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Nitrated apolipoprotein AI/apolipoprotein AI ratio is increased in diabetic patients with coronary artery disease

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Abstract

Aims/Hypothesis—Recent studies have suggested that determination of HDL function may be more informative than its concentration in predicting its protective role in coronary artery disease (CAD). Apolipoprotein AI (apoAI), the major protein of HDL, is nitrated *in vivo* to nitrated apoAI (NT-apoAI) that might cause dysfunction. We hypothesized that NT-apoAI/apoAI ratio might be associated with diabetes mellitus (DM) in CAD patients.

Methods—We measured plasma NT-apoAI and apoAI levels in 777 patients with coronary artery disease (CAD) by ELISA. Further, we measured plasma cholesterol efflux potential in subjects with similar apoAI but different NT-apoAI levels.

Results—We found that median NT-apoAI/apoAI ratio was significantly higher in diabetes mellitus (DM) (n=327) versus non-diabetic patients (n=450). Further analysis indicated that DM, thiobarbituric acid-reactive substances and C-reactive protein levels were independent predictors of higher NT-apoAI/apoAI ratio. There was negative correlation between NT-apoAI/apoAI and use of anti-platelet and lipid lowering drugs. The cholesterol efflux capacity of plasma from 67 individuals with differing NT-apoAI but similar apoAI levels from macrophages *in vitro* was negatively correlated with NT-apoAI/apoAI ratio.

Conclusions—Higher NT-apoAI/apoAI ratio is significantly associated with DM in this relatively large German cohort with CAD and may contribute to associated complications by reducing cholesterol efflux capacity.

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Keywords

apolipoprotein AI; lipoproteins; diabetes; cardiovascular disease

Introduction

Diabetes mellitus (DM), a well known risk factor of atherosclerosis, is responsible for a three to four fold increase in cardiovascular events ¹. It is known that hyperglycemia stimulates the production of advanced glycosylated end products, activates protein kinase C, and enhances the polyol pathway leading to increased superoxide anion formation ^{2, 3}. Superoxide anion interacts with nitric oxide to form a strong oxidant peroxynitrite, which reacts with tyrosines in various proteins to generate nitrotyrosine (NTyr) residues. Therefore, levels of total plasma NTyr provide a composite index of reactive nitrogen species. Another pathway of the NTyr production involves reaction of nitrogen dioxide (NO₂) with tyrosine residues ⁴. ·NO₂ is generated by myeloperoxidase from hydrogen peroxide (H₂O₂). Several small population studies have shown that NTyr levels are increased in DM ^{5–10} and coronary artery disease (CAD) ^{11, 12}.

Of various proteins present in plasma, apolipoprotein AI (apoAI) has been shown to be a preferred substrate for modification by reactive nitrogen species in human artery wall ¹³. Circulating nitrated apoAI (NT-apoAI) levels are 7–70-fold higher in comparison with other nitrated proteins ^{13, 14}. In the apoAI molecule, tyrosine residues 166 and 192 have been shown to be the predominant sites of nitration by myeloperoxidase ^{15–17}. *In vitro* nitration of apoAI impairs lipid binding and ATP-binding cassette family A protein 1 (ABCA1)-dependent cholesterol efflux functions ¹⁶. However, physiologic effects of *in vivo* NT-apoAI are unknown.

In a small case-control study, the circulating NT-apoAI/apoAI ratio was significantly increased in diabetic patients compared with controls ¹⁸. In addition, higher levels of NT-apoAI were found in patients with cardiovascular disease in both the circulation and in atheroma ^{13, 14, 16}. Recently, Vazquez et al. reported a significantly higher level of circulating NT-apoAI in obese women ¹⁹. Although there appears to be a tendency towards higher plasma NT-apoAI levels in diabetes and CAD subjects, the studies were small and used gas chromatography-mass spectrometry methods that are not suitable for larger cohort studies. Previously, we developed a novel ²⁰ enzyme-linked immunosorbent assay (ELISA), which was confirmed by both Western blot analysis ²⁰ and mass spectrometry ¹⁹. This sensitive and specific ELISA method is suitable to quantify human NT-apoAI levels in a large population. In the present study, we hypothesized that NT-apoAI would be increased in diabetic patients with CAD.

