Pro-oxidant HDL predicts poor outcome in patients with ST-elevation acute coronary syndrome

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Summary
Oxidative stress affects clinical outcome in patients with ST-elevation acute coronary syndrome (STE-ACS). Although high-density lipoprotein (HDL) particles are generally considered protective, deleterious properties of HDL have been observed in patients with acute myocardial infarction. Here, we analysed the association between pro-oxidant HDL and all-cause mortality in STE-ACS patients. We determined the antioxidant function of HDL in 247 prospectively enrolled patients undergoing primary percutaneous coronary intervention for STE-ACS. Patients were stratified as by a pro-oxidant serum HDL oxidant index (HOI≥1) or with an antioxidant serum HOI (HOI<1) capacity. Multivariate regression analysis was used to relate HOI to survival. The median follow-up time was 23 months (IQR 14.4–40.0 months). Pro-oxidant HDL was observed in 44.1 % of STE-ACS patients and was independently associated with all-cause mortality with a hazard ratio of 3.30 (95% CI 1.50–7.27, \( p = 0.003 \)).

Mortality rates were higher in patients with baseline pro-oxidant HDL compared to patients with preserved HDL function at 30 days (11.9% vs 2.2%, \( p=0.002 \)), and at 4 years (22.9% vs 8.7%, \( p=0.002 \)). Elevated neutrophil counts were a strong and independent predictor for pro-oxidant HDL with an odds ratio per standard deviation of 1.50 (95% CI 1.11–2.03, \( p=0.008 \)), as was history of prior acute myocardial infarction, elevated triglyceride levels and reduced glomerular filtration rate. In conclusion, pro-oxidant HDL represents a strong and independent predictor of long-term as well as short-term all-cause mortality in STE-ACS patients. Elevated neutrophil counts predicted the presence of serum pro-oxidant HDL. The maintenance of HDL functions might be a promising therapeutic target in STE-ACS patients.

Keywords
STE-ACS, HDL, antioxidant function, outcome

Introduction
Despite significant advances in the management of acute myocardial infarction (AMI), cardiovascular mortality remains the leading cause of death worldwide. Adverse ventricular remodelling and dysfunction are observed in a substantial proportion of AMI patients and are associated with poor clinical outcome (1). Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) production and antioxidant defences, has been identified to be crucially involved in adverse pathophysiological processes occurring after AMI. The excessive formation of ROS, produced either by the ischaemic myocardium itself or by infiltrating leukocytes (2, 3), triggers thrombosis, myocardial injury and cardiomyocyte death through a number of mechanisms including direct damage to proteins and membranes or indirect damage through the activation of inflammatory pathways (4–8).

High-density lipoprotein (HDL) is well known for antioxidant properties by binding and removing oxidant molecules (9). Notably, the ability of HDL to prevent generation of oxidised low-density lipoproteins (oxLDL) has been well examined over the last years (10–12). Considering the pivotal role of oxLDL in activating platelets during thrombosis (4, 7, 8), the protective role of HDL might be of major importance in AMI patients. However, it has become evident that protective functions of HDL are attenuated in patients with AMI (11, 13, 14). Particularly, HDL particles may amplify pro-oxidant processes during AMI (11). The impact of impaired or reversed antioxidant function of HDL on clinical outcome in AMI patients has not been investigated. Therefore, we determined the antioxidant capacity of HDL in patients with ST-elevation acute coronary syndrome (STE-ACS) with a simple serum-based assay, and analysed its association with all-cause mortality and cardiovascular events. An established functional assay that measured the capacity of HDL to prevent oxidation of LDL in a cell-free environment was utilised (10).
Materials and methods

Study population and design

Between December 2006 and December 2011 we prospectively enrolled all consentable patients with the diagnosis of STE-ACS (ICD-10: I21.0–3), into this observational, single-centre study. Patients were admitted to the catheter laboratory of the Vienna General Hospital, a university-affiliated tertiary centre, and treated with aspiration thrombectomy during primary percutaneous coronary intervention (pPCI). Blood samples were drawn from the femoral sheath during pPCI. The study protocol was approved by the Ethics Committee of the Medical University of Vienna (approval reference number 303/2005).

Study objectives and definitions

The primary study endpoint long-term all-cause mortality was obtained in collaboration with government authorities. Data on rehospitalisations were checked in the Krankenanstaltenverbund (KAV) der Stadt Wien - an integrated database connecting Viennese hospital records. The combined endpoint major adverse cardiac events (MACE) was defined as all-cause death, non-fatal myocardial infarction or coronary revascularisation. Cardiovascular risk factors were recorded according to the respective guidelines. Thrombolysis in myocardial infarction (TIMI) risk score, developed to predict in hospital mortality in patients with STE-ACS, was calculated for each patient at the time of presentation (15). TIMI risk score variables for STE-ACS are eight parameters, including age, diabetes mellitus/hypertension/angina, blood pressure, heart rate, Killip class, body weight, anterior ST-elevation or left bundle branch block and time delay to treatment.

