Oral N-acetylcysteine reduces plasma homocysteine concentrations regardless of lipid or smoking status\textsuperscript{1,2}

Wulf Hildebrandt,\textsuperscript{5,6,10} Roland Sauer,\textsuperscript{5,7,10*} Gabriel Bonaterra,\textsuperscript{4,8} Klaus A Dugi,\textsuperscript{4,9} Lutz Edler,\textsuperscript{6} and Ralf Kinscherf\textsuperscript{5,8}

Departments of\textsuperscript{3}Anatomy and Cell Biology III and \textsuperscript{4}Internal Medicine I, University of Heidelberg, Heidelberg, Germany; Departments of\textsuperscript{5}Imunochemistry and \textsuperscript{6}Biostatistics, Deutsches Krebsforschungszentrum, Heidelberg, Germany; and \textsuperscript{7}Department of Neurology, University Hospital Erlangen, Erlangen, Germany

\textbf{ABSTRACT}

Background: Elevated total plasma homocysteine (tHcy) is considered to be an independent cardiovascular disease risk factor, although tHcy lowering by B-vitamins improves only certain clinical endpoints. N-acetylcysteine (NAC), a thiol-containing antioxidant, acutely lowers tHcy and possibly also blood pressure. However, to our knowledge, at present no conclusive long-term evaluation exists that controls for factors such as hyperlipidemia, smoking, medication, and disease stage, all of which affect the thiol redox state, including tHcy.

Objective: We reanalyzed 2 double-blind, placebo-controlled trials in unmedicated middle-aged men, one in a hyperlipidemic group (HYL group; n = 40) and one in a normolipidemic group (NOL group; n = 42), each stratified for smokers and nonsmokers.

Design: We evaluated the effect of 4 wk of oral NAC (1.8 g/d) on tHcy (primary endpoint), plasma thiol (cysteine), and intracellular glutathione concentrations as well as on blood pressure. The HYL group had total cholesterol >220 mg/dL or triglycerides >150 mg/dL.

Results: NAC treatment significantly (P = 0.001, multivariate analysis of variance for repeated measures) lowered postabsorptive plasma concentrations of tHcy by −11.7% ± 3.0% (placebo: 4.1% ± 3.6%) while increasing those of cysteine by 28.1% ± 5.7% (placebo: 4.0% ± 3.4%) with no significant impact of hyperlipidemia or smoking. Moreover, NAC significantly decreased systolic (P = 0.003) and diastolic (P = 0.017) blood pressure within all subjects with a significant reduction in diastolic pressure in the HYL group (P = 0.008) but not in the NOL group. An explorative stepwise multiple regression analysis identified 1) post-treatment cysteine as well as 2) pretreatment tHcy and 3) albumin plasma concentrations as being significant contributors to tHcy reduction.

Conclusions: Four weeks of oral NAC treatment significantly decreased plasma tHcy concentrations, irrespective of lipid or smoking status, and lowered systolic blood pressure in both normolipidemic and hyperlipidemic men, with significant diastolic blood pressure reductions in the HYL group only. Increased oral intake of cysteine may therefore be considered for primary or secondary prevention of vascular events with regard to the 2 independent risk factors of hyperhomocysteinemia and arterial hypertension. \textit{Am J Clin Nutr} doi: 10.3945/ajcn.114.101964.

\textbf{Keywords:} homocysteine, N-acetylcysteine, atherosclerosis, oxidative stress, blood pressure

\textsuperscript{1}Supported by the German Cancer Research Center (DFKZ), Heidelberg, Germany.

\textsuperscript{2}Supplemental Table 1 is available from the “Supplemental data” link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

\textsuperscript{3}Present address: Institute of Anatomy and Cell Biology, University of Marburg, Marburg, Germany.

\textsuperscript{4}Present address: Boehringer Ingelheim Pharma GmbH, Biberach, Germany.

\textsuperscript{5}These authors contributed equally to this work.

*To whom correspondence should be addressed. E-mail: roland.sauer@uk-erlangen.de.

\textsuperscript{11}Abbreviations used: GSH, glutathione; GSSG, oxidized glutathione; HYL group, hyperlipidemic group; MANOVA, multivariate ANOVA; MI, myocardial infarction; NAC, N-acetylcysteine; NOL group, normolipidemic group; oxLDL, oxidized LDL; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; tHcy, total plasma homocysteine.

Received October 26, 2014. Accepted for publication August 27, 2015. doi: 10.3945/ajcn.114.101964.
in which B-vitamins are ineffective [e.g., in renal disease (25)] or if a reduction in tHcy of >25% is intended.

N-acetylcysteine (NAC), a common, mucolytic, thiol-containing drug, rapidly increases plasma concentrations of cysteine, i.e., the predominant plasmatic thiol-antioxidant that is a precursor of glutathione (GSH) and thus determines the intracellular redox state (26–29). Because tHcy is a thiol-containing compound as well, there is an ongoing exchange between its plasmatic free fraction (~20–25%, including disulfides with tHcy itself and cysteine) and its protein (albumin)-bound fraction (~75–80%), which depends on the competing cysteine-cystine redox couple (32). In fact, acute NAC application reduces tHcy by replacing homocysteine in its disulfide-binding to albumin, thus enhancing its free plasma fraction and therefore its renal clearance (27–29). In patients with end-stage renal disease, the rapid tHcy lowering through intravenous NAC was associated with ameliorated endothelial dysfunction (12, 25). Several months of oral NAC intake improved cardiovascular endpoints, with unknown effects on tHcy (30). The actual potential of NAC intake has remained unclear because reports on rather large and dose-dependent tHcy reductions of 45% (31, 32) contrast with negative studies (33, 34). More conclusive studies are warranted to overcome methodologic shortcomings that originate from small-sized and noncontrolled designs or when confounding factors such as medication, smoking, arterial hypertension, hyperlipidemia, or exercise are not controlled for (7). Moreover, stratification is urgently needed for conditions that reportedly alter the thiol/disulfide state, such as smoking and hyperlipidemia (35–37). Therefore, we reanalyzed 2 previous randomized, double-blind, placebo-controlled trials (35) for the effect of oral NAC on tHcy with effective control of the above-mentioned confounders. Two groups with different intra-/extracellular thiol redox states (all middle-aged healthy, normotensive, unmedicated men) were enrolled, with either normolipidemia (n = 42) or hyperlipidemia (n = 40), and stratified for age-matched nonsmokers and smokers.

