Decline in Serum Cholinesterase Activities Predicts 2-Year Major Adverse Cardiac Events

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Parasympathetic activity influences long-term outcome in patients with cardiovascular disease, but the underlying mechanism(s) linking parasympathetic activity and the occurrence of major adverse cardiovascular events (MACEs) are incompletely understood. The aim of this pilot study was to evaluate the association between serum cholinesterase activities as parasympathetic biomarkers and the risk for the occurrence of MACEs. Cholinergic status was determined by measuring the cumulative capacity of serum acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) to hydrolize the AChE substrate acetylthiocholine. Cholinergic status was evaluated in randomly selected patients undergoing cardiac catheterization. The patients were divided into two groups of 100 patients in each group, with or without occurrence of MACEs during a follow-up period of 30 months. Cox regression models adjusted for potential clinical, metabolic and inflammatory confounders served to evaluate associations with clinical outcome. We found that patients with MACE presented lower cholinergic status and AChE values at catheterization (1,127 ± 422 and 359 ± 153 nmol substrate hydrolyzed per minute per milliliter, respectively) than no-MACE patients (1,760 ± 546 and 508 ± 183 nmol substrate hydrolyzed per minute per milliliter, p < 0.001 and p < 0.001, respectively), whose levels were comparable to those of matched healthy controls (1,622 ± 303 and 504 ± 126 nmol substrate hydrolyzed per minute per milliliter, respectively). In a multivariate analysis, patients with AChE or total cholinergic status values below median showed conspicuously elevated risk for MACE (hazard ratio 1.85 (95% confidence interval [CI] 1.09–3.15, p = 0.02) and 2.21 (95% CI 1.22–4.00, p = 0.009)) compared with those above median, even after adjusting for potential confounders. We conclude that parasympathetic dysfunction expressed as reduced serum AChE and AChE activities in patients compared to healthy controls can together reflect impaired parasympathetic activity. This impairment predicts the risk of MACE up to 40 months in such patients. Monitoring these parasympathetic parameters might help in the risk stratification of patients with cardiovascular disease.

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INTRODUCTION

Imbalanced sympathetic-parasympathetic activity has been associated with poor cardiovascular outcome, calling for identifying readily measurable biomarkers of parasympathetic activity for predicting future risks. Supporting this notion, indirect measures of cardiac parasympathetic dysfunction such as elevated resting heart rate, delayed heart rate recovery after exercise and attenuated heart rate increase during exercise have all been shown to be independent predictors for adverse cardiovascular outcome (1-3). Abnormalities in these parameters (4) have been shown in diverse study populations to be associated with sudden cardiac death (1,5) as well as all-cause mortality (2,3,6,7), but clinically validated biomarkers to assess the parasympathetic system are not yet available. Of note, the parasympathetic neurotransmitter, acetylcholine (ACh), is extremely labile and difficult to use for clinical measurements (8). ACh is hydrolyzed in the serum by two homologous enzymes with unique features: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). BChE is the major ACh hydrolyzing enzyme in the circulation (9). Correspondingly, most previous evaluations of ACh hydrolyzing capacity in the serum used butyrylthiocholine (BTCh), a butyrylcholine (BCh) analog as a substrate. A recent study demonstrated a strong inverse relation between serum BTCh hydrolyzing activity (which would negate parasympathetic
potency (8)) and long-term mortality in a cohort of stable coronary artery disease patients (10). However, BCh is not physiologically available in the body and is only hydrolyzed by BChE but not AChE. Moreover, AChE is 20-fold faster than BChE in hydrolyzing ACh, and studies demonstrate a causal link between inflammatory pathways and cholinergic signaling (11,12). Specifically, the so-called cholinergic antiinflammatory pathway inhibits cytokine synthesis and release (13,14), predicting direct associations between cholinesterase activities and inflammation.

On the basis of these considerations, we hypothesized that using the ACh analog acetylthiocholine (ATCh) as a substrate that is hydrolyzed by both enzymes might reflect the cholinergic status and would be physiologically more meaningful and practically advantageous. ATCh can better reflect the full parasympathetic potency in inactivating ACh and might offer insight into the nature of its relevance for cardiovascular diseases.

In the present pilot study, we have taken this approach to evaluate the association between parasympathetic system activity and major adverse cardiac events (MACEs).

