Carotid artery intima-media thickness correlates with oxidative stress in chronic haemodialysis patients with accelerated atherosclerosis

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Abstract
Background. Accelerated atherosclerosis is the major cause of mortality in patients on chronic haemodialysis (HD). Increased oxidative stress might be the major factor leading to high cardiovascular mortality rate in HD patients. The aim of our study was to clarify effects of uraemia and dialysis on oxidative stress parameters and explore the relation between oxidative stress markers and carotid artery intima-media thickness (CIMT) as an indicator of atherosclerosis.

Methods. Twenty chronic HD patients, 20 predialytic uraemic patients and 20 healthy subjects were included in the study. Serum thiobarbituric acid reactive substances (TBARS), protein carbonyl content (PCO) and nitrite/nitrate levels were determined as oxidative stress markers. Serum vitamin E, plasma sulphhydryl (P-SH), glutathione peroxidase (GPx) activities were measured as antioxidants. CIMT was assessed by carotid artery ultrasonography.

Results. Both chronic HD and predialytic uraemic patients had enhanced oxidative stress indicated by higher levels of nitrite/nitrate, TBARS and PCO, and lower levels of P-SH, SOD, CAT and GPx compared to controls. HD patients had significantly higher CIMT and nitrite/nitrate while significantly lower P-SH, vitamin E, SOD, CAT and GPx compared to predialytic uraemic patients. There was a significant positive correlation between CIMT and TBARS ($r = 0.38$, $P = 0.003$) and nitrite/nitrate levels ($r = 0.41$, $P = 0.001$), while there was a significant negative correlation between CIMT and SOD ($r = -0.35$, $P = 0.01$), CAT ($r = -0.65$, $P < 0.001$) and P-SH levels ($r = -0.50$, $P < 0.001$). A linear regression analysis showed that TBARS were still significantly and positively correlated with CIMT ($P = 0.001$), while CAT and P-SH were significantly and negatively correlated with CIMT ($P = 0.002$ and $P = 0.048$, respectively).

Conclusions. HD exacerbates oxidative stress and disturbances in antioxidant enzymes in uraemic patients. We propose that serum TBARS and nitrite/nitrate can be used as positive determinants, while erythrocyte SOD, CAT and P-SH may be used as negative determinants of atherosclerosis assessed by CIMT in uraemic and HD patients.

Keywords: carotid artery intima media thickness; chronic haemodialysis patients; oxidative stress

Introduction
Atherosclerosis is accepted as a common mechanism underlying all cardiovascular diseases (CVDs) and atherosclerotic CVD is a significant cause of morbidity and mortality in patients with end-stage renal disease (ESRD) [1–3]. Evidence showed that there is an increased incidence and accelerated worsening of atherosclerosis in patients on chronic haemodialysis (HD). A marked increase in coronary artery disease (CAD) incidence and death rates has been reported in HD patients when compared with an age-matched general population or non-uraemic populations with hyperlipidaemia and hypertension [2]. In patients on maintenance HD, 40–50% of deaths are attributed to lethal cardiac events, and thus the rate of cardiac mortality is ∼5–20 times higher than that in the normal population [4]. Even in predialytic uraemic patients who are not on maintenance HD, it has been shown that the intima-media thickness (IMT) of the carotid and femoral arteries is increased [5]. Damage of large arteries, characterized by increased IMT and arteriosclerosis, is a contributing factor to mortality in patients with ESRD [6,7].

The reason for the rapid clinical progression of CAD in patients with ESRD is not completely understood. It has been suggested that uraemia itself may pose a specific risk for this patient group and several studies have focused
on oxidative stress in uraemia. The association between oxidative stress and atherosclerosis was recognized by Glavind et al. [8] as early as 1952. Oxidative stress, which results from an imbalance between reactive oxygen (ROS) and nitrogen species (RNS) production and antioxidant defence mechanisms, is now well recognized in chronic kidney disease and HD patients. Increased oxidative stress could be involved in accelerated atherosclerosis in these patients [9–11]. A number of recent studies, both in rats and uraemic patients, have raised the hypothesis that an impaired NO (nitric oxide) synthetic pathway could have a key role in mediating the complex renal haemodynamic and non-haemodynamic disorders associated with progression of RD [12,13].