METHODS

Subjects and study design

Two previous randomized placebo-controlled trials (35; in that study denoted as study 2) on the effect of 4 wk of oral NAC intake on insulin sensitivity (primary endpoint) were reanalyzed for effects on tHcy plasma concentrations (primary endpoint) and plasma thiol (cysteine), cystine, and intracellular GSH concentrations as well as on blood pressure (secondary endpoints) within a group with hyperlipidemia (HYL group; trial A) and a group with normolipidemia (NOL group; trial B), both with balanced stratification for nonsmokers and smokers as shown in Figure 1.

A total of 155 outpatients (out of 1250 screened for the exclusion criteria given below) with hyperlipidemia at the Department of Endocrinology and Metabolism of the University Clinic of Heidelberg were found to be eligible for trial A and were contacted (Figure 1A). Forty-seven patients with hyperlipidemia, defined as total cholesterol >220 mg/dL or triglycerides >150 mg/dL, were included in trial A and randomly allocated to the NAC or placebo treatment arm and further stratified as nonsmokers (n = 26) and smokers (n = 21). Of these, 21 nonsmokers and 19 smokers were analyzed. In total, 110 healthy subjects were recruited to trial B by public announcement and screened for lipid profile (i.e., according to the inclusion and exclusion criteria of the NOL group given below) (Figure 1B). Fifty-two subjects with confirmed normolipidemia were randomly

FIGURE 1 Trial profiles for trial A (A) in hyperlipidemic outpatients and trial B (B) in normolipidemic subjects. Both groups were stratified for smokers and nonsmokers. For the present retrospective analysis, subjects were excluded (in addition to those excluded from the previously published study (35; the 2 present trials are denoted there as study 2) if 1) ≥1 main variables such as pre- or post-treatment homocysteine or thiol plasma concentration or blood pressure values were missing; 2) systolic or diastolic blood pressure exceeded 160 or 100 mm Hg, respectively, at any pre- or post-treatment occasion besides screening; or 3) any additional drug or vitamin intake was reported.
assigned to the NAC or placebo treatment arms and further stratified as smokers \((n = 27)\) and nonsmokers \((n = 25)\), of whom 21 each were included in the final analysis. Smoking in the HYL and NOL groups was defined as inhalative smoking of \(\geq 15\) cigarettes/d \((>0.5\) mg nicotine and \(>6\) mg tar/cigarette) for \(\geq 8\) y. In nonsmokers any present and former cigarette consumption or regular passive smoking was excluded by a detailed initial interview.

Informed oral and written consent was obtained from all subjects before inclusion into the study, which was approved by the Ethical Committee of the University of Heidelberg (L-264/2001) and performed according to the Declaration of Helsinki (1996) and to Good Clinical and Laboratory Practice. Note that the trials conducted between 2001 and 2002 did not need to be registered in one of the currently available International Committee of Medical Journal Editors–approved public trial registries (i.e., www.clinicaltrials.gov, www.actr.org.au, www.isrctn.org, www.umin.ac.jp, www.trialregister.nl). However, the randomization and blinding code was created and kept by the Department of Biostatistics of the German Cancer Research Center (head: Lutz Edler), complying with Good Clinical Practice. All subjects underwent baseline and follow-up medical check-ups after 4 wk of trial medication intake, including medical history with assessment of physical activity (h/wk, at baseline only), a physical examination, routine laboratory venous blood variables, arterial blood pressure measurement, pulmonary function analysis, and a 12-lead electrocardiogram at rest. Pre- and post-treatment venous blood variables, blood pressure, and body composition were measured between 0800 and 1000 h after a \(\geq 12\)-h overnight fast including abstinence from trial medication (post-treatment), nicotine in smokers, and 24-h avoidance of strenuous exercise. Bilateral brachial blood pressure was measured in duplicate after 15 min in a semi-reclined position by means of an automated oscillometric upper arm device (Omron HEM 705 CP), which has been validated (38). Trial medication was handed out in weekly doses and the protocol-conform intake was confirmed by personal telephone call at least 3 times within the first trial week.

The main exclusion criteria in our study were as follows: any known or suspected intolerance to NAC; any history of migraine, chronic headache, or tinnitus; arterial hypertension at initial screening (systolic blood pressure \(>160\) mm Hg, diastolic blood pressure \(>100\) mm Hg); hypotension (systolic blood pressure \(<90\) mm Hg); any history or symptoms of cardiovascular disease or events (MI, coronary artery or peripheral vascular disease, stroke, transient ischemic attack); any other major cardiac, respiratory, intestinal, hepatic, renal, dermatologic, neurologic, or psychiatric disease; any alcohol or drug abuse; insufficient cooperation and missing oral or written consent; and any concomitant medication or ongoing or recent (within the past 3 mo) intake of NAC. Additional exclusion criteria for the present reanalysis (trials A and B) of our previous trials (35) were as follows: 1) missing measurements for main variables \((tHcy, plasma thiol concentration, or blood pressure)\), 2) arterial hypertension (systolic blood pressure \(>160\) mm Hg, diastolic blood pressure \(>100\) mm Hg) at any pre- or post-treatment occasion in addition to screening, and 3) any other antioxidant or vitamin supplementation including vitamins C, B-6, and B-12 or folate. Five and 4 subjects were excluded due to meeting the exclusion criteria in numbers 1 and 2, respectively, in trials A or B, with one of these subjects also meeting the exclusion criteria in number 3 (see Figure 1).