Determination of HDL Oxidant Index (HOI) and myeloperoxidase (MPO) plasma levels

HDL antioxidant capacity was determined using a 2,7′-dichlorodihydrofluorescein diacetate (DCF)-based cell free fluorescent assay that evaluated the capacity of HDL to inactivate previously oxidised LDL (10). This well validated assay is based on the characteristics of non-fluorescent DCF diacetate that converts into its fluorescent form (DCF) in the presence of oxLDL (11, 12). In accordance with prior studies we used apolipoprotein B-depleted serum, which includes HDL, apo-A1, apo-A2, and HDL-associated particles for this test (11, 12). The assay was performed as previously described (10). Briefly, after polyethylene glycol depletion of apolipoprotein B from previously unthawed patient sera, HDL containing supernatant was used in this assay. Serum samples were stored at –80°C until performance of this assay. LDL (Merck Millipore, Darmstadt, Germany) was diluted in phosphate-buffered saline (PBS) to a final cholesterol concentration of 100 µg/ml and oxidised at 37°C in 100 µM CuSO₄ (Merck) for 6 hours (h) by dialysis. The organic phospholipid DCF (Molecular Probes, Eugene, OR, USA) was prepared as previously described (11). oxLDL (final concentration: 1.4 µg/ml), DCF (final concentration: 2.9 µg/ml), and 15 µl of apolipoprotein B-depleted patient serum samples were incubated with PBS to a final volume of 175 µl in individual wells of 96-well black microplates with clear bottom (Corning, Amsterdam, Netherlands). After 1 h of incubation at 37°C, fluorescence signal was measured at an excitation of 485 nm and emission wavelength of 530 nm using a Synergy H1 Hybrid Microplate Reader (Biotek, Winooski, VT, USA). Patient samples were run in duplicate. The HOI was determined by calculating the ratio of fluorescence in the presence of HDL patient samples divided by the fluorescence in the absence of HDL. A HOI <1.0 designates antioxidant HDL, whereas a HOI ≥1.0 indicates pro-oxidant HDL (16, 17). This definition is based on functional properties of HDL and indicates whether the respective HDL patient sample attenuates or aggravates experimental oxidative stress. The intra-assay and inter-assay coefficients of variation were 5.9% and 7.8%. To control for inter-assay variation across different plates, sample values were normalised to a pooled control sample run on each plate. Plasma concentrations of MPO were determined in EDTA plasma by commercially available ELISA kits (Immundiagnostik AG, Bensheim, Germany). The intra-assay and inter-assay coefficients of variation for MPO measurements were 2.6% and 4.2%.

Statistical analyses

Discrete data were described by absolute and relative frequencies and compared between groups using Chi-square tests. Continuous data were presented as either means (± standard deviation) or medians (with interquartile ranges) and compared between groups using unpaired t-test or the Mann-Whitney U-tests as appropriate. Pearson correlation was performed to assess the relationship between quantitative variables. Potential risk factors for having pro-oxidant serum HDL (HOI ≥1) were evaluated using a stepwise binary logistic regression model. The regression model included cardiovascular risk factors and blood parameters that were different between patients with antioxidant and pro-oxidant serum HDL. Median follow-up time was estimated using the inverse Kaplan–Meier method. The influence of having pro-oxidant HDL on all-cause mortality was investigated by univariate and multivariable Cox regression models. Adjustments were made for TIMI risk score (15), which includes all classical cardiovascular risk factors, and all variables associated with pro-oxidant serum HDL. Kaplan-Meier analyses were applied to measure the effect of pro-oxidant HDL on mortality and MACE and compared using log-rank test. Thirty-day, one-year and four-year mortality rates were compared between subgroups using a Chi-square test. Two-sided p-values less than 0.05 were used to indicate statistical significance. SPSS 21.0 (SPSS/IBM) was used for all analyses.

Results

Study population

We prospectively enrolled 247 STE-ACS patients. HOI analyses revealed that 44.1% of STE-ACS presented with pro-oxidant serum HDL, whereas 55.9% of patients had a preserved anti-oxidant function of serum HDL. An overview of patient characteristics...
comparing patients with pro-oxidant and antioxidant HDL function is presented in ▶ Table 1. Patients with pro-oxidant HDL had a higher prevalence of diabetes and a history of prior myocardial infarction. Furthermore, differences in estimated glomerular filtration rate (GFR), neutrophil levels and triglycerides were observed. There was no difference in serum HDL cholesterol levels between the two groups. Measurements of MPO plasma levels showed a trend towards higher concentrations in patients with pro-oxidant HDL (429 ± 318 ng/ml) compared to patients with antioxidant HDL (370 ± 194 ng/ml, p=0.087). The Pearson correlation analysis revealed a significant positive relationship between MPO plasma levels and neutrophil counts (r=0.367, p< 0.001). During a median follow-up time of 23 months (interquartile range [IQR] 14.4–40.0 months), 15 % of patients (n=37) died. Causes of death were cardiovascular disease (78 %), malignancy (19 %) and liver failure (3 %).