Research Design and Methods

Study Population

A total of 777 subjects with CAD admitted to the Department of Medicine III (Angiology and Cardiology) and the Department of Heart and Thoracic Surgery, University Clinic Halle, Germany between January, 2008 and December, 2010 were prospectively studied. As

expected, very few normal subjects were admitted during this period precluding comparison of different parameters between normal and CAD patients. The data presented include a retrospective analysis of measured NT-apoAI and apoAI levels. CAD was diagnosed as the presence of a luminal diameter stenosis $\geq 50\%$ in at least one major coronary artery (left anterior descending, left circumflex or right coronary artery or their major branches) by angiography or a history of myocardial infarction, among which 380 patients had stable angina pectoris and 397 had acute coronary syndrome (ACS). Multi-vessel CAD was defined as a disease stage in which at least two of the major coronary arteries is involved with atherosclerosis of significant severity. Within this cohort, 327 patients had DM and 450 patients had no DM. DM was diagnosed at the time of admission by past clinical history of a fasting blood glucose level > 7.0 mmol/L (> 125 mg/dL) and/or the two-hour value in the 75 g oral glucose tolerance test (OGTT) > 11.1 mmol/L (200 mg/dL) or use of hypoglycemic medications. HbA1c and insulin levels were not determined. At the time of admission, the history of current smoking, hypertension, dyslipidemia, peripheral artery disease (PAD), previous stroke or transient ischemic attack (TIA), New York heart association (NYHA) functional classification, renal failure, and use of anti-platelet drugs and lipid lowering drugs was collected. Hypertension was defined as patients receiving anti-hypertensive treatment or having a previous diagnosis of hypertension (blood pressure $\geq 140/90$ mmHg). The study was approved by the ethics committee of the medical faculty of the Martin Luther-University Halle-Wittenberg.

Laboratory Evaluation

Subjects were instructed to fast for at least eight hours prior to blood sampling. Serum samples were collected and stored at -80°C prior to use. Laboratory tests were performed at the time of hospital admission for triglyceride (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C) and C-reactive protein (CRP) as described before ²¹. For the evaluation of renal function, estimated glomerular filtration rate (eGFR) was calculated by the abbreviated Modification of Diet in Renal Disease (MDRD) ²² Study equation: $186 \times (\text{Creatinine}/88.4)^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$. Plasma glucose levels were measured (Autokit glucose, Wako, #439–90901) in 71% (550 subjects) of the population, among which 249 patients had DM and 308 patients had no DM.

Measurement of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation in human plasma was quantified by measuring the formation of TBARS using a kit. Plasma (10 μL) was mixed with 10 μL SDS solution. Then 125 μL TBARS solution (0.53 g trichloroacetic acid dissolved in 100 ml of sodium hydroxide and 100 ml of sodium dodecyl sulfate) was added to the sample. After incubation at 95°C for 60 min and keeping at 4°C for 15 min, the sample was centrifuged at 3,000 rpm for 15 min. The amount of TBARS produced was measured at 532 nm. Malondialdehyde was used to construct a standard curve in parallel.

Standards used for the quantification of NT-apoAI and apoAI

Purified human HDL (MyBioSource, # MBS173147, total protein 40 mg/ml, standard curve range 1–12 ng/ml) was used as standard for the determination of plasma concentrations of

apoAI. For NT-apoAI standard, purified human apoAI (Biomedical Technologies, # BT-927) was nitrated with sodium nitrite^{23, 24}. Briefly, purified apoAI (~ 1 mg/ml) was adjusted to pH 3.5 and mixed with 200 mM sodium nitrite and then incubated at 37°C for 24 h under low agitation. Samples were dialyzed against phosphate buffer saline (PBS) for 24 h at 4°C, and protein concentration was determined by the Coomassie Reagent (Thermo Scientific, #23236) and bovine serum albumin (BSA) as a standard²⁵. The amounts of nitrated tyrosines in the modified apoAI were determined using a commercially available NTyr ELISA kit based on nitrated albumin (Cell Biolabs, # STA-305) as described by the manufacturer. The nitrated apoAI (0.7 mg/ml protein) contained 2000 nM NT-apoAI giving a concentration of 80 mmol of nitrated tyrosine per mol of apoAI.