Common carotid IMT (CIMT) as measured by ultrasoundography represents a marker of structural atherosclerosis. Increased CIMT has been shown to be correlated with CV risk [14] and severity of coronary atherosclerosis [15] and is helpful in predicting CV events in population groups [16,17]. Increased CIMT is considered as an early phase of atherosclerosis and might be seen even in patients with mild hypertension and normal serum cholesterol [18]. Assessment of CIMT using high-resolution B-mode ultrasonography is a reliable, reproducible and non-invasive method for detecting and monitoring the progression of atherosclerosis [19].

The aim of our study was to clarify the respective effects of uraemia and HD on oxidative stress parameters and explore any relation between CIMT and oxidative stress markers and antioxidants in ESRD patients. We determined CIMT as an indicator of atherosclerosis; serum thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation; plasma protein carbonyl content (PCO) as a marker of oxidative protein damage and serum nitrite/nitrate levels as indicators of RNS production. We measured erythrocyte glutathione level (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, and plasma sulfhydryl (P-SH) and serum vitamin E concentrations as antioxidant markers.

Subjects and methods

The investigation conforms to the principles outlined in the Declaration of Helsinki. The study was approved by the local ethics committee and all participants gave written informed consent.

Twenty predialytic uraemic patients (uraemic group) and 20 HD patients (HD group) attending the Nephrology Department of Akdeniz University Medical Faculty were included in the study. Patients in the uraemic group had creatinine clearance between 15 and 60 ml/min/1.73 m². Patients in the HD group had creatinine clearance <10 ml/min/1.73 m² and had been on chronic HD treatment for >6 months (the mean duration of HD treatment was 5.8 ± 0.8 years). HD patients were dialyzed three times a week with synthetic or hemisynthetic membrane, each session lasting 4 h with bicarbonate dialysate.

Twenty healthy individuals who proved to be in a good state of health and free from any signs of chronic disease after a careful clinical examination and laboratory check-up were included in the study as the control group. All controls were non-smokers and did not consume alcohol regularly.

The intake of analgesics, vitamins or anti-inflammatory drugs was stopped 4 weeks before blood sampling in both patient groups and controls.

Determination of the oxidative stress markers

Fasting blood samples were obtained from all subjects. In the HD group, blood samples were collected before the first weekly HD treatment.

Serum was obtained by centrifugation at 3000 g for 10 min. The serum TBARS level was determined by the fluorometric method of Wasowicz et al. and the results were expressed as nmol/ml [20]. Serum nitrite and nitrate levels were measured by the methods of Kader et al. [21] and Bories et al. [22], respectively. Serum vitamin E concentration was measured as described by Desai using a standard curve of known concentrations of α-tocopherol acetate [23].

Haemolysates were prepared from samples collected into vacuum tubes containing 1.7 mg/ml K3 EDTA as anticoagulant. After centrifugation at 3000 g for 15 min, plasma and buffy coat were removed from the pellet. P-SH concentration was determined by the method of Koster et al. [24]. PCO of oxidatively modified proteins was determined by the method of Reznick et al. [25]. Absorbance of the sample was measured at 360 nm and the result was given as µmol/l carbonyl by using εmax = 22 000 M⁻¹ cm⁻². Erythrocytes were washed three times in an ice-cold isotonic sodium chloride solution (1:10, v/v) and were resuspended in a washing solution to give 50% suspension. Haemolysis of washed cell suspension was achieved by mixing 1 volume with 9 volumes of distilled water. Haemolysate obtained was divided into two aliquots. The first aliquot was used to determine haemoglobin concentration using the cyanomethaemoglobin method [26]. The second aliquot was used for determination of reduced GSH and enzymatic activities of SOD [27], CAT [28] and GPx [29]. Erythrocyte GSH concentration was measured by the method of Fairbanks and Klee and expressed as mg/gHb [26]. Erythrocyte Cu, Zn–SOD activity was assayed using the spectrophotometric indirect inhibition technique of Misra and Fridovich, based on the ability of SOD to inhibit auto-oxidation of adrenalin to adenochrome at alkaline pH [27]. CAT activity was measured using the Aebi method based on the decomposition of substrate hydrogen peroxide, as indicated by the decrease in absorbance at 240 nm [28]. Gpx activity was measured by the coupled method of Paglia and Valentine using t-butyldihydroperoxide as a substrate [29]. The results for SOD and GPx were expressed as U/gHb, and for CAT as k/gHb, where k is the rate constant for the CAT activity.