**Risk factors for having pro-oxidant HDL**

Potential risk factors for having pro-oxidant serum HDL in the setting of STE-ACS were evaluated using a stepwise binary logistic regression model. History of prior AMI, elevated neutrophil counts, increased triglyceride levels and reduced estimated GFR remained independent risk factors for having pro-oxidant HDL (HOI ≥ 1, ▶ Table 2).

**Survival analyses**

We identified a significant effect of having pro-oxidant serum HDL on all-cause mortality with a crude hazard ratio (HR) of 2.88 (95 % confidence interval [CI] 1.46–5.75; p = 0.003). To account for potential confounding effects, we adjusted the risk of all-cause death by TIMI risk score, history of myocardial infarction, GFR,
triglyceride levels and neutrophil counts. The results persisted after multivariate adjustment with an adjusted HR of 3.30 (95% CI 1.50–7.27, p = 0.003) for all-cause mortality. The same results were found when the associations with absolute values of HOI were analysed (HR 1.31, 95% CI 1.06–1.63, p=0.012; adjusted HR 1.27, 95% CI 1.00–1.61, p=0.048) instead of dichotomous variables (pro-oxidant versus antioxidant HDL). Kaplan–Meier analysis revealed a significant increase of all-cause mortality (p = 0.001, log-rank test; ▶ Figure 1A) and MACE (p = 0.002, log-rank test; ▶ Figure 1B) in patients with pro-oxidant HDL. Short-term mortality was increased in patients with pro-oxidant HDL after 30 days (11.9% vs 2.2%, p=0.002) compared to patients with preserved anti-oxidant HDL function. The same trend was observed in patients with pro-oxidant HDL for long-term mortality after 1 year (19.3% vs 5.1%, p<0.001) and 4 years (22.9% vs 8.7%, p=0.002).

**Discussion**

The current study demonstrates that impaired antioxidant function of serum HDL is strongly associated with long-term as well as short-term mortality in STE-ACS patients. This association was even more pronounced after adjustment for potential confounders. In 44.1% of STE-ACS patients, serum HDL was pro-oxidant. This switch of HDL from a protective to a deleterious particle occurred independently of serum HDL cholesterol levels. This finding is in line with other studies showing an impaired anti-apoptotic (14), antithrombotic (13), and antioxidant function (11) of HDL in AMI patients. In contrast to previous studies, the present data reveal for the first time that poor HDL antioxidant capacity is associated with higher mortality and increased rate of MACE in STE-ACS patients. The HDL functional assay (10) has been validated by numerous studies (11, 12, 16). Our study population represents a well selected STE-ACS population that was treated with aspiration thrombectomy during pPCI, based on the

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*variable was scaled to 1 standard deviation.

Table 2: Risk factors associated with an increased odds of having pro-oxidant HDL (HOI≥1) in univariate and multivariable models.

Figure 1: Kaplan-Meier estimates of mortality and major adverse cardiac events (MACE). Kaplan-Meier plots showing mortality A) and MACE B) for STE-ACS patients with pro-oxidant and antioxidant HDL. Indicated p-values were derived from log rank test.
angiographic evidence of a thrombotic occlusion of an epicardial coronary artery. The observed short-term mortality as well as long-term mortality were comparable to the outcome of STE-ACS patients in other clinical thrombectomy trials (18–21).

There exists strong evidence on the adverse effects of oxidative stress during AMI, including direct cytotoxic, negative inotropic, prothrombotic, cytokine stimulating and apoptotic effects (4, 6–8, 13). Neutrophils represent the major source of ROS generation during AMI (3). OxLDL has been shown to be directly involved in platelet activation during thrombosis (4, 7, 8). Therefore, it is tempting to speculate that the preservation or restoration of antioxidant serum HDL function during AMI may be a therapeutic target. This hypothesis is supported by experimental data showing a reduction of myocardial ischaemic injury by the exogenous administration of HDL in animals (22, 23). Interestingly, pharmacological elevation of HDL has failed to show beneficial effects on morbidity and mortality in AMI patients (24). Understanding of the molecular mechanisms underlying the loss of HDL function during AMI should be a stimulus for ongoing research.

