 activity. On multiple logistic regression analysis, risk factors associated with CAD included high CP and low albumin levels.
CONCLUSIONS: CAD patients and controls were matched for age and sex, and high CP and low albumin levels were found to be independent risk factors for CAD. The present data gathered from the study group living in the Antalya region verifies that in CAD patients, CP impairs the oxidant-antioxidant balance in favour of the oxidants.

Key Words: *Albumin; Ceruloplasmin; Coronary artery disease; Lipid peroxidation; Lipids; Paraoxonase; Vitamin C*

Un taux de céruloplasmine sérique élevé est-il relié à une augmentation du risque de coronaropathie

HISTORIQUE : Un déséquilibre entre le processus de peroxydation lipidique et la protection anti-oxydantes s'associe à la physiopathologie de la coronaropathie. Les auteurs cherchaient à déterminer le lien entre les éléments contributifs de la protection anti-oxydante, comme l'activité de la paraoxonase-1 (PON1), les taux d'albumine, de vitamine C et de céruloplasmine (CP) et les indicateurs de peroxydation lipidique.
MÉTHODOLOGIE : Dans la présente étude, les auteurs ont mesuré l'activité de la PON1, de même que les concentrations sériques de divers composants lipidiques, les taux d'albumine, de vitamine C et de CP et les indicateurs de peroxydation lipidique (diènes conjugués [DC] et substances thiobarbituriques acido-réactives [STBAR]). Ils ont colligé les données auprès de 26 patients turques non diabétique atteints d'une coronaropathie démontrée par angiographie et de 26 sujets témoins en santé vivant dans la région d'Antalya, en Turquie.
RÉSULTATS : Les patients atteints d'une coronaropathie présentaient une activité considérablement plus faible de la PON1 et de concentrations de cholestérol à lipoprotéine de haute densité, de vitamine C et d'albumine ainsi que des concentrations de CP, de DC et de STBAR plus élevées que les sujets témoins. Dans toute la population à l'étude (n=52), les taux de CP sériques étaient corrélés positivement avec les taux de STBAR et de DC, et négativement avec les taux d'albumine et de vitamine C, de même qu'avec l'activité de la PON1. À l'analyse de régression logistique multiple, les facteurs de risque associés à la coronaropathie incluaient des taux de CP élevés et d'albumine faibles.
CONCLUSIONS : Les patients atteints d'une coronaropathie et les sujets témoins étaient appariés selon l'âge et le sexe, et on a déterminé que les taux de CP élevés et d'albumine faibles étaient des facteurs de risque indépendants de coronaropathie. Les données recueillies auprès du groupe à l'étude habitant dans la région d'Antalya confirment que, chez les patients atteints d'une coronaropathie, la CP compromet l'équilibre oxydant-anti-oxydant en faveur des oxydants.

The balance between oxidant production and sophisticated enzymatic and nonenzymatic antioxidant defense systems maintains physiological homeostasis and counteracts the oxidative damage of proteins, lipids and DNA (1). An imbalance between oxidative damage and antioxidative protection is associated with the pathophysiology of coronary artery disease (CAD) (2). Low-density lipoprotein (LDL) must be modified to oxidized LDL to trigger the pathological events leading to CAD (3). Studies indicate that oxidative modification of LDL is challenged by various antioxidant defense systems (4), as well as by paraoxonase-1 (PON1) (5), albumin (6) and vitamin C (7).

The high-density lipoprotein (HDL)-associated enzyme PON1 catalyzes the breakdown of phospholipid and cholesteryl ester lipid peroxides in both LDL and HDL, as well as in arteries ex vivo (8). PON1 protects lipoproteins against oxidation, probably by hydrolyzing specific lipid peroxides (5). Paraoxonases reduce oxidative stress in serum and tissues, protecting against cardiovascular diseases (8).