Determination of the CIMT

Ultrasoundographic scanning of the carotid artery was performed using a high-resolution ultrasonographer (Toshiba Corevision high-resolution B-mode ultrasonography) provided with a 7.5 MHz linear transducer. All the measurements were carried out blindly by the same operator.
Systolic blood pressure (mmHg) 129.29 ± 0.75
Smoking 0 11/9 12/8 0.75

Table 2. Post hoc catalase, GPx: glutathione peroxidase, CIMT: carotid artery intima-media thickness. TBARS: serum thiobarbituric acid reactive substances, P-SH: plasma sulfhydryl, GSH: erythrocyte glutathione, SOD: superoxide dismutase, CAT: TBARS (nmol/ml) 1.20 ± 0.48
Nitrite + nitrate (μmol/l) 138.34 ± 27.85
Carbonyl (nmol/mg protein) 0.55 ± 0.11
P-SH (μmol/l) 28.83 ± 5.48
GSH (mg/gHb) 1.74 ± 0.33
Vitamin E (μmol/l) 60.29 ± 10.47
SOD (U/gHb) 1646.24 ± 151.98
CAT (k/gHb) 541.37 ± 145.35
GPx (U/gHb) 2.33 ± 0.90
CIMT (mm) 0.52 ± 0.08

BMI: body mass index, PTH: parathormone.
Post hoc comparisons: *P < 0.05 versus control group; ††P < 0.001 versus uraemic group.

Each subject was examined in the supine position in a semi-dark room. The carotid artery was investigated bilaterally and scanned at the level of the bifurcation of the common carotid arteries. The image was focused on the far wall of the artery. CIMT was taken as the distance from the common carotid arteries. The image was focused on the far wall of the bilateral distal common carotid arteries (1–3 cm proximal to the carotid bifurcation) at the diastolic phase. CIMT was expressed as the mean of six measurements (three on each side) [30]. No measurement was made on the sites where a plaque existed.

Statistical analysis
All statistical tests were performed with a commercially available statistical analysis program (SPSS 11.0 for Windows). Continuous variables were expressed as mean ± standard deviation while categorical variables were expressed in ratio. The Kruskal–Wallis and Mann–Whitney U tests were used to compare oxidative stress markers and CIMT measurements among the groups. Spearman’s correlation test was performed to explore the correlation between oxidative stress markers and CIMT. A multiple linear regression model with oxidative stress markers, age, gender, systolic and diastolic blood pressure and lipid profiles correlated with CIMT was performed. P-values <0.05 were interpreted as statistically significant.

Results
Clinical and demographic characteristics of the patients and controls are given in Table 1. There were no significant differences in age, BMI and gender among the groups.

Oxidative stress and antioxidant parameters measured are shown in Table 2. The nitrite and nitrate levels were significantly higher in the HD group compared to the uraemic group and healthy controls (P < 0.001). The HD group had significantly higher TBARS levels compared to controls (P = 0.05). Plasma PCO content was significantly higher in both the HD and uraemic groups compared to controls (P < 0.001).

The HD group had significantly lower P-SH concentration than the uraemic group, while the difference in P-SH concentration between the uraemic group and controls was also significant (P < 0.001). The HD group had significantly higher GSH levels compared to controls and the uraemic group, while the uraemic group had significantly higher GSH levels than controls (P = 0.006). The uraemic group had significantly higher serum vitamin E levels compared to the haemodialysis group and control group (P < 0.001).
Both HD and uraemic patients had significantly lower erythrocyte SOD levels compared to controls, while the HD group had even lower erythrocyte SOD levels than the uraemic group ($P < 0.001$). Both the HD and uraemic groups had significantly lower CAT levels compared to controls, while the lowest CAT levels were found in the HD group ($P < 0.001$). The erythrocyte GPx level in HD patients was significantly lower than the levels in uraemic patients and control subjects ($P = 0.001$).