**Trial medication**

For 4 wk, 1.8 g NAC (Fluimucil 200-mg capsules; Zambon) or placebo (lactose, 200-mg capsules) per day were administered at 3 portions of 600 mg/d. To achieve effective blinding, capsules (size 2, white) were kept identical and trial medication boxes were equipped with an extra capsule with the characteristic odor of NAC.

**Measurements and equipment**

**Blood sampling**

Blood samples from an antecubital vein were analyzed in the central laboratory of the Medical University Clinic of Heidelberg for plasma concentrations of albumin; total, VLDL, LDL, and HDL cholesterol; triglycerides; folic acid; insulin (by radioimmunoassay; Schering); and glucose (by the hexokinase method of Beckman-Coulter). The CV for plasma lipids was between 1.98% and 2.93% with regard to 3 different target values including those relevant for hyperlipidemia. \(tHcy\) was determined immediately after sampling by HPLC with fluorometric detection (Abbott Laboratories), with CVs of 6.2% and 4.45% for the presently relevant target values of 8.76 and 21.2 \(\mu\)mol/L, respectively.

The plasma acid-soluble thiol concentration (mainly cysteine or NAC) was measured photometrically (412 nm) without delay after sampling, as previously described (26), by using 5,5-dithiobis-2-nitrobenzoate (Sigma Aldrich). Cystine (cysteine-disulfide) as well as methionine and cystathionine plasma concentrations were determined from the same supernatant by HPLC (Amino Acid Analyzer LC 3000; Eppendorf), with a CV \(<10\%\).

After immediate isolation of peripheral blood mononuclear cells by density gradient centrifugation (Histopaque; Sigma) and subsequent freezing at \(-80^\circ\)C, reduced glutathione (GSH), oxidized glutathione (GSSG), and the sum of both after reduction (total GSH) were measured according to Hildebrandt et al. (26) by using the method of Tietze (39), with normalization for intracellular protein content according to Lowry et al. (40).

Commercially available ELISA kits were used to determine concentrations of TNF-\(\alpha\), soluble intercellular and vascular cell adhesion molecules (sICAM and sVCAM, respectively; IBL International), as well as oxidized LDL (oxLDL; Mercodia) in plasma samples in EDTA-coated tubes centrifuged at 1300 \(\times\) \(g\) for 10 min (\(4^\circ\)C) and stored at \(-75^\circ\)C.

**Body composition**

Body composition was analyzed by measurement of electrical impedance and reactance under standardized conditions by using the TVI-10 body composition analyzer (FM Service GmbH), as previously described (35).

**Statistical analysis**

This analysis re-evaluated the data of 2 previously published trials (35; denoted in that study as study 2) for the effect of NAC compared with placebo on \(tHcy\) as the primary endpoint following the design of Hildebrandt et al. (35), given in Figure 1A (trial A) and Figure 1B (trial B), by replacing the previous
primary endpoints [i.e., HOMA-IR index and glucose concentration(s) by tHcy]. Secondary endpoints such as plasma thiols (cysteine), cystine, and systolic and diastolic blood pressures were analyzed accordingly, including the intracellular thiold redox state (GSH, GSSG, and the sum of both after reduction, as well as the GSH:GSSG ratio). The study population was stratified according to hyperlipidemia and normolipidemia and smoking or nonsmoking status. Descriptive statistical analyses report means ± SEMs of I) baseline values in the HYL and NOL groups and their smoking or nonsmoking status (Table 1) and 2) the pre- and posttreatment values of the primary and secondary endpoints separately for the 2 treatment arms (NAC or placebo) in strata defined by the absence or presence of hyperlipidemia and/or smoking (Figures 2–4, Supplemental Table 1). Differences between I) the HYL and NOL groups or 2) the NAC and placebo arms at baseline were tested by using unpaired t test or Mann-Whitney U test when the t test was inadequate.

The 4-wk treatment-related effects on the primary endpoint tHcy were primarily analyzed by using a multivariate ANOVA (MANOVA) for repeated measurements (pre- and post-treatment tHcy values) to test the impact of the factors treatment (NAC or placebo), hyperlipidemia (hyperlipidemia or normolipidemia), and/or smoking (smokers or nonsmokers) [i.e., to detect a relevant time-by-factor (treatment, hyperlipidemia, or smoking) interaction]. This analysis included testing for higher-order interactions such as time × treatment × hyperlipidemia or time × treatment × smoking. The individual pre- to posttreatment changes within the NAC or within the placebo arms of the total population, the HYL and NOL groups, and the smoking and nonsmoking strata were tested by using paired Student’s t test or Wilcoxon’s Signed Rank test when the t test was inadequate. The corresponding (post hoc, unadjusted) P values are shown in Figures 2–4 and Supplemental Table 1 and reported together with the outcome of the overall analysis using MANOVA. All P values and significance concentrations were reported without adjustment for multiplicity as part of a detailed descriptive statistical analysis accompanied by the respective means and SEs in tables or figures. All statistical results are reported as significant when P < 0.05. Secondary endpoints were analyzed accordingly. Pairwise correlations between patients’ characteristics

### TABLE 1

Baseline characteristics of the normo- and hyperlipidemic groups, their nonsmoking and smoking strata, and all nonsmokers and all smokers