In humans, albumin is the most abundant serum protein. Its antioxidant activity is essential in maintaining physiological homeostasis because it binds and transports endogenous substances (such as lipids, ascorbate and divalent cations), scavenges oxygen free radicals and preserves microvascular integrity (9). The albumin molecule has been demonstrated to inhibit copper ion-dependent generation of hydroxyl radicals and lipid peroxidation, thereby preventing the oxidative injury of lipoproteins (6).

Vitamin C is an extremely effective antioxidant and an efficient scavenger of many reactive oxygen species (10). Vitamin C strongly inhibits copper-induced LDL oxidation (7).

Ceruloplasmin (CP) is a copper-containing alpha-2-glycoprotein with a molecular weight of approximately 132 kDa. CP has diverse functions. It is essential for iron homeostasis, is involved in angiogenesis and acts as a pro-oxidant or an antioxidant (11). The known functions of CP include copper transportation, iron metabolism, antioxidant defense, and involvement in angiogenesis and coagulation (12). It has been shown that CP catalyzes the oxidation of iron(II) to iron(III), with a catalytic cycle that involves four of the six atoms of copper associated with CP, and uses dioxygen as an electron acceptor without the mediation of an incompletely reduced reactive oxygen species, such as a superoxide anion or hydrogen peroxide (12).

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Several studies have reported an increase in the levels of thiobarbituric acid-reactive substances (TBARS) containing lipid peroxidation end products in the plasma of CAD patients (13,14). It is suggested that elevated levels of plasma TBARS and CDs may be independent risk factors and predictors of CAD (13).

We have not been able to find any reports about the correlation of PON1 activity, albumin, vitamin C and CP levels, and lipid peroxidation indicators in CAD. For this reason, we aimed to determine the relationship between CP and the contributors of antioxidant protection, such as PON1 activity, albumin and vitamin C levels, and lipid peroxidation indicators in age- and sex-matched CAD patients and healthy controls living in the Antalya region (Turkey).

PATIENTS AND METHODS

Subjects

The study was approved by the Akdeniz University Ethics Committee of the Faculty of Medicine (Akdeniz University, Antalya, Turkey). All patients gave written, informed content. The study participants (n=52) included 26 CAD patients (13 male, mean [± SEM] age 57.77±2.62 years, and 13 female, mean age 56.77±1.68 years) and 26 healthy controls (13 male, mean age 56.31±2.74 years and 13 female, mean age 54.23±1.55 years) from the Antalya region who underwent coronary angiography for the presence of CAD at the Hospital of Akdeniz University. Exclusion criteria included diabetes mellitus, active smoking, myocardial infarction, and infectious and autoimmune diseases.

The standard Judkins technique was used in coronary arteriographic examinations, and images were interpreted by a panel of experienced cardiologists who were blinded to data from all biochemical analyses. Subjects with stenosis estimated to be greater than 25% in at least one major coronary artery were considered to be CAD patients, and subjects with no detectable coronary artery anomalies were classified as controls.

Serum samples

Venous blood samples were obtained in the morning after a 12 h fast. Serum was separated immediately by low-speed centrifugation (600 *g* for 10 min at 4°C). Fresh serum samples were analyzed by biochemists who were blinded to the classification of subjects as CAD patients and controls.

Lipid assays

Total cholesterol, HDL cholesterol (HDL-C) and triglycerides were measured enzymatically using commercial kits (Abbott AEROSET system; Abbott Laboratories, USA). The LDL cholesterol (LDL-C) fraction was calculated indirectly using the Friedewald equation (15). The factor (triglyceride level)/5 was used to estimate very-low density lipoprotein cholesterol concentration (15).

PON1 activity assay

Serum PON1 activity was assayed according to the methods of Furlong et al (16) and Mackness et al (17), with minimal modifications. In brief, the assay mixture consisted of 1500 µL of a 6 mmol/L paraoxon substrate solution in 0.1 mol/L Tris Hydrochloride buffer (pH 8.0) containing 1 mmol/L calcium chloride and 60 µL of fresh serum. Absorbance was measured at 405 nm and 37°C in 1 min intervals on a spectrophotometer (photometer 4010; Boehringer Mannheim GmbH, Germany). The molar extinction coefficient for *p*-nitrophenol was calculated as 3030 L/(mol × cm) to determine enzymatic activity. One unit of PON1 activity was defined as 1 µmol of *p*-nitrophenol formed per minute, and the activity is expressed in units per litre of serum.