HD patients had significantly higher CIMT than uraemic patients and healthy subjects, while the uraemic group had also significantly higher CIMT values than control subjects ($P < 0.001$).

There was a positive correlation between CIMT levels and TBARS levels ($r = 0.38$, $P = 0.003$, Figure 1A). Similarly, a positive correlation was found between CIMT and nitrite and nitrate values ($r = 0.41$, $P = 0.001$, Figure 1B). In contrast, there was a negative correlation between CIMT and antioxidants; SOD ($r = −0.35$, $P = 0.01$, Figure 2A); CAT ($r = −0.65$, $P < 0.001$, Figure 2B) and P-SH values ($r = −0.50$, $P < 0.001$, Figure 2C). No significant correlation was found between CIMT and other measured oxidative stress and antioxidant parameters. However, there was a significant correlation between CIMT and systolic blood pressure ($r = 0.42$, $P = 0.002$).

We modelled a multiple linear regression analysis to define the independent determinants of CIMT. CIMT was taken as a dependent variable. Age, gender, systolic and diastolic blood pressure, creatinine, HDL cholesterol and LDL cholesterol were incorporated into the model as independent variables, in addition to TBARS, nitrite and nitrate values, SOD, CAT and P-SH levels. The adjusted $R^2$ of the model was 0.725 with $P < 0.001$. The linear regression model revealed that TBARS were still significantly and positively correlated with CIMT (standardized beta = 0.349, $P = 0.001$) while CAT and P-SH were significantly and negatively correlated with CIMT (standardized beta = −0.384, $P = 0.002$ and standardized beta = −0.184, $P = 0.048$, respectively) (Table 3).

Discussion

In our study, we assessed the relationship between the specific markers of oxidative stress, antioxidants and the presence of early sub-clinical atherosclerosis in humans determined by CIMT. We found a significant increase in CIMT in the uraemic and HD groups compared to healthy controls. The HD group had the highest CIMT values indicating a higher risk for atherosclerotic diseases. Our results are in accordance with the previous studies that evaluated atherosclerosis in HD patients using carotid ultrasonography [3,19,31,32].

Our first novel contribution from this study was the demonstration of a positive correlation between CIMT and two oxidative stress markers: serum TBARS and nitrite/nitrate levels. The second novel finding of this study was the negative correlation between CIMT and three antioxidants: erythrocyte SOD, CAT and P-SH levels. We suggest the use of TBARS and nitrite/nitrate levels as positive determinants and erythrocyte SOD, CAT and P-SH levels as negative determinants of atherosclerosis. To the best of our knowledge, no previous report for correlations between CIMT and oxidative stress markers and antioxidants in chronic renal disease patients exists in the literature. We demonstrated that serum TBARS, erythrocyte SOD and P-SH were still significantly correlated with CIMT when adjusted by certain other factors affecting CIMT.

In our study, higher TBARS, PCO and nitrite/nitrate levels indicate increased production of ROS and RNS, in both HD and uraemic groups compared to healthy controls. Our results are in accordance with the following data in the current literature. A high prevalence of increased oxidative stress was reported to be associated with increased risk of CV morbidity and mortality in adult HD patients [9,33–37]. We found increased oxidative stress and CIMT in HD and predialyitic uraemic patients, which supported the
Oxidative stress and CIMT in haemodialysis patients

Table 3. Linear regression analysis for defining the independent determinants of CIMT

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>$\beta$ (coefficient)</th>
<th>$t$-test value</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS</td>
<td>0.349</td>
<td>3.693</td>
<td>0.001</td>
</tr>
<tr>
<td>CAT</td>
<td>-0.384</td>
<td>-3.578</td>
<td>0.002</td>
</tr>
<tr>
<td>P-SH</td>
<td>-0.184</td>
<td>-1.675</td>
<td>0.048</td>
</tr>
<tr>
<td>Age</td>
<td>0.237</td>
<td>2.406</td>
<td>0.021</td>
</tr>
</tbody>
</table>

$R^2 = 0.725, P < 0.001$.