<table>
<thead>
<tr>
<th></th>
<th>Normolipemic</th>
<th>Hyperlipidemic</th>
<th>All (normo- or hyperlipidemic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>42</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>33.7 ± 1.3</td>
<td>33.7 ± 1.9</td>
<td>33.7 ± 2.0</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>81.3 ± 2.0</td>
<td>79.6 ± 2.6</td>
<td>82.9 ± 3.2</td>
</tr>
<tr>
<td>Body height, cm</td>
<td>180.8 ± 1.2</td>
<td>180.5 ± 1.8</td>
<td>181.0 ± 1.6</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.8 ± 0.5</td>
<td>24.4 ± 0.6</td>
<td>25.3 ± 0.8</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>20.2 ± 0.9</td>
<td>20.1 ± 1.4</td>
<td>20.2 ± 1.2</td>
</tr>
<tr>
<td>Physical activity, h/wk</td>
<td>2.70 ± 0.40</td>
<td>3.00 ± 0.54</td>
<td>2.39 ± 0.83</td>
</tr>
<tr>
<td>Systolic blood</td>
<td>127.9 ± 1.5</td>
<td>125.9 ± 2.1</td>
<td>130.0 ± 2.2</td>
</tr>
<tr>
<td>Diastolic blood</td>
<td>82.0 ± 1.4</td>
<td>80.3 ± 2.2</td>
<td>83.6 ± 1.6</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>84.1 ± 5.0</td>
<td>71.7 ± 6.5</td>
<td>96.4 ± 6.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>185.4 ± 3.8</td>
<td>178.3 ± 5.6</td>
<td>192.5 ± 4.6</td>
</tr>
<tr>
<td>Total, mg/dL</td>
<td>124.4 ± 4.3</td>
<td>120.3 ± 6.2</td>
<td>128.5 ± 5.9</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>73.1 ± 6.9</td>
<td>58.7 ± 5.9</td>
<td>87.4 ± 11.7</td>
</tr>
<tr>
<td>VLDL, mg/dL</td>
<td>18.3 ± 1.8</td>
<td>13.8 ± 1.2</td>
<td>22.9 ± 3.1</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>43.6 ± 1.3</td>
<td>44.1 ± 2.0</td>
<td>43.1 ± 1.9</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>47.6 ± 0.9</td>
<td>47.0 ± 1.1</td>
<td>48.2 ± 1.3</td>
</tr>
<tr>
<td>tHcy, μmol/L</td>
<td>9.08 ± 0.35</td>
<td>8.75 ± 0.49</td>
<td>9.41 ± 0.51</td>
</tr>
<tr>
<td>Methionine, μmol/L</td>
<td>25.1 ± 0.7</td>
<td>25.7 ± 1.1</td>
<td>24.6 ± 1.1</td>
</tr>
<tr>
<td>Cystathionine, μmol/L</td>
<td>0.74 ± 0.05</td>
<td>0.71 ± 0.06</td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>Folic acid, mg/mL</td>
<td>10.01 ± 0.79</td>
<td>9.45 ± 1.18</td>
<td>10.57 ± 1.07</td>
</tr>
<tr>
<td>Thiol (cysteine), μmol/L</td>
<td>7.57 ± 0.22</td>
<td>7.75 ± 0.33</td>
<td>7.39 ± 0.31</td>
</tr>
<tr>
<td>Cystine, μmol/L</td>
<td>42.9 ± 0.9</td>
<td>41.7 ± 1.3</td>
<td>44.2 ± 1.3</td>
</tr>
<tr>
<td>Total GSH, nmol/mg</td>
<td>21.3 ± 1.5</td>
<td>18.6 ± 1.8</td>
<td>24.0 ± 2.4</td>
</tr>
<tr>
<td>GSH, nmol/mg</td>
<td>18.2 ± 1.5</td>
<td>16.0 ± 2.0</td>
<td>20.5 ± 2.2</td>
</tr>
<tr>
<td>GSSG, nmol/mg</td>
<td>3.12 ± 0.37</td>
<td>2.69 ± 0.40</td>
<td>3.54 ± 0.61</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>12.9 ± 2.5</td>
<td>10.4 ± 1.8</td>
<td>15.4 ± 4.6</td>
</tr>
<tr>
<td>siCAM, ng/mL</td>
<td>468.2 ± 26</td>
<td>397.2 ± 28</td>
<td>538.3 ± 39</td>
</tr>
<tr>
<td>sVCAM, ng/mL</td>
<td>732 ± 33</td>
<td>727 ± 51</td>
<td>737 ± 44</td>
</tr>
<tr>
<td>TNF-α, ng/mL</td>
<td>22.5 ± 1.4</td>
<td>21.4 ± 2.0</td>
<td>23.5 ± 2.1</td>
</tr>
</tbody>
</table>

1Values are means ± SEMs. Smoking was defined as ≥5 cigarettes for ≥8 y (>0.5 mg nicotine, >6 mg tar). **Different from normolipidemic (unpaired Student’s t test); *P < 0.05, **P < 0.01, ***P < 0.001, GSH, glutathione; GSSG, oxidized glutathione; siCAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; tHcy, total plasma homocysteine; total GSH, sum of glutathione and oxidized glutathione after reduction.
are described by using the Pearson correlation coefficient, denoted by \( r \), together with the \( P \) values of the test of the null hypothesis of noncorrelation.

As an exploratory analysis, we additionally searched for possible predictors of absolute pre-to post-treatment \( \text{tHcy} \) changes to be expected in the combined NAC and placebo arms as well as in the NAC arm alone. To this end, we performed a stepwise multiple regression with adjustment for a hypothesis-driven (according to references 27–29) selection of variables as follows: 1) post-treatment plasma thiol (cysteine), 2) pre-treatment \( \text{tHcy} \), 3) pretreatment plasma albumin concentration, 4) age, and 5) plasma concentration of methionine (as a precursor.