Vitamin C assay

Serum vitamin C was immediately assessed by the spectrophotometric method described by McCormick et al (18). Serum samples (0.5 mL) were added to freshly prepared metaphosphoric acid (60 g/L, 2.0 mL) and were treated with a dinitrophenylhydrazine-thiourea-copper-sulphate reagent (0.4 mL). Thus, the ascorbic acid was oxidized by copper(II) to form dehydroascorbic acid, which reacted with the acidic 2,4-dinitrophenylhydrazine to form red bis-hydrazone, which was measured at 520 nm. Levels of vitamin C were expressed as mg/L.

Albumin assay

Serum albumin levels were measured using the bromocresol green dye binding method (19). Stock bromocresol green reagent solution was prepared by dissolving 0.419 g bromocresol green in 10 mL sodium hydroxide (0.1 N) and diluting it to 1 L with distilled water. Serum (20 µL) was added to the bromocresol working solution (5 mL), which was prepared by mixing the stock bromocresol green solution (250 mL) with a citrate buffer (pH 4.2, 750 mL) and polyoxyethylene lauryl ether (30%, 4 mL) solution. The absorbance of this mixture was measured at 628 nm. Albumin concentrations were expressed as g/L using a graphic albumin standard.

CP oxidase activity assay

CP oxidase activity was determined according to the method described by Schosinsky et al (20), using *o*-dianisidine dihydrochloride as a substrate. 0.75 mL of an acetate buffer (0.1 mol/L, pH 5.0) and 0.05 mL of serum were pipetted into two tubes (one marked '5 min' and the other '15 min'). The tubes were placed into a water bath (30°C) and allowed to sit for 5 min for temperature equilibration before pipetting. The timer was started as 0.2 mL of *o*-dianisidine dihydrochloride (7.8 mmol/L) reagent (preincubated at 30°C) was added into each tube. After exactly 5 min, the '5 min' tube was removed from the water bath, and 2.0 mL of sulphuric acid (9 mol/L) was added and mixed immediately. After exactly 15 min, the '15 min' tube was removed, and 2.0 mL of sulphuric acid (9 mol/L) was added and mixed immediately. The absorbances of the purplish-red solutions were measured at 540 nm. Activity was estimated in U/L, using $\varepsilon_{max}=96\times10^4$ L/(mol × cm), in terms of substrate consumed.

Lipid peroxidation product assays

CD assay: CD levels were measured using the method described by Recknagel and Glende (21). Lipids were extracted with 2:1 (by volume) chloroform-methanol; the extract was evaporated to dryness under a stream of nitrogen and then redissolved in cyclohexane. The cyclohexane solution was assayed at 234 nm. The results were expressed as µmol/L using $\varepsilon_{max}=2.52\times10^4$ L/(mol × cm).

TBARS assay: TBARS levels were measured by the fluorometric method described by Wasowicz et al (22), using 1,1,3,3-tetramethoxypropane as a standard. The fluorescence of the butanol extract was measured in a spectrofluorometer (Shimadzu RF-5000; Shimadzu Corp, Japan) using wavelengths of 525 nm for excitation and 547 nm for emission. The results are presented in µmol MDA/L.

Statistics

Student's *t* test was used for comparison of means. Pearson's correlation coefficient was used to test the strength of any associations between variables. Multiple logistic regression analysis was used to identify associations between CAD and the independent parameters. Data were expressed as mean ± SEM, and $P<0.05$ was considered to be statistically significant.

RESULTS

Table 1 reports data of the general lipid profiles of CAD patients and control subjects. Compared with the control group, CAD patients had lower HDL-C levels ($P<0.01$) (Table 1).