ELISA procedure for the measurement of NT-apoAI and apoAI

Serum NT-apoAI was measured by a sandwich ELISA as described before²⁰. Briefly, microtiter plates were coated with capturing monoclonal anti-NTyr antibodies (1 µg/mL, Upstate, # 05–233, clone 1A6) in PBS overnight at 4°C. The non-specific sites were blocked by incubating the wells with 200 µL of phosphate buffered saline containing 0.05% Tween (PBST) and 3% BSA for 1 h at room temperature. After washing three times with PBST, patient samples (1:25,000 dilution in PBST containing 3% BSA) or standard controls (10–100 ng/mL, nitrated human apoAI) were added to each well and incubated for 1 h at 37°C, enabling the binding of all the nitrated proteins to immobilized monoclonal antibodies. The wells were washed three times with PBST and then incubated for 1 h at 37°C with 100 µL of goat anti-human apoAI (1:1,000 dilution in PBST containing 3% BSA; Academy Bio-Medical Company, # 11S-G2) to specifically identify NT-apoAI. After washing three times with PBST, 100 µL of swine anti-goat IgGs conjugated to alkaline phosphatase (1:1,000 dilution in PBST containing 3% BSA; Southern Biotech, # 6300–04) were added to each well and incubated for 1 h at 37°C, followed by the addition of p-nitrophenyl phosphate solution (1 mg/mL; 150 µL/well). After 10–20 min incubation at 37°C, the absorbance was measured at 405 nm and background values obtained from wells incubated without serum or standards were subtracted from the total values. The standard curve was linear between 0–100 ng/mL. Total apoAI levels were also measured in these plasma samples by ELISA (see below) and the NT-apoAI/apoAI ratios are reported as mmol/mol of apoAI.

Total apoAI levels were measured by the ELISA procedure^{26, 27}. This method is similar to that described above for the measurement of NT-apoAI except that the coating antibody used for apoAI was anti-human apoAI monoclonal antibody 4H1 (University of Ottawa Heart Institute, # A13). Serum samples were diluted 1:250,000 for apoAI quantifications in PBST containing 3% BSA. The quantification of apoAI was linear within 1–12 ng/mL.

Cholesterol efflux from macrophage cell line

To study the effect of plasma NT-apoAI levels on the cholesterol efflux capacity of plasma, we selected 67 subjects with a narrow range of apoAI levels (1.4–1.6 mg/dL) and widely variable NT-apoAI levels. Plasma from these subjects was used to measure cholesterol efflux according to the established procedures²⁸. Briefly, J774 macrophages were plated in 24-well plates. After 16 hours, cells were loaded with 0.2 µCi/ml [³H]-cholesterol in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum for 24 hours in

37°C, 5% CO₂. After labeling, cells were washed three times with serum free DMEM containing 0.2% BSA and incubated with 0.2% BSA in DMEM for 1 hour to equilibrate. Cells were then incubated with different human plasma samples (2%, v/v) in serum free DMEM with 0.2% BSA. Radioactivity was measured in both medium and cells after 4 hours using a scintillation counter (Beckman Coulter, LS 6500). The % cholesterol efflux was calculated as follows: radioactivity in medium/(radioactivity in medium + cells)×100.

Statistical Methods

Continuous variables were reported as means ± standard deviation (SD). For variables that did not show normal distribution, median values with their 25–75th percentiles were reported. Normally distributed variables were compared by Student's t-test. The Kolmogorov-Smirnov test was used to test for normality. Comparisons among more than two groups were performed by analysis of variance (ANOVA), followed by least significant difference (LSD) test for multiple comparisons. For skewed distribution variables, the differences among groups were performed by non-parametric Kruskal Wallis test. The Pearson's χ^2 test was used to assess statistical differences in dichotomous and categorical variables. For univariate analyses, Spearman's correlation was used to determine correlation of NT-apoAI/apoAI ratio with various clinical parameters. As NT-apoAI/apoAI ratio and cholesterol efflux in selected 67 subjects were both normally distributed by Kolmogorov-Smirnov test, their relationship was analyzed by Pearson correlation. To further investigate the relationship between NT-apoAI/apoAI ratio and clinical characteristics or laboratory parameters, multivariate linear regression analysis was performed after log₁₀ transformation of skewed distributed continuous variables, using log₁₀ transformed NT-apoAI/apoAI ratio as the dependent variable and gender, smoking, hypertension, dyslipidemia, PAD, previous stroke or TIA, ACS, NYHA functional classification III-IV, renal failure, multi-vessel CAD, current aspirin-use, current statin use, as well as log₁₀ transformed age, BMI, TG, TC, LDL-C, HDL-C, and CRP levels as independent variables. Attempts were also made to predict DM by multivariate logistic regression analysis, using DM as the dependent variable, and age, gender, BMI, smoking, hypertension, dyslipidemia, CRP, TG, TC, LDL-C, HDL-C, TBARS and NT-apoAI/apoAI ratio as independent variables. P values < 0.05 were considered statistically significant. All analyses were done using SPSS version 16 analytical software (SPSS Inc., Chicago, IL).