**FIGURE 2** Plasma concentrations of acid-soluble thiol (cysteine; A), cystine (B), and (\( \text{tHcy} \)) (C) before (pre) and after (post) 4 wk of placebo (open bars) or NAC (closed bars) treatment for all subjects (left), normo- and hyperlipidemic subjects (middle), and their combined strata of nonsmokers and smokers (right). Values are means ± SEMs. By using MANOVA for repeated measures applied to the total study population, the impact of the factor treatment (NAC vs. placebo) was significant (time by treatment) for thiol (\( P = 0.001 \)) and homocysteine (\( P = 0.001 \)) plasma concentrations, whereas no significant impact was detected for the factors hyperlipidemia (hyperlipidemia vs. normolipidemia) and/or smoking (smokers vs. nonsmokers) or higher-order interaction (see Methods). ****Post- vs. pretreatment by paired Student’s \( t \) test within each treatment arm: *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \). MANOVA, multivariate ANOVA; NAC, N-acetylcysteine; \( \text{tHcy} \), total plasma homocysteine.
of homocysteine, which may be subjected to nutritive variation). The resulting model is reported and shown in Figure 5 of individually measured tHcy changes against the individually predicted tHcy changes based on the regression equation together with the 95% prediction interval for all subjects in the 2 arms. SPSS software (version 14.0) was used for all statistical analyses.

RESULTS

Pretreatment values for NOL and HYL groups and nonsmokers/smokers

Due to inclusion criteria, the HYL group had significantly higher pretreatment plasma concentrations of triglycerides and total, LDL, oxLDL, and VLDL cholesterol, as well as lower HDL cholesterol, but similar albumin concentrations compared with the NOL group (Table 1). Furthermore, the HYL group was characterized by statistically significantly older age and higher body weight, BMI, and systolic and diastolic blood pressure than the NOL group, with increased systolic values (140–160 mm Hg) present in 23 and 6 subjects within the HYL and NOL groups, respectively. Physical activity had a tendency to be, but was not significantly (P = 0.149), lower in HYL compared with NOL groups. Because of the strict exclusion criteria (especially medication), the number of eligible hyperlipidemic outpatients (out of a total of 1250) was too small to allow for matching or comparability with regard to age, BMI, or blood pressure between NOL and HYL groups. However, within each of the separately randomized NOL and HYL groups, we achieved a good comparability of anthropometric and cardiovascular disease risk factors between strata of nonsmokers and smokers, facilitating data analysis of all nonsmokers and all smokers.

Pretreatment postabsorptive tHcy was normal to moderately elevated (5.1–28.7 μmol/L) (43) and not statistically different between the NOL and HYL groups and nonsmokers and smokers (Table 1). The same applied for the homocysteine precursor methionine, folic acid as a determinant of homocysteine remethylation, and cystathionine, an intermediate product of the vitamin B-6–dependent homocysteine trans-sulfuration. As reported in Table 1, postabsorptive plasma concentrations of thiol (cysteine) were significantly lower in HYL compared with NOL groups. In line with the pro-oxidative shift in the plasma compartment, plasma concentrations of cystine (cysteine-disulfide; Table 1) in the HYL group significantly exceeded those in the NOL group. Nevertheless, thiol and cystine plasma concentrations were not found to be different between the total groups.

FIGURE 3  Systolic (A) and diastolic (B) blood pressure before (pre) and after (post) 4 wk of placebo (open bars) and NAC (closed bars) treatment is shown for all subjects (left), normo- and hyperlipidemic subjects (middle), and their combined strata of nonsmokers and smokers (right). Values are means ± SEMs. By using MANOVA for repeated measures applied to the total study population, the impact of the factor treatment (NAC vs. placebo) was significant (time by treatment) for systolic (P = 0.003) and diastolic (P = 0.017) blood pressure, whereas no significant impact was detected for the factors hyperlipidemia (hyperlipidemia vs. normolipidemia) and/or smoking (smokers vs. nonsmokers) or higher-order interaction (see Methods). * *** Post- vs. pretreatment by paired Student’s t test within each treatment arm: * P < 0.05, ** P < 0.01, *** P < 0.001. MANOVA, multivariate ANOVA; NAC, N-acetylcysteine.
of nonsmokers and smokers (Figure 2A, B) or their strata within the NOL and HYL groups. The intracellular thiol redox state (represented by total and GSH as well as GSSG and the GSH:GSSG ratio) did not reflect the extracellular oxidative shift in the HYL compared with the NOL group at baseline. In fact, concerning the glutathione state, there were no significant differences between HYL and NOL groups, except for GSSG, which was significantly lower in the HYL group (Table 1). Pretreatment plasma concentrations of sVCAM or TNF-α were found to be similar between HYL and NOL groups as well as between related strata or the total groups of smokers and nonsmokers, whereas sICAM was significantly higher in the HYL group (Table 1).

A weak but significant negative correlation was found for all subjects between pretreatment thiol plasma concentrations and VLDL (r = −0.30), oxLDL (r < −0.30), triglycerides (r < −0.30), or HDL (r < 0.30), whereas pretreatment Hcy plasma concentrations correlated significantly with the total and the HYL groups’ plasma concentrations of methionine (r = 0.38 and 0.55, P < 0.001), sICAM (r = 0.31 and 0.38, P < 0.05), and sVCAM (r = 0.29 and 0.41, P < 0.01).
FIGURE 5  Multiple regression–based prediction of measured changes in tHcy concentrations (y axis, dependent variable) by 3 identified independent variables: 1) pretreatment tHcy, 2) interventionally reached (post-treatment) thiol (cysteine), and 3) pretreatment albumin plasma concentrations. The x-variable (predicted tHcy changes) were individually calculated by means of the multiple regression equation term (given below the x-axis) and plotted together with the 95% prediction interval and the regression for the total group including the NAC (closed circles; n = 37) and placebo (open circles; n = 45) treatment arms. The correlation coefficients (multiple r) and P values were as follows: total group: r = 0.58, P < 0.001; NAC treatment arm: r = 0.75, P < 0.001; placebo treatment arm: r = 0.33, P < 0.05. NAC, N-acetylcysteine; tHcy, total plasma homocysteine.