In CAD patients, PON1 activity ($P<0.01$), and vitamin C ($P<0.001$) and albumin ($P<0.001$) levels were significantly lower than in the control subjects (Table 2). On the other hand, CAD patients had elevated levels of CP, CDs and TBARS ($P<0.001$) (Table 2).

In the study population (n=52), PON1 activity was positively correlated with albumin ($r=0.305$, $P<0.05$) and HDL-C ($r=0.312$,

TABLE 1
General lipid profile of coronary artery disease (CAD) patients and control subjects

Parameter	CAD patients (n=26)	Control subjects (n=26)	P
Total cholesterol (mmol/L)	5.24±0.26	5.16±0.21	NS
HDL-C (mmol/L)	0.97±0.04	1.13±0.04	<0.01
LDL-C (mmol/L)	3.51±0.23	3.21±0.18	NS
VLDL-C (mmol/L)	0.36±0.03	0.33±0.02	NS
Triglycerides (mmol/L)	1.71±0.14	1.57±0.10	NS
Age (years)	57.27±1.53	55.27±1.55	NS

Values are expressed as mean ± SEM. HDL-C High-density lipoprotein cholesterol; LDL-C Low-density lipoprotein cholesterol; NS Nonsignificant; VLDL-C Very low-density lipoprotein cholesterol

TABLE 2
Paraoxonase-1 activity, vitamin C, albumin, ceruloplasmin, conjugated diene and TBARS levels in coronary artery disease (CAD) patients and control subjects

Parameter	CAD patients (n=26)	Control subjects (n=26)	P
Paraoxonase-1 (U/L)	214.63±24.43	309.70±25.40	<0.01
Vitamin C (mg/L)	8.50±0.50	11.50±0.70	<0.001
Albumin (mg/L)	30.40±0.90	40.1±0.50	<0.001
Ceruloplasmin (U/L)	211.62±4.74	131.08±5.84	<0.001
Conjugated dienes (μmol/L)	2.30±0.10	1.68±0.13	<0.001
TBARS (μmol MDA/L)	1.93±0.04	1.51±0.10	<0.001

Values are expressed as mean ± SEM. TBARS Thiobarbituric acid-reactive substances

P<0.05) levels, and negatively correlated with CD (r=–0.375, P<0.01) and TBARS (r=–0.418, P<0.005) levels. Serum CP levels were positively correlated with TBARS (r=0.587, P<0.0001) and CD levels (r=0.675, P<0.0001) and negatively correlated with albumin (r=–0.793, P<0.001) and vitamin C levels (r =–0.420, P<0.005), as well as PON1 activity (r=–0.373, P<0.01). Levels of the lipid peroxidation products TBARS and CDs were negatively correlated with albumin levels (r=–0.417, P<0.005 and r=–0.532, P<0.001, respectively).

On multiple logistic regression analysis, risk factors associated with CAD included high CP and low albumin levels.

DISCUSSION

In the present study, we observed that serum PON1 activity was significantly decreased in CAD patients compared with healthy controls. This result is in agreement with studies of different populations (23,24) and our previously published data (25). However, in a population of Iranian descent, it was found that PON1 activity was not statistically different in premature CAD (26). PON1 activity is influenced genetically and by environmental factors such as diet, lifestyle, and exposure to environmental chemicals and diseases. The decreased activity observed in CAD patients may be explained by the fact that PON1 loses its activity in the oxidative environment (27), which is associated with the progression of CAD.

We have demonstrated decreased vitamin C concentrations in the serum of patients with CAD. Thus, the findings of the present study were consistent with the concept that the scavenging of increased oxygen-derived free radicals may account for the beneficial effects of vitamin C in these settings (28). The salient finding of the present study was the positive correlation between vitamin C and albumin, and the negative correlation with CP, which were independent predictors of a higher risk of cardiovascular events. This observation supports the concept that oxidative stress contributes to the progression of CAD and may, therefore, be an important determinant of clinical events.