Results

Clinical characteristics and parameters

Among 777 subjects with CAD, 327 had DM. Plasma apoAI was normally distributed ($p > 0.05$) and was reported as means ± SD. The distributions of age, BMI, glucose, TC, TG, HDL-C, LDL-C, CRP, eGFR, TBARS, and NT-apoAI were skewed ($p < 0.05$). Clinical characteristics and parameters of the two groups and significant differences are shown in Table 1. The DM group was older, had greater BMI, higher incidence of hypertension, PAD, previous stroke or TIA, NYHA functional classification III-IV, multi-vessel CAD, renal failure, higher TG, glucose, CRP, and lower TC, HDL-C, LDL-C, eGFR, and a lower rate of anti-platelet drugs use than the non-DM group.

There was a trend towards lower apoAI in the diabetic group ($p=0.096$). NT-apoAI levels were not significantly different in these two groups ($p=0.452$) but NT-apoAI/apoAI ratio was significantly higher in the DM group ($p=0.016$) (Table 1, Fig 1).

Correlations between NT-apoAI/apoAI and different clinical characteristics or parameters

Correlations between NT-apoAI/apoAI and the various clinical parameters are shown in Table 2. Although significant ($p = 0.016$), the correlations ($r = 0.087$) between NT-apoAI/apoAI and DM, male gender, previous stroke or TIA, TBARS, and use of anti-platelet and lipid lowering drugs in the entire cohort were weak. In non-DM subjects, NT-apoAI/apoAI ratio was significantly associated with male gender, hypertension, use of anti-platelet and lipid lowering drugs and TBARS. The NT-apoAI/apoAI ratio was significantly correlated in DM subjects with PAD and with previous stroke or TIA (Table 2). When analyzed for association with different genders, significant correlations were found between NT-apoAI/apoAI and DM ($r = 0.121$, $p = 0.005$), hypertension, previous stroke or TIA, renal failure, use of anti-platelet drugs, and TBARS in the male subjects (Table 3). Such associations, however, were not seen in the females perhaps due to smaller number. These studies indicated that NT-apoAI/apoAI is correlated with DM in male subjects.

Lower plasma NT-apoAI/apoAI ratio in subjects using aspirin and statins

There was a significant negative correlation between NT-apoAI/apoAI and use of anti-platelet or lipid-lowering medications (Fig 2). Post-hoc analyses showed significant heterogeneity in NT-apoAI/apoAI ratio in subjects taking four specific drugs (Fig 2A), with aspirin use is appearing to have the lowest plasma NT-apoAI/apoAI. Similarly, lipid lowering drug classes showed heterogeneity with regard to plasma NT-apoAI/apoAI (Fig 2B). In particular, subjects taking statins had significantly lower NT-apoAI/apoAI than those not taking lipid-lowering drugs.

TBARS are an independent predictor of higher NT-apoAI/apoAI ratio

TBARS were an independent predictor of higher NT-apoAI/apoAI ratio after adjusting for gender, age, BMI, smoking, hypertension, dyslipidemia, PAD, previous stroke or TIA, ACS, NYHA functional classification III-IV, renal failure, multi-vessel CAD, current aspirin use, current statin use, TG, TC, LDL-C and HDL-C levels (Table 4). However, multivariate logistic regression analysis showed that TBARS were not independently associated with DM (Table 5). These data suggest that TBARS are predictors of NT-apoAI/apoAI but not DM.

DM is an independent predictor of higher NT-apoAI/apoAI ratio

Using multivariate linear regression analysis, DM, TBARS and CRP levels were independently associated with NT-apoAI/apoAI ratio in a positive manner. Subjects with previous stroke or TIA ($B=0.100$, $p=0.051$) or taking aspirin ($B=-0.076$, $p=0.067$) had a trend towards a higher or a lower NT-apoAI/apoAI ratio, respectively (Table 3).

NT-apoAI/apoAI ratio is independently associated with DM

Using multivariate logistic regression analysis, variables that were independently associated with DM were: age, BMI, smoking, hypertension, plasma TG, TC and NTyr-AI/apoAI ratio (Table 4).