Pretreatment values for NAC and placebo treatment arms

No significant differences were detected between the NAC and the placebo arm (within all subjects or any group or strata) with regard to pretreatment (baseline) values of tHcy or any other variable shown, with the exception of pretreatment sVCAM, which was significantly higher in the NAC arm within all subjects.

NAC-related changes

Four weeks of oral NAC compared with placebo intervention led to a significant increase in postabsorptive thiol (cysteine) plasma concentrations (Figure 2A, Supplemental Table 1) in all subjects (P = 0.001 when testing for time-by-treatment interaction; P > 0.3 for the impact of time by hyperlipidemia, time by smoking, or higher-order interactions by MANOVA). According to the paired t test, increase in thiol within the NAC treatment arm was significant in both NOL (P = 0.004) and HYL (P = 0.006) groups and their respective nonsmoking strata (P < 0.05) as well as in all nonsmokers (P = 0.023) and all smokers (P = 0.001) (Figure 2A, Supplemental Table 1). A concomitant increase in cystine plasma concentrations was not significant by MANOVA (P > 0.05 for treatment by time; however, P = 0.005 for the factor time) but reached significance within the NAC treatment arm for all subjects (P = 0.017, paired t test), although significance was not detectable within the NOL or HYL groups (or their nonsmokers and smokers) or within all nonsmokers or smokers (Figure 2B, Supplemental Table 1). No significant changes in plasma thiol or cystine concentrations were observed within the placebo treatment arm by paired t test.

As a main finding (Figure 2C, Supplemental Table 1), 4 wk of oral NAC compared with placebo treatment led to a significant decrease in tHcy plasma concentrations (P = 0.001 for time by treatment; P > 0.35 for time by hyperlipidemia, time by smoking, or higher-order interactions by MANOVA) within the total group. In detail, the paired t test detected significant tHcy reductions within the NAC arm but not within the placebo arm in all subjects (NAC: -11.7% ± 3.0%, P < 0.001; placebo: 4.1% ± 3.6%, P > 0.05) in the NOL group (NAC: -11.5% ± 5.0%, P = 0.009; placebo: -0.5% ± 4.6%, P > 0.05) and the HYL group (NAC: -11.9% ± 3.7%, P = 0.009; placebo: 9.3% ± 5.5%, P > 0.05) as well as in all nonsmokers (NAC: -11.3% ± 4.4%, P = 0.009; placebo: 6.3% ± 4.2%, P > 0.05) and smokers (NAC: -12.1% ± 4.3%, P = 0.009; placebo: 1.8% ± 5.9%, P > 0.05). Notably, these NAC-related tHcy changes occurred in the absence of any significant changes in plasma concentrations of methionine, cystathionine, or folic acid (Supplemental Table 1).

There was a significant NAC-related reduction in systolic blood pressure (Figure 3A, Supplemental Table 1) in all subjects (P = 0.003 for time by treatment; P > 0.40 for time by hyperlipidemia, time by smoking, or higher-order interactions by MANOVA). Thereby, the reduction within the NAC treatment arm for all subjects was found to be highly significant (−5.2 ± 1.5 mm Hg; P < 0.001, paired t test). Although this effect was marginal (although significant) within the NOL group (−3.2 ± 1.5 mm Hg; P = 0.042, paired t test within the NAC treatment arm), with no significant changes within its nonsmoker or smoker strata, the reduction in systolic blood pressure was most pronounced and highly significant in the HYL group (−7.1 ± 2.5 mm Hg; P = 0.001, paired t test within the NAC treatment arm) and its nonsmoking (P = 0.008) and smoking (P = 0.034) strata. Moreover, this decrease was detectable in both all nonsmokers (P = 0.007) and smokers (P = 0.008). Systolic blood pressure changes in the HYL group were moderately but significantly related to thiol plasma concentrations that were reached on intervention (NAC and placebo arm) in the total HYL group (r = −0.35, P < 0.025) as well as to changes in tHcy plasma concentrations (r = 0.33, P < 0.025).

The effect of NAC on diastolic blood pressure (Figure 3B, Supplemental Table 1) showed a marginal but significant reduction within the total group (P = 0.017 for time by treatment; P > 0.45 for time by hyperlipidemia, time by smoking, or higher-order interactions by MANOVA), which was detectable within the NAC treatment arm of all subjects (P = 0.037, paired t test) and within the HYL group (−3.3 ± 1.1 mm Hg, P = 0.008) and its nonsmoking stratum (P = 0.015), but not within the NOL group.

Moreover, there was an intracellular increase in total GSH (i.e., the sum of GSH and GSSG after reduction) and GSH, which failed to be significant with MANOVA (P < 0.084 and P > 0.079 for time by treatment, respectively; P > 0.55 for other interactions), but reached high significance within the NAC treatment arm (P = 0.008 and P = 0.002, respectively; paired t test) in all subjects (Figure 4A, B, Supplemental Table 1).
Together with a significant decrease in GSSG ($P = 0.001$, paired $t$ test), this yielded an improved redox state (GSH:GSSG ratio, $P > 0.001$; Figure 4C, D; Supplemental Table 1). The increase in GSH and its ratio to GSSG was significant in both NOL ($P = 0.039$) and HYL ($P = 0.03$) groups. Although only smokers showed a significant increase in total GSH ($P = 0.027$) and GSH ($P = 0.012$) together with a decrease in GSSG ($P = 0.014$), a significantly improved redox state was detectable in all non-smokers ($P = 0.049$) and all smokers ($P = 0.004$) (Figure 4A–D). According to MANOVA, there was a significant interaction of smoking by time ($P = 0.046$) on GSSG. The placebo treatment led to no significant changes in any of these variables.