HDL particles, composed of lipids and more than 100 different proteins, exert their protective functions by the collaboration of essential apolipoproteins and associated enzymes (25, 26). There is increasing evidence that alterations in HDL protein composition and quality are involved in loss of HDL function (14, 25, 27, 28). Particularly, myeloperoxidase (MPO), a lysosomal protein stored in azurophilic granules of neutrophils, is known to affect HDL function by promoting oxidative damage on HDL associated proteins (25, 28). MPO is largely secreted by activated neutrophils during AMI (29, 30). The crucial role of neutrophils and their impact on clinical outcome in patients with AMI has been well established (31–34). In the present study, we identified increased neutrophil counts as independent predictor for having pro-oxidant HDL. Elevated neutrophil counts explain a pro-inflammatory and pro-oxidant environment that entails dysfunctional serum HDL. Notably, we observed a significant correlation between neutrophil counts and MPO plasma levels. Although the enzymatic activity of MPO does not necessarily correspond to MPO protein and expression levels (35, 36), the hypothesis of MPO as mediator of impaired antioxidant HDL function is plausible. The effect of MPO activity and MPO-specific oxidation products on HDL-associated proteins (25, 37) is to be addressed by future studies. Further independent risk factors for having pro-oxidant HDL could be identified. i) History of prior AMI, considering the pivotal role of inflammation in the development of dysfunctional AMI (26) and the persistent inflammatory state after AMI (38), it is not surprising that patients with prior AMI are more prone to develop dysfunctional HDL. This observation might at least partially explain why survivors of AMI remained at a significantly higher cardiovascular risk compared to the general population (39). ii) Reduced GFR is a finding which is in line with prior studies (40, 41) and might be explained by a higher inflammatory and oxidative stress level in patients with chronic kidney disease (42). iii) Elevated triglyceride levels were a strong risk factor for pro-oxidant HDL, which supports other studies showing that individuals with metabolic syndrome have more dysfunctional HDL (43, 44).

Interestingly, although statins were found to improve antioxidant function of HDL (11), ongoing statin therapy at time of hospital admission seemed to not affect antioxidant capacities of HDL in the present study. One may speculate that the protective effects of statins are obscured because they are more often prescribed in patients with a prior cardiovascular event, representing a high-risk population with generally more reduced antioxidant function. Indeed, sub-analyses confirmed that the percentage of patients with a history of prior AMI, which was identified as a risk factor for impaired antioxidant HDL function in the present study, was significantly higher in patients treated with statins than in statin-naive patients (40% vs 8%, p<0.001). The knowledge that pro-oxidant HDL is an independent predictor of short-term and long-term outcomes is valuable to detect high-risk STE-ACS patients that could be given intensified therapy. However, clinical studies, evaluating the beneficial effects of anti-oxidant therapies in STE-ACS patients have been controversial (39). We propose that the maintenance of HDL functions should become a major therapeutic target in AMI patients. High-risk patients may benefit from an intensified treatment of selected co-morbidities, close check-ups after hospital discharge and guided lifestyle modifications. Particularly, intensified exercise training has shown to significantly improve HDL functions in patients with chronic heart failure (40).

A potential limitation of our study is that the antioxidant assay was performed with apolipoprotein B–depleted patient serum samples instead of isolated HDL. Although polyethylene glycol precipitation is generally considered as a convenient, reproducible, and rapid method to extract HDL from patient serum (11), we cannot exclude that other non-HDL associated proteins exert additional antioxidant effects that may interfere with antioxidant capacities of HDL. However, prior studies revealed a strong correlation of results for study samples collected by different HDL isolation techniques, including ultracentrifugation, fast-performance liquid chromatography, and isolation of HDL using precipitation with dextran sulfate or polyethylene glycol (47, 48). Therefore, the use of apolipoprotein B–depleted serum samples is suitable for describing antioxidant HDL function as long as this method is consistently applied for all samples in a single study (48).

What is known about this topic?

- Oxidative stress is known to be crucially involved in adverse pathophysiological processes occurring after acute myocardial infarction (AMI).
- High-density lipoprotein (HDL) is well known for its antioxidant properties by binding and removing oxidant molecules.
- Protective functions of HDL are attenuated in patients with AMI.

What does this paper add?

- Pro-oxidant HDL represents a strong and independent predictor of long-term as well as short-term all-cause mortality in patients with ST-elevation acute coronary syndrome.
- Elevated neutrophil counts predicted the presence of serum pro-oxidant HDL.
Conclusion
We identified pro-oxidant HDL as a strong and independent predictor of long-term as well as short-term all-cause mortality in STE-ACS patients. Elevated neutrophil counts were an independent predictor for having pro-oxidant HDL.

Conflicts of interest
None declared.

References