Plasma samples containing high NT-apoAI/apoAI ratio show reduced cholesterol efflux from macrophages

To understand physiological consequences of high NT-apoAI/apoAI ratio, we hypothesized that this might affect basal cholesterol efflux capacity of plasma from macrophages based on the report that *in vitro* nitration of apoAI reduces its capacity for cholesterol efflux¹⁶. As apoAI levels affect cholesterol efflux, we wanted to study the effect of NT-apoAI independent of apoAI effects. Therefore, we thought that the analyses of all the 777 samples would not provide information about NT-apoAI independent of apoAI. Hence, we selected plasma samples that had similar apoAI levels (1.4–1.6 mg/dL). There were 67 subjects (non-DM, n=43; DM, n=24) that had apoAI levels in this narrow range. In these subjects NT-apoAI/apoAI ratio ranged from 4.9 to 121.5 mmol/mol and was significantly correlated with cholesterol efflux in a negative manner ($r = -0.704$, $p < 0.0001$) in both non-DM and DM groups (Table 2, Fig 3, Table 6).

Discussion

In the present study, we found that NT-apoAI/apoAI ratio was positively associated with DM and DM was an independent predictor of higher NT-apoAI/apoAI ratio even after adjusting for several clinical characteristics and parameters in this relatively large cohort with CAD. Moreover, this study demonstrated a negative association between NT-apoAI/apoAI ratio and cholesterol efflux from macrophages independent of total apoAI levels. Additionally, we observed a positive correlation between TBARS and NT-apoAI/apoAI.

Few studies have found increased total (free and protein associated) NTyr residues in diabetic subjects^{7, 9, 29}. Significant correlations have also been observed between NTyr immunostaining intensity in microvasculature and fasting blood glucose or hemoglobin A1C³⁰. However, NT-apoAI/apoAI ratio seems to be better correlated with DM than the total NTyr. One small study, which used a semi-quantitative Western blot assay to measure NT-apoAI, showed that NT-apoAI/apoAI ratio, glucose, HbA1c, lipoperoxides and advance glycation end products were significantly higher in DM patients with different complications than healthy controls¹⁸. We also found that, as expected, DM subjects had more prevalent clinical conditions such as hypertension, PAD, previous stroke or TIA, renal failure, severe heart failure, or multi-vessel CAD. Further, DM subjects had significantly higher NT-apoAI/apoAI ratios in their plasma. Although there was a trend towards lower apoAI in the DM group in comparison to non-DM ($p=0.096$), neither NT-apoAI nor apoAI was significantly different in these two groups, which was consistent with the previous report¹⁸. However, multivariate linear regression showed DM was independently associated with NT-apoAI/apoAI ratio in this cohort of patients with CAD.

The reasons for an association between NT-apoAI/apoAI ratio and DM are unknown. However, we tested the possibility that this ratio might indicate HDL dysfunction and that it may contribute to DM and other clinical conditions. Indeed, we observed that plasma from individuals that have higher amounts of NT-apoAI with similar apoAI levels is defective in basal cholesterol efflux. It is known that decreased cholesterol efflux is correlated with the risk of DM ^{31, 32} and CAD ^{33, 34}. In our study of 67 selected CAD subjects with similar apoAI but differing NT-apoAI/apoAI, cholesterol efflux was significantly associated with NT-apoAI/apoAI ratio (Table 2). Comparison between DM and non-DM subjects showed a trend towards a lower cholesterol efflux in DM ($p=0.09$) (Table 1). Further, cholesterol efflux showed similar co-morbidities and laboratory parameters including NT-apoAI/apoAI ratio (Table 6) between non-DM and DM group in these selected subjects. Thus, NT-apoAI/apoAI ratio is associated with lower cholesterol efflux in both non-DM and DM groups and that this ratio may contribute to several co-morbidities seen in CAD subjects.

In this study, we used a subset of 67 individuals with similar levels of apoAI but different levels of NT-apoAI to measure cholesterol efflux. Comparison between DM and non-DM subjects showed a trend towards a lower cholesterol efflux in DM ($p=0.09$; Table 1). Lack of significant association between DM and non-DM subgroups might be due to small size of the two groups. This trend may become significant if all the samples were analyzed for cholesterol efflux measurement.

Besides DM, we also found history of previous stroke or TIA had an associative trend with a higher NT-apoAI/apoAI ratio ($p=0.051$). High levels of NTyr were detected during the ischemia and reperfusion of brain in animal experiments ^{35, 36} and in plasma of cerebrovascular ischemia patients after stenting/angioplasty ³⁷. Although the number of subjects with previous stroke or TIA in our CAD cohort was relatively small ($n=98$), it provided an indication that NT-apoAI/apoAI ratio might be associated with severe systemic atherosclerosis including cerebrovascular and cardiovascular disease. On the contrary, PAD was not an independent predictor of NT-apoAI/apoAI in our study perhaps due to underestimation of PAD in comparison with severe cerebrovascular or cardiovascular disease.