None of the factors or their interaction tested by MANOVA in all subjects affected changes in sVCAM; sICAM; TNF-α; triglycerides; total, LDL, VLDL, HDL, or oxLDL cholesterol; or albumin. However, NAC treatment significantly decreased sICAM in all nonsmokers ($-8.00\%\pm 1.9\%$; $P = 0.002$, paired $t$ test) and sVCAM in the HYL group ($-9.5\%\pm 2.5\%$; $P = 0.001$, paired $t$ test) and its nonsmoking ($P = 0.045$) and smoking ($P = 0.009$) strata. However, notably, pretreatment sVCAM concentrations were significantly different between the NAC and the placebo arm (see Supplemental Table 1). TNF-α remained largely unchanged in all groups or strata. As shown in Supplemental Table 1, there were no significant NAC-related changes in subgroups or strata with regard to postabsorptive plasma lipid variables (i.e., triglycerides; total, LDL, VLDL, HDL, or oxLDL cholesterol; or albumin). However, absolute changes in HDL were positively related to changes in plasma thiols in both HYL and NOL groups ($r = 0.35, P < 0.01$) and the NAC treatment arm ($r = 0.31, P = 0.062$), with this association being stronger in the HYL group ($r = 0.50, P = 0.001$) than in the NOL group ($r = 0.23, P = 0.15$).

**Determinants of NAC-related tHcy changes (explorative multiple regression analysis)**

The NAC-induced absolute tHcy changes were found to be significantly inversely related to 3 factors: 1) the pretreatment concentration of tHcy (all subjects: $r = -0.47, P = 0.001$; NAC treatment arm: $r = -0.66, P < 0.001$; placebo treatment arm: $r = -0.20, P = 0.19$; i.e., the higher the pretreatment tHcy concentration, the larger the decrease in tHcy); 2) pretreatment plasma albumin concentrations (all subjects: $r = 0.25, P < 0.05$; NAC-treatment arm: $r = 0.33, P < 0.025$; placebo treatment arm: $r = 0.30, P < 0.05$); 3) post-treatment, postabsorptive tHcy plasma concentrations (all subjects: $r = -0.26, P < 0.05$; NAC treatment arm: $r = -0.28, P = 0.1$; placebo treatment arm: $r = 0.00, P = 1.0$). According to a stepwise multiple regression analysis (including the above 3 factors, age, and methionine), only the 3 above-mentioned factors independently and significantly contributed to the absolute treatment-related tHcy-lowering effect (Figure 5; showing measured vs. predicted tHcy changes), with multiple $r = 0.58$ ($P < 0.001$), $r = 0.75$ ($P < 0.001$), and $r = 0.33$ ($P < 0.05$) for the total groups, the NAC treatment arm, and the placebo treatment arm, respectively.

**DISCUSSION**

The present reanalysis of 2 randomized, controlled, double-blind trials (35; denoted in that study as study 2 and presently addressed as trials A and B) intended to clarify the potential of oral NAC intake to lower tHcy in groups with variable prevailing postabsorptive plasma thiol concentrations. Our study design considered hyperlipidemia and normolipidemia as conditions with different prevailing plasma (cysteine) concentrations and intracellual (GSH) tHcy concentrations (35, 36) in addition to a possible impact of smoking (37, 41). As a main finding, we demonstrated a significant tHcy-lowering effect (~12%) over 4 wk of 1.8 g oral NAC/d (Supplemental Table 1, Figure 2C), regardless of lipid or smoking status. It is known that similar or lower NAC doses may significantly reduce tHcy concentrations by up to 45% within 2–4 wk (31, 32). However, these studies involved higher mean pretreatment tHcy concentrations (~14 μmol/L), which affects the NAC-related changes in absolute or even percentage terms as actually shown by our present explorative multiple regression analysis. As another important point, the presently studied, strictly postabsorptive conditions are likely to underestimate the tHcy-lowering potential of oral NAC within the first hours after intake, given the previously published (26) time course of plasma thiol (cysteine) increase induced by the capsule preparation of NAC that was also presently used.

The present data do not allow a final conclusion concerning the mechanism behind NAC-induced decreases in tHcy. However, previous studies and in vitro models suggested a thiol-exchange mechanism, whereby homocysteine is replaced in disulfide-binding to albumin, allowing for renal clearance of a thus increased free plasma homocysteine fraction (27–29). Accordingly, Ventura et al. (32) showed a dose-dependent effect of NAC together with an impact of pretreatment tHcy concentrations on resulting tHcy reductions. In line with this, our multiple regression analysis identified the interventionally reached thiol (cysteine) and pretreatment tHcy concentrations in combination with albumin concentrations as predictors of the decrease in tHcy by NAC (Figure 5).

To our knowledge, no evaluation of NAC-related tHcy-lowering exists that controls for various relevant clinical confounders of the intra-/extracellular thiol redox states, such as hyperlipidemia, smoking, medication, or renal function. Thus, it has remained unclear whether the well-documented acute tHcy-lowering effect of NAC (12, 25, 27, 29, 42) is reflected in long-term oral NAC intake (31, 32). There are numerous possible confounders in previous negative studies (33, 34) that might have blurred or biased the outcome, such as small-sized designs, sex, medication use (vitamins, angiotensin-converting enzyme inhibitors), and progressed clinical conditions such as arterial hypertension, renal failure, or cardiovascular events. The present trials A and B enrolled, in contrast, only healthy normotensive to conditionally slightly hypertensive middle-aged men ($n = 82$) without any known previous cardiovascular events or medication use. Furthermore, we monitored established factors of tHcy such as methionine, folic acid, and cystathionine, an indicator for possible limitations of the nonmethylating homocysteine metabolism. In addition, we sampled blood during strictly postabsorptive conditions with regard to nutrition, smoking, and trial medications after 24-h abstinence from strenuous exercise (27, 43, 44).