MATERIALS AND METHODS

Study Design and Patient Selection

The dataset included in this study was collected as part of the Tel Aviv Prospective Angiographic Survey (TAPAS), a prospective, single-center registry that enrolls all patients undergoing cardiac catheterization at the Tel Aviv Medical Center (15–18). The registry includes 4,500 consecutive admitted patients who had no MACEs (a negative outcome group) and group 2 consisted of 100 randomly selected patients who had no MACEs (a positive outcome group) at follow-up of up to 40 months. Eight patients were excluded because of incomplete data, and 192 patients remained for the final analysis. In addition, an age-, sex- and body mass index–matched control group of 264 apparently healthy patients was used.

Control group characteristics were previously described (19). For the control group, the exclusion criteria included a history of cerebrovascular or cardiac events during the previous 12 months; known inflammatory diseases, history of acute febrile disease or infection during the previous 3 months; and known malignancy, pregnancy, steroidal or nonsteroidal treatment during the previous 6 months.

All patients signed a written informed consent for participation in the study, which was approved by the Tel Aviv Medical Center institutional ethics committee (IEC) according to the Declaration of Helsinki (IEC approvals 07-396, 00-116).

Cardiovascular Endpoints and Follow-up

An MACE was defined as follows: all-cause mortality, myocardial infarction (MI) and hemorrhagic or ischemic stroke. Occurrence of the individual components of the composite endpoint was ascertained by medical chart review and by telephone interview at regular intervals up to 40 months after coronary angiography. All patient files were coreviewed by an independent physician for accurate analysis of endpoint definitions. End of follow-up for MACE outcome was defined as the first from the following events: MACE, or January 1, 2012.

Laboratory Tests

All of the participants underwent angiography after a night of fasting, except for patients with ST-elevation MI. Arterial blood was obtained via the arterial access puncture site as part of the coronary angiography procedure. Serum samples were kept frozen at ~80°C until analysis of cholinesterase activities. To compare the effects of AChE, BChE and cholinergic status changes on cardiovascular risk, we used ATCh as a substrate with or without tetraisopropyl pyrophosphoramide (iso-OMPA, a specific BChE inhibitor), thus identifying AChE alone, mimicking the total impact of ACh hydrolysis and enabling a calculation of BChE contribution. This method has been reliably used in several recent studies (14,19–23).

Cholinesterase activities. In brief, total cholinergic status is the total capacity for ACh hydrolysis (that is, the summation of AChE and BChE activities).

AChE activity levels were assessed in the serum samples by using a microtiter plate assay by measuring rates of ATCh (1 mmol/L; Sigma-Aldrich, St. Louis, MO, USA) hydrolysis after 20-min preincubation in the dark with 500 μmol/L iso-OMPA (Sigma-Aldrich), a specific BChE inhibitor. Enzyme activities were calculated using the 405 absorbance value for 5-thio-2-nitrobenzoate produced from dithionitrobenzoate reaction with the hydrolysis product, thiocholine (13,600 [mol·L]/cm). The nonenzymatic breakdown of substrate was subtracted from the total rate of hydrolysis.

Cholinergic status. Cholinergic status was determined by measuring the total serum rates of ATCh hydrolysis without cholinesterase inhibition (14,19,21). To eliminate any assay-by-assay variations, we reanalyzed 10 arbitrary control samples in different plates and at different measurement times.

Calculated BChE. Calculated BChE activity values were determined by subtracting AChE values from the measured cholinergic status values.

Cholinesterase activity assays were performed in triplicate by a single blinded technician for each cohort. In an attempt to minimize intratest variations, exclude day-of-measurement effects and ascertain the authenticity of the observed differences, we used the same reagents throughout the series and remeasured selected samples each test day. Freezing and defrosting did not affect the enzyme activities.
Enzymatic stability and reproducibility criteria for our measurements were based on the use of highly purified human recombinant cholinesterases as reference enzymes (24, 25).

Crystallographic Structure

The crystallographic structure PDB file of recombinant human acetylcholinesterase in complex with donepezil (PDB ID: 4EY7 [26]) and the X-ray structure PDB file of human butyrylcholinesterase (PDB ID: 2XQG [27]) were obtained from the RCSB Protein Data Bank (www.pdb.org) (28). One of the monomers in the crystallographic structure of human BChE and all water and ligand molecules besides donepezil were extracted from the PDB files using the PyMOL Molecular Graphics System, Version 1.3 (Schrödinger LLC, New York, NY, USA). Then the two crystallographic structures were aligned and the width of the active gorge site was measured using PyMOL.

Statistical Analysis

Categorical variables were compared by using a χ² test and continuous variables by using a t test (presented as means with standard deviations [SDs]) or by Kruskal Wallis/Mann-Whitney test (medians with interquartile range [IQR]). Continuous variables were tested for normal distribution by using a Kolmogorov-Smirnov test and Q-Q plots.