Plasma CRP levels were previously found to be associated with NTyr levels ³⁸. We found that CRP levels were also independent predictors of higher NT-apoAI/apoAI ratio. Elevated CRP was found in patients with DM and those with cardiovascular disease ³⁹, and it carries prognostic value for cardiovascular events in CAD patients with DM ⁴⁰. Further investigations are needed to evaluate whether NT-apoAI/apoAI ratio can be an inflammatory biomarker in cardiovascular disease or inflammation contributes to higher NT-apoAI/apoAI ratio.

TBARS, biomarkers of oxidative stress, have been shown to be higher in pre-diabetic and diabetic patients than in normal subjects ^{41–44}. Since we did not have control subjects we could not evaluate whether TBARS found in our CAD subjects are higher than normal values. However, our study did not show any significant difference in TBARS levels between DM and non-DM subjects. This might be partly explained as our subjects, both DM and non-DM, had CAD, which is associated with elevated levels of TBARS ⁴⁵.

Nevertheless, CRP and TBARS were found to be a predictor of higher NT-apoAI/apoAI ratio. Therefore, inflammatory response and oxidative stress might contribute to increases in NT-apoAI/apoAI ratio.

Another observation made in this study was the negative correlation between NT-apoAI/apoAI and anti-platelet or lipid lowering drug use. To our knowledge, there are no prior reports as to the association between cardiovascular medications and NT-apoAI/apoAI ratio. Our analyses identified that aspirin and statins consuming subjects had significantly lower NT-apoAI/apoAI ratio than those not on any drugs (Fig 2). It is known that long-term administration of aspirin significantly reduces vascular inflammation and atherosclerotic lesions by lowering macrophage-foam cell content^{46–48}. Aspirin, predominantly existing in the form of salicylic acid in plasma, might play an anti-inflammatory role by scavenging free radicals^{49, 50}. Statins, besides their inhibitions on HMG-CoA reductase to lower serum cholesterol levels, have been shown to display anti-thrombotic, anti-inflammatory, and antioxidant activities^{51–53}. Statins have been shown to be related to lower total NTyr^{54, 55}. It is likely that anti-inflammatory and antioxidant effects of aspirin and statins might have contributed to lower NT-apoAI/apoAI ratio in individuals that were taking these drugs. However, neither aspirin (trend, $p=0.067$) nor statin ($p=0.625$) use was an independent predictor of NT-apoAI/apoAI ratio on multivariate analysis. This might be secondary to the high prevalence of subjects taking aspirin ($n=563$) and statins ($n=639$) as well as the use of other cardiovascular medications that might have limited statistical significance in detecting such differences.

This cross-sectional study provides a glimpse about different parameters at a given time point. It does not provide understanding about the progressive and/or causal relationships. A prospective study may overcome this shortcoming. This study was based on patients admitted to the clinic and therefore does not include normal subjects. Instead, this study allows differentiating between DM and non-DM subjects that were admitted to hospital for CAD. A major outcome of this study, therefore, is that it establishes a relationship between NT-apoAI/apoAI levels and DM in a relatively large German cohort with CAD.

In conclusion, in this relatively large German cohort with CAD, we report that (1) increased circulating NT-apoAI/apoAI ratio is associated with DM; (2) DM is an independent predictor of higher NT-apoAI/apoAI ratio and this ratio is independently associated with DM; (3) higher NT-apoAI/apoAI ratio is associated with higher CRP levels and TBARS levels; (4) there was a negative correlation between NT-apoAI/apoAI ratio with the use of anti-platelet and lipid-lowering drugs; and (5) higher NT-apoAI/apoAI ratio from subjects with similar apoAI levels is associated with reduced cholesterol efflux capacity from macrophages in vitro.

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statistical evaluation, discussion and edited the manuscript; AS contributed to collection of samples, critically read the manuscript and provided comments; MMH supervised the studies and edited the manuscript.

Abbreviations

ABCA1	ATP-binding cassette family A protein 1
ACS	acute coronary syndrome
ANOVA	Analysis of Variance
apoAI	apolipoprotein AI
BMI	body mass index
BSA	bovine serum albumin
CAD	coronary artery disease
CRP	C-reactive protein
DM	diabetes mellitus
ELISA	enzyme-linked immunosorbent assay
HDL-C	high density lipoprotein-cholesterol
LDL-C	low density lipoprotein-cholesterol
LSD	least significant difference
NTyr	nitrotyrosine
NT-apoAI	nitrated apoAI
NYHA	New York Heart Association
PAD	peripheral artery disease
PBS	phosphate buffer saline
PBST	phosphate buffered saline containing Tween
TBARS	thiobarbituric acid-reactive substances
TC	total cholesterol
TG	triglyceride
TIA	transient ischemic attack

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Highlights

- There are significant amounts of nitrated apoAI in the plasma of coronary artery disease patients
- NT-apoAI/apoAI ratio is significantly higher in diabetes versus non-diabetic patients
- This ratio is negatively correlated with macrophage cholesterol efflux independent of apoAI

Figure 1. NT-apoAI, apoAI and NT-apoAI/apoAI levels in non-DM (n = 327) and DM (n = 450) subjects

(A) Median NT-apoAI levels were similar in non-DM and DM subjects ($p = 0.452$).

(B) Mean apoAI levels were not significantly different between non-DM and DM subjects ($p = 0.096$).

(C) Median NT-apoAI/apoAI levels were significantly elevated in DM group than non-DM group (* $p = 0.016$).

Figure 2. Comparisons of NT-apoAI/apoAI levels in patients taking different drugs

(A) Anti-platelet drugs: There were 140, 219, 70 and 344 individuals taking no anti-platelet drugs, aspirin, clopidogrel, and aspirin + clopidogrel, respectively. Median NT-apoAI/apoAI levels among the 4 groups were significant different by ANOVA test ($P = 0.001$), indicating significant effects of drugs on plasma NT-apoAI/apoAI levels. By LSD test, there were significant differences between no drugs vs. aspirin (* $p = 0.01$) and aspirin vs. clopidogrel ($\dagger p = 0.007$).

(B) Lipid lowering drugs: Subjects were divided into four groups. There were 70 individuals who did not take any lipid lowering drugs, whereas 639 and 37 individuals were on statins and ezetimibe, respectively. There were 28 individuals on other drugs. Median NT-apoAI/apoAI levels among the 4 groups were significant different by ANOVA test ($p = 0.008$), indicating significant effects of drug use on plasma NT-apoAI/apoAI levels. Sub-group comparisons by LSD test showed significant differences between no drugs and statins (* $p = 0.029$), no drugs and other drugs ($\dagger p = 0.001$), and statins and other drugs ($\ddagger p = 0.014$).

Figure 3. Relationship between cholesterol efflux and NT-apoAI/apoAI ratio

Linear regression analysis showed a negative correlation between cholesterol efflux and NTyr-apoAI/AI levels in 67 subjects with similar apoAI levels (1.4–1.6 mg/dL).

Table 1
Group comparisons for different variables between Non-DM and DM groups

Values for variables that show normal distribution are given as means $\pm$ SD and evaluated using Student t-test. Values for variables that do not show normal distribution are provided as median (25–75th percentiles) and evaluated using Kruskal Wallis test. Dichotomous and categorical variables are given as percentages and compared using Pearson's χ^2 test.