We presently observed another NAC-related effect relevant to cardiovascular prevention, namely a moderate but significant reduction in systolic blood pressure (Supplemental Table 1, Figure 3). This effect was most pronounced in nonsmokers and hyperlipidemic subjects, the group with prevailing low plasma thiol.
concentrations and possible endothelial dysfunction. NAC may increase nitric oxide availability by scavenging hydrogen peroxide (H₂O₂) and hydroxyl radicals (45). On the other hand, acute NAC-related blood pressure lowering has been reported in animal models of nitric oxide–deficient arterial hypertension (46) as well as in patients with arterial hypertension, type 2 diabetes or renal disease (12, 34). Because these conditions with endothelial dysfunction improved through NAC with or without tHcy-lowering, it is possible that the present NAC-dependent lowering of both tHcy and blood pressure may be coincidental and that health benefits of NAC treatment, as reported in reference 30, might primarily be due to blood pressure lowering. Because we examined only participants once after 4 wk of trial medication intake, without 24-h blood pressure measurement, the real influence of NAC on the course of blood pressure should be investigated more precisely in the future.

Another secondary aim was the influence of NAC on plasma lipid fractions, among which HDL cholesterol has previously been suggested to increase along with NAC intake, at least in healthy subjects (47). However, none of these variables revealed a relevant change in NAC in our present trials, although a significant positive correlation existed between changes in plasma thiol and HDL concentrations in the NAC or even both treatment arms (data not shown).

Furthermore, we studied the response of circulating sICAM, sVCAM, and TNF-α to NAC, because they have been implicated in cardiovascular risks (48) and are induced on activation of nuclear transcription factor κB under pro-oxidative conditions. Nuclear transcription factor κB can be attenuated by NAC, at least in certain clinical conditions (49). Although there was no such effect for TNF-α and sICAM, it was evident for sVCAM within the HYL group (Supplemental Table 1); however, we noted a difference between the NAC and the placebo arm for sVCAM (P = 0.02) at baseline, which may have contributed to that effect, although it was the only unbalanced factor among a large number of comparisons.

By using state-of-the-art analytic methods for assessing thiol redox behavior in vivo in the present trials, it seems that the effect of NAC on intracellular GSH state may not be uniform among the cardiovascular disease risk groups. Although smokers showed a smaller increase in plasma thiol concentrations, they showed a more substantial and significant improvement in their intracellular GSH status compared with nonsmokers (Figures 2 and 4, Supplemental Table 1). Intra- and extracellular compartments (i.e., enhancement of thiol compounds between smokers and nonsmokers) may therefore have to be considered for targeted antioxidant supplementation. This may reflect findings of an increased GSH synthesis in smokers by upregulation of rate-limiting enzymes (γ-glutamyl-cysteine-synthetase), at least in the airways (50), at a lower antioxidative capacity in the plasmatic compartment (37, 42). With regard to intracellular effects, there is indeed a difference in the response of insulin sensitivity to NAC treatment between smokers and nonsmokers, as previously reported for the presently reanalyzed 2 trials (35).

Methodologic limitations of our 2 trials include its retrospective design and limited participant number. The predominant aim of the present reanalysis of 2 placebo-controlled randomized trials was the assessment of significance of the overall effects (by using MANOVA for repeated measures), although the statistical outcomes of effects in subgroups/strata are of a descriptive nature. Thus, their significance should not be overinterpreted but should help to generate new hypotheses. However, an increase in type 1 error cannot be excluded for specific comparisons (when using the paired t test/Wilcoxon’s Signed Rank test). There were no indications for relevant imbalances at baseline between NAC and placebo groups in the combined data set of the total 82 subjects, except for sVCAM as discussed above. In the future, more time points should be chosen for improved monitoring of the time course in NAC effects on plasma thiol and tHcy concentrations, renal homocysteine excretion, and blood pressure.

In summary, 4 wk of oral 1.8 g NAC/d significantly decreases tHcy in hyperlipidemic and normolipidemic smokers and nonsmokers to a clinically relevant extent, which is probably underestimated with regard to our strictly postabsorptive conditions of ≥12 h abstinence from medication. In addition, a significant reduction in systolic blood pressure after NAC intake was found in all subjects, together with a reduction in diastolic blood pressure in the HYL group. Future studies might evaluate whether oral NAC could substantially enhance the ~25% tHcy-lowering effect of folate/B-vitamins alone, which has previously resulted in rather disappointing outcomes for some but not all cardiovascular endpoints (4, 15–18). NAC may offer an alternative option in conditions in which folate therapy is ineffective (12, 25). Upcoming trials on thiol antioxidants should also monitor both intra- and extracellular compartmental changes that might respond differently between clinical conditions.

We acknowledge the expert laboratory assistance of Helge Lips, Ulrike Traut, Silke Vorwald, and Ute Winter; furthermore, we thank Ellen Essen and Gabriella Stauch for preparation of the manuscript. We are deeply indebted to the late Wulf Droge, who mentored and supported this research as the head of the former Department of Immunoochemistry at the German Cancer Research Center.

The authors’ responsibilities were as follows—WH, LE, and RK: designed the research; LE: conducted randomization and blinding; WH, RS, and KAD: conducted the research; GB: provided essential reagents; WH, RS, GB, and LE: analyzed data; WH, RS, LE, and RK: wrote the manuscript; and WH, RS, and RK: had primary responsibility for final content. All authors read and approved the final manuscript. None of the authors had a financial conflict of interest to disclose.

REFERENCES