In the present study, albumin levels of controls were in the normal range (35 g/L to 55 g/L), while 23 of the CAD patients (88.5% of CAD group) had hypoalbuminemia (less than 35 g/L). We found lower albumin levels in CAD patients than in controls, similar to a study in Ankara, Turkey (29). Even though serum albumin concentration is reduced in advanced age, fasting and in some diseases (9), we could not find any report elucidating the association between decreased albumin levels and CAD. The oxidative stress hypothesis of vascular injury suggest that hypoalbuminemia increases the risk of cardiovascular toxicity, because hypoalbuminemia results in reduced scavenging capacity for oxidants (6). One explanation may be that the free radicals formed during CAD exceed the antioxidant capacity of the albumin, resulting in lipid peroxide accumulation.

In the present study, we found increased CP levels in CAD patients. The present data on the serum CP concentrations are also supported by Fox et al (30) and McMurray et al (31). Enbergs et al (32) and Halevy et al (33) did not find changes in plasma CP levels in angiographically detected CAD patients. A possible cause of this increase in CP in CAD patients is the impairment of the oxidant-antioxidant balance in favour of the oxidants. Serum CP levels were reported to be an independent risk factor for cardiovascular diseases (30). We also found CP to be an independent risk factor for CAD.

We observed that CP levels positively correlated with CD and TBARS levels in CAD patients. We could not find comparable reports in the literature studing the correlation between CP and lipid peroxidation products (CDs and TBARS) in CAD patients. Our data suggest that the oxidative effects of CP on serum lipids predominate in CAD patients in association with decreased antioxidant protection. Therefore, elevated serum CP levels may signal abnormally high oxidant stress (34) and contribute to increased lipid peroxidation in CAD patients.

In the present study, CD and TBARS levels were measured as indicators of lipid peroxidation and were found to be higher in CAD patients than in healthy control subjects. Our previous findings are in agreement that CAD patients have increased levels of serum lipid peroxidation products (35). The increase in TBARS levels may be attributed to the hydroperoxides arising from the polyunsaturated fatty acids of LDL (14), decreased vitamin C, albumin, HDL-C and PON1 activity associated with decreased antioxidant protection, as well as to increased CP levels.

In the study groups, we observed that PON1 activity positively correlated with albumin and HDL-C levels, and negatively correlated with levels of CP, TBARS and CDs. No reports are available in the literature regarding their correlation; therefore, we suggest that the interactions between PON1, albumin and CP are worth studying.

We also observed that albumin levels negatively correlated with CP, TBARS and CD levels, and positively correlated with PON1 activity, and vitamin C and HDL-C levels. We have not been able to find comparable reports describing the correlation between CP and albumin levels of CAD patients in the literature. However, the observation that albumin-inhibited, CP-induced LDL oxidation (36) may make it easier to understand the restrictive effects of albumin on CP pro-oxidant activity in the CAD process. Samokyszyn et al (37) reported that in vitro CP can act as a pro-oxidant or an antioxidant, depending on its concentration, but longitudinal studies are needed to confirm the nature of the association of low albumin levels and CAD, as well as the role of CP in their association.

CP has diverse functions. It is essential for iron homeostasis, is involved in angiogenesis and acts as a pro-oxidant or an antioxidant (11).

Our study does not support the hypothesis that CP protects tissues against reactive oxygen species depending on its ferroxidase activity, which depends on its capacity to act as a preventive antioxidant (38), but it supports the notion that high CP levels are cardiovascular risk factors in humans (30). On the contrary, a low level of the other

copper-carrying protein, albumin, was found to be associated with an increased risk of CAD. This association may be related to lipid peroxidation or a number of still largely unknown factors that increase the level of serum CP.

CONCLUSIONS

This is the first report presenting these parameters in age- and sex-matched CAD patients and control subjects living in the Antalya region of Turkey. It is likely that the oxidative effects of CP on serum lipids, in combination with decreased antioxidant protection, predominate in CAD patients. An imbalance in the antioxidant capacities of PON1 and albumin, and the oxidation reactions because of CP, may be related to the pathophysiology of CAD. It is noteworthy to establish a possible interaction between PON1, albumin and CP in future studies.

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