Variable	Non-DM (n=450)	DM (n=327)	p value
Male (N%)	307 (68.2%)	220 (67.3%)	0.781
Age (years)	68 (61–75)	71 (65–76)	0.001 [†]
BMI (kg/m ²)	26.7 (24.4–29.7)	28.7 (26.0–32.4)	<0.0001 [†]
Smoking (N%)	82 (18.5%)	45 (14.1%)	0.111
Clinical complications or conditions			
Hypertension (N%)	381 (85.2%)	311 (95.1%)	<0.0001 [†]
Dyslipidemia (N%)	296 (67.4%)	224 (69.1%)	0.616
PAD (N%)	62 (13.8%)	71 (21.3%)	0.004 [†]
Previous stroke or TIA (N%)	46 (10.3%)	52 (15.9%)	0.020 [†]
NYHA functional classification III–IV (N%)	106 (24.4%)	116 (36.7%)	<0.0001 [†]
Multi-vessel CAD (N%)	302 (69.1%)	253 (79.8%)	0.001 [†]
ACS (N%)	237 (52.8%)	160 (49.1%)	0.308
Renal Failure (N%)	96 (23.7%)	138 (45.7%)	<0.0001 [†]
Anti-platelet drugs			
Aspirin (N%)	131 (29.2%)	88 (27.2%)	0.050
Clopidogrel (N%)	40 (8.9%)	30 (9.3%)	
Aspirin and Clopidogrel (N%)	211 (47.0%)	133 (41.0%)	
Lipid lowering drugs			
Statins (N%)	373 (83.1%)	266 (81.8%)	0.153
Ezetimibe (N%)	25 (5.6%)	12 (3.7%)	
Other lipid lowering drugs (N%)	18 (4.0%)	10 (3.1%)	
Laboratory parameters			
TC (mmol/L)	4.4 (3.8–5.2)	4.3 (3.5–5.1)	0.036 [†]
TG (mmol/L)	1.3 (1.0–1.8)	1.5 (1.1–2.2)	0.001 [†]
HDL-C (mmol/L)	1.0 (0.8–1.3)	0.9 (0.7–1.1)	<0.0001 [†]
LDL-C (mmol/L)	2.7 (2.1–3.4)	2.7 (1.9–3.2)	0.044 [†]
CRP (mg/L)	6.95 (5.0–23.53)	8.9 (5.0–31.3)	0.019 [†]
Glucose (mg/dL)	113.5 (87.2–138.9)	137.9 (106.4–182.0)	<0.0001 [†]
eGFR (mL/min·1.73m ²)	74.6 (52.6–94.1)	68.5 (45.9–89.6)	0.007 [†]
ApoAI (mg/mL)	1.33 $\pm$ 0.68	1.24 $\pm$ 0.72	0.096
TBARS (nmol/mL)	1.17 (0.51–2.57)	1.26 (0.50–2.87)	0.314
NT-apoAI (nmol/L)	1868 (1039–3050)	1951 (1131–2966)	0.452

Variable	Non-DM (n=450)	DM (n=327)	p value
NT-apoAI/apoAI (mmol/mol)	46.74 (24.26–70.90)	53.58 (25.38–88.08)	0.016 [‡]
Cholesterol efflux * (%)	20.77 ± 3.71	19.15 ± 3.64	0.09

DM, diabetes mellitus; BMI, body mass index; PAD, peripheral artery disease; TIA, transient ischemic attack; NYHA, New York Heart Association; CAD, coronary artery disease; ACS, acute coronary syndrome; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; TBARS, thiobarbituric acid-reactive substances; ApoAI, apolipoprotein AI; NT-apoAI, nitrated apoAI.

* Cholesterol efflux was measured in 67 subjects with similar apoAI level (1.4–1.6 mg/dL), among which 24 subjects had DM and 43 subjects had non-DM.

[‡] p<0.05

Table 2
Univariate correlates of NT-apoAI/apoAI (mmol/mol) among total cohort, non-DM group and DM group

Spearman's correlation was used to determine correlation of NT-apoAI/apoAI with various clinical parameters.

Variable	Total population			Non-DM			DM		
	r	p		r	p		r	p	
Male gender	-0.073	0.042 [†]		-0.117	0.013 [†]		-0.010	0.856	
Age (years)	0.008	0.831		0.051	0.284		-0.704	0.180	
BMI (kg/m ²)	-0.049	0.181		-0.07	0.142		-0.065	0.247	
Smoking	-0.01	0.784		-0.042	0.376		0.051	0.367	
Hypertension	-0.055	0.124		-0.109	0.021 [†]		0.005	0.935	
Dyslipidemia	-0.013	0.721		-0.077	0.109		0.066	0.236	
PAD	0.038	0.295		-0.081	0.088		0.152	0.006 [†]	
Previous stroke or TIA	0.097	0.007 [†]		0.065	0.172		0.120	0.030 [†]	
NYHA functional classification III-IV	-0.001	0.974		-0.049	0.311		0.031	0.586	
Renal Failure	0.049	0.196		0.019	0.696		0.044	0.443	
ACS	-0.051	0.156		-0.052	0.275		-0.045	0.422	
Multi-vessel CAD	0.005	0.881		-0.012	0.807		0.009	0.879	
Anti-platelet Drugs	-0.103	0.004 [†]		-0.183	<0.0001 [†]		0.011	0.844	
Lipid lowering drugs	-0.085	0.018 [†]		-0.098	0.037 [†]		-0.060	0.281	
TC (mmol/L)	0.034	0.398		0.061	0.254		0.020	0.749	
TG (mmol/L)	-0.017	0.683		-0.056	0.303		-0.005	0.942	
HDL-C (mmol/L)	0.027	0.515		0.057	0.292		0.035	0.583	
LDL-C (mmol/L)	0.047	0.256		0.097	0.073		0.004	0.949	
CRP (mg/L)	0.059	0.102		0.054	0.255		0.046	0.409	
Glucose (mg/dL)	