We analyzed the distributions of the different parasympathetic variables by using histograms. To evaluate the significance of the differences of the two principal components of the parasympathetic system for MACE, we redivided our combined cohort including patients with and without MACEs into two new groups by AChE medians and examined the clinical and laboratory characteristics (Supplementary Table S1). Next, we examined the effect of both AChE/cholinergic status medians on the risk of the composite myocardial infarction, stroke and all-cause mortality (MACE) endpoint. MACE was evaluated using univariate and multivariate Cox proportional hazard regression. Survival curves divided by AChE medians and MACE are presented. We adjusted our model for age, sex, conventional risk factors (diabetes mellitus, hypertension, dyslipidemia, peripheral vascular disease, known ischemic heart disease, prior stroke and acute coronary syndrome [ACS] status) by using highly sensitive troponin (29) and laboratory variables (estimated glomerular filtration rate, hemoglobin A1c, triglyceride, total cholesterol, low- and high-density lipoprotein, high-sensitivity C-reactive protein (hs-CRP) and white blood cell counts). The influence of adding each block to the model was checked. A two-tailed p < 0.05 was considered statistically significant. All analyses were performed with the SPSS 19.0 software (SPSS, Chicago, IL, USA).

All supplementary materials are available online at www.molmed.org.

RESULTS

A total of 192 patients referred for coronary angiography at the Tel Aviv Medical Center were included in the final analysis. A total of 98 patients suffered a MACE during a mean follow-up period of 369 ± 250 d (median 411, range 2–1,199). The mean age was 67.9 ± 12.2 years (range 32–95); 67% of the patients were males. Approximately two-thirds of the cohort underwent angiography because of acute coronary syndrome and the other one-third because of stable angina. Coronary angiography revealed normal or nonobstructive coronary artery disease in 18% of the patients, and one-, two- or three-vessel coronary artery disease was found in 21%, 25% and 36% of the patients, respectively (Supplementary Table S1). During follow up, 39 (44%) patients died. Seventeen of those deaths were due to cardiac causes.

Serum AChE, BChE and Cholinergic Status Values Show Inverse Associations with MACE

To enable in-depth interrogation of the cholinergic parameters, we analyzed the distributions of measured AChE and BChE as well as cholinergic status in MACE patients. Histograms of enzymatic levels of AChE (red), BChE (yellow) and cholinergic status (green) are presented for MACE, no-MACE and age-, sex- and body mass index–matched controls. The MACE histograms are left-shifted, reflecting reduced enzymatic activities in all three measurements, whereas no-MACE patients present similar distribution to that of matched controls. Hydrolyzed/min × mL, hydrolyzed per minute per milliliter.

Figure 1. Decreased serum levels of AChE, BChE and cholinergic status in MACE patients. Histograms of enzymatic levels of AChE (red), BChE (yellow) and cholinergic status (green) are presented for MACE, no-MACE and age-, sex- and body mass index–matched controls. The MACE histograms are left-shifted, reflecting reduced enzymatic activities in all three measurements, whereas no-MACE patients present similar distribution to that of matched controls. Hydrolyzed/min × mL, hydrolyzed per minute per milliliter.
cholinergic status values and of calculated BChE in patients with and without MACEs compared with matched controls (19) without cardiac disease. These distributions clearly demonstrated drastic decline in AChE, BChE and cholinergic status values in patients with MACEs compared with those without MACE or in healthy controls (Figure 1). The mean normalized serum AChE activity (359 ± 153 nmol substrate hydrolyzed per minute per milliliter) was lower in MACE patients than the measured levels in patients without MACE (508 ± 183; p < 0.001), who presented indistinguishable activities from those of apparently healthy individuals (1,118 ± 217 and 1,622 ± 303).

Cardiac patients presented with a wider distribution pattern of serum AChE, BChE and cholinergic status values, reflecting larger individual variability in these values compared with matched controls. Using individual enzyme measurements demonstrated that the parasympathetic changes in MACE patients reflected a mutual decline in serum AChE and BChE activities that together led to reduced cholinergic status.