Out of the model: gender ($P = 0.16$); systolic and diastolic blood pressure ($P = 0.18$ and $P = 0.81$, respectively); creatinine ($P = 0.13$); HDL cholesterol ($P = 0.23$) and LDL cholesterol ($P = 0.07$); nitrite/nitrate ($P = 0.07$) and SOD ($P = 0.29$).

role of oxidative stress in the development of accelerated atherosclerosis in these patients.

There might be various mechanisms contributing to high oxidative stress in chronic renal diseases. Increased ROS and RNS generation and decreased antioxidant defences have been implicated in the pathogenesis of oxidative stress in uraemia [9,33–35]. Chronic inflammation, as evidenced by increased levels of pro-inflammatory cytokines, is a common feature in HD patients. Activation of polymorphonuclear neutrophils and monocytes has been described during HD, which results in the release of ROS and cytokines [38]. Activated neutrophils generate superoxide anions ($O_2^-$) that, combining with endothelial-derived NO, form the highly cytotoxic hydroxyl radical [39]. Accumulation of tumour necrosis factor-α and interleukin-1β in supranormal amounts in uraemic plasma, being released by monocytes activated on dialysis membrane, potently induces NO synthesis [40–43].

Uraemia is associated with excessive systemic NO release, both in experimental model and in human beings. In the systemic circulation of uraemic rats, as well as uraemic patients, NO is formed in excessive amounts [12]. Data are available in humans showing that platelets from uraemic patients on HD generate more NO than healthy subjects [40,41]. Plasma L-arginine was higher in uraemic patients than in controls, and intraplatelet levels of cGMP (second messenger of NO) were also higher in uraemic than in control platelets [40]. In addition, uraemic patients have higher levels of NO in the exhaled air and higher plasma levels of NO metabolites than normal humans [44–47]. It was also demonstrated that uraemic plasma, unlike normal plasma, was a potent inducer of NO in umbilical or microvascular endothelial cells [40,41]. Another possible cause of the increased NO levels is higher release from systemic vessels due to the augmented expression of both iNOS and endothelial NOS. A putative cause for excessive NO production in uraemia can be guanidinosuccinate, a uraemic toxin that accumulates in the circulation of uraemic patients and upregulates NO synthesis. Heparin, which is used as anticoagulant during HD, might contribute to increased NO production in HD patients, as indicated by its capability to promote NO production by cultured human endothelial cells [40,48].

We found a decrease in SOD, CAT, GPx and P-SH levels in both HD and uraemic patients compared to the healthy controls. Our data indicate that HD and predialytic uraemic patients have an impaired antioxidant response, which may...
be attributed in part to their antioxidant enzyme deficiency. Our data led us to conclude that oxidative stress and disturbances in antioxidant enzymes occur at the early stages of chronic uraemia and are exacerbated by HD, which may lead to the development of atherosclerosis and other long-term complications in ESRD patients.

Limitations of the study
The major limitation of the study was definitely the small sample size. The study was a cross-sectional study; a prospective study following the uraemic patients until they require and undergo HD treatment might yield better results in exploring the effects of HD on the oxidative stress parameters in renal failure patients. However, our study is the first to demonstrate a positive correlation between CIMT and serum TBARS, and nitrite/nitrate levels and a negative correlation between CIMT and erythrocyte SOD, CAT and P-SH levels.

Conclusion
There is increased oxidative stress in HD and predialytic uraemic patients compared to healthy subjects, with increased risk for development of atherosclerotic diseases as indicated by the presence of higher CIMT in these patients. There was a significant positive correlation between CIMT, and serum TBARS and nitrite/nitrate levels and a significant negative correlation between CIMT, and SOD, CAT and P-SH levels. We propose, for the first time in ESRD patients, that serum TBARS and nitrite/nitrate levels can be used as positive determinants while erythrocyte SOD, CAT and P-SH levels may be used as negative determinants of atherosclerosis assessed by CIMT.

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Conflict of interest statement. None declared.

References
30. Poli A, Tremoli E, Colombo A et al. Ultrasonographic measurement of the common carotid artery wall thickness in hypercholesterolemic


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