To evaluate the significance of the differences in these two principal components of the parasympathetic system for MACE, we redivided our combined cohort including patients with and without MACE into two new groups that differ by their serum AChE levels (Supplementary Table S1). The two groups, above and below median AChE activities, were similar in risk factors and clinical presentation. Nevertheless, the lower AChE group suffered from 68 MACEs (70%) compared with patients in the higher AChE group, who experienced 30 events (31.6%) (p < 0.001). The crude hazard ratio for MACE was 1.68 (95% confidence interval [CI] 1.06–2.65, p = 0.026) in patients within the lower median of AChE compared with patients in the higher median AChE. To compare AChE to cholinergic status as a predictive biomarker, we further reclassified the combined cohort into patients with cholinergic status values above or below median. The crude hazard ratio for MACE was 1.83 (95% CI 1.13–2.99, p = 0.015) for the patients within the lower cholinergic status median compared with the patients in the higher median. Figure 2 presents the hazard ratio (HR) of the two tested biomarkers in relation to MACE.

Serum Cholinesterase Values Predict Multivariate MACEs

In the multivariate regression, the addition of AChE to the model was significant (p = 0.02). After controlling for all the risk factors detailed in Materials and Methods, the lower AChE median remained significantly associated with adverse outcome, with HR = 2.21 (95% CI 1.22–4.00, p = 0.009). The addition of the cholinergic status parameter was also highly significant (p = 0.008). After controlling for all the above-mentioned risk factors, the lower cholinergic status median was still highly significantly associated with adverse outcome: HR = 2.21 (95% CI 1.22–4.00, p = 0.009). Figure 3 demonstrates the survival curves by medians for AChE and cholinergic status.

In patients with troponin-positive acute coronary syndrome, cholinergic status significantly predicted MACE: HR = 2.0 (95% CI 1.38–3.89, p = 0.05). AChE showed a trend toward significance: HR = 1.88 (95% CI 0.99–3.6, p = 0.054). The troponin-negative ACS group was too small for conducting this analysis.

![Figure 2](image_url) **Figure 2.** HR for MACE. Means and CIs for MACE of the cardiac catheterization group are presented by using univariate and multivariate Cox proportional hazard regression for lower medians of AChE or total cholinergic status. Adj, adjusted model for conventional risk factors (see Materials and Methods).

![Figure 3](image_url) **Figure 3.** Lower AChE and total cholinergic status medians predict worse outcome. Cumulative (Cum) survival curves for cardiac catheterization patients in the higher and lower median groups of AChE or total cholinergic status activity values are shown.
ACETYLCOLINE AND MACE

Cholinergic Status Correlates with Inflammation and Anemia

Higher cholinergic status and AChE values were associated with higher hemoglobin levels and lower hs-CRP values, suggesting a protective power for cholinesterase increases against anemia and inflammation (Figure 4). Cholinergic status values were directly and significantly correlated with hemoglobin levels \( (r = 0.35, p < 0.001) \) and inversely with hs-CRP values \( (r = -0.27, p < 0.001). \) Similarly, AChE measures were directly and significantly correlated with hemoglobin levels \( (r = 0.35, p < 0.001) \) and inversely with hs-CRP values \( (r = -0.19, p = 0.008). \)

The above results raised the question of what fraction of the cholinergic status value is contributed by serum AChE. A representative kinetic chart of cholinesterase activity during reaction time in serum samples is presented in Figure 5A. The apparent linearity and minimal deviation in ATCh hydrolysis rates (reflected in the slopes and SDs presented for these curves) adds significance to the measured values of both enzyme activities. This analysis predictably demonstrated a considerably faster rate of ATCh hydrolysis in the cholinergic status measurement (no inhibition), which reflects the cumulative activity of both BChE and AChE. Although the AChE protein amounts to a small part of the total cholinesterase content in the serum \( (9), \) net AChE activity was about 40% of the total cholinergic status value, due to its rapid hydrolysis rate of ATCh, as can been seen by the slope differences (Figure 5A). The BChE and AChE proteins share 50% of their sequence and resemble each other in their structure (Figures 5B, C). However, the active site space is considerably wider in BChE compared with AChE \( (30) \) (Figures 5D, E), explaining the differences observed in their substrate specificity.

DISCUSSION

By performing individual serum cholinesterase activity measurements to...
yield distinct AChE, BChE and cholinergic status values, we discovered cumulative changes in the AChE and BChE values, which shed new light on our understanding of parasympathetic contributions to the prediction of MACEs in coronary patients. To the best of our knowledge, this study is the first to demonstrate the distinct and additive value of these biomarkers in coronary artery disease patients.

The main distinction and advantage of our study is the use of ATCh as a substrate, with and without the inclusion of a selective BChE inhibitor, compared with previous studies that used the butyrylthiocholine analog (10,31) and viewed BChE alone, neglecting the contribution of AChE and without ascertaining specificity through the use of a selective inhibitor. BChE is largely produced in the liver, but AChE is released, among other sources from peripheral leukocytes (32). Consequently, our measurements provide a closer view of the physiology of the cholinergic balance in the circulation and compare the different components of the total capacity for ACh hydrolysis in the circulation. Figure 6 demonstrates this principle graphically.

We identified a positive correlation between the potential for ACh hydrolysis and decreased inflammation, compatible with the notion that ACh blocks inflammatory pathways (33). These findings are compatible with previous reports of ACh antinflammatory properties that inhibit innate immune responses. This mechanism depends on the α7 nicotinic ACh receptor, which inhibits nuclear factor κB nuclear translocation and suppresses cytokine release by monocytes and macrophages as well. Hence, parasympathetic vagus activation initiates as an antinflammatory reflexlike process (34). Activation of this “cholinergic reflex” has been shown to alleviate inflammatory disease, including endotoxemia and septic peritonitis (34). However, hyperactivation of this suppressive effect may also increase the susceptibility to subsequent infection (35,36).

Our study demonstrated that patients with lower serum cholinesterase activities had elevated inflammation (hs-CRP) and more anemia, suggesting additional possible causes for their adverse events. Other studies have also shown an association between parasympathetic activity and inflammation (37), providing a possible mechanistic explanation for our study’s results. However, treatments used in cardiac patients, such as β blockers, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers, have been shown to augment parasympathetic activity and reduce inflammation as part of their beneficial mechanisms (38–40). Furthermore, reduction in both cholinergic status and AChE levels was found to be significantly associated with adverse outcome also after adjusting for the differences in hemoglobin and hs-CRP, suggesting that increased inflammation or reduced hemoglobin levels alone could not fully account for the observed effects.

Our study thus strengthens the recently published article by Goliasch et al. (10) while further offering a refinement of the test, differentiating between parasympathetic and inflammatory risks and providing a biological explanation and an improved procedure for using serum cholinesterase measurements as significant MACE predictors for coronary patients. This method involves the use of an additional substrate to the one used routinely in clinical settings, but is otherwise straightforward and readily amenable for use in the clinic.

Indirect measures of cardiac parasympathetic dysfunction obtained during exercise testing have been shown to be independent predictors for adverse cardiovascular outcome (1–3). The prognostic value of the currently studied biomarkers was shown to predict the 12-month outcome in stroke patients (19). Ischemic stroke and MI share similar
risk factors, and both stroke and MI patients share decreases in serum AChE activity that may avoid excessive neuroinflammation. Moreover, most stroke patients show increases in serum BChE that aid to balance their cholinergic status values, whereas poststroke patients with drastic AChE decline are at added risk of adverse outcome. In comparison, cardiac patients present drastically reduced AChE, BChE and cholinergic status measures, the cumulative power of which predicts subsequent MACE risks. In past studies, we demonstrated the close correlation between our biomarkers and parallel physiological variables such as resting and recovery heart rate after stress testing (21,22,36).

Future studies will be required to fully assess the predictive value of each of these parameters alone, compared with both of them together. Other regulatory mechanisms changing cholinesterase activities may also contribute to this decline, such as the AChE mRNA-targeted microRNA-132 (41), which can block AChE gene expression and is upregulated under stress (42) and in intestinal bowel disease (43), yet is drastically reduced in the Alzheimer’s disease brain (44). Also relevant are the stress-induced alternative splicing changes (45) and epigenetic potentiation of AChE gene expression, which may explain the long-lasting nature of such reactions (46).

Limitations

This study has several limitations: first, the healthy controls included in the present analysis were taken from a previous set of samples. However, over the past several years, we performed extensive AChE and cholinergic status tests on parallel control groups of patients with similar characteristics (19,21). Thus, our data were validated in a large number of volunteers, all of whom showed activities in the same range. Also, the current cohort was relatively small, but randomly selected patients with and without MACE showed significant differences. Additionally, we did not measure other variables associated with parasympathetic activity such as heart rate variability or heart rate values. However, we demonstrated the close correlation between our biomarkers and parallel physiological variables in past studies (21,22). And lastly, Figure 6 represents the current knowledge about the two cholinesterases (AChE and BChE) and their contribution to the cholinergic status and its effect on the heart and inflammatory profile of MACE patients. Future study will be needed to obtain a more detailed perspective of the scheme shown in Figure 6.

CONCLUSION

In our pilot study, parasympathetic dysfunction expressed as decreased cumulative capacity of serum AChE and BChE to hydrolyze AThc predicts long-term MACEs even after adjusting for potential clinical, metabolic and inflammatory confounders. Future studies are needed to evaluate these parasympathetic parameters and their risk stratification capabilities of patients with cardiovascular disease.

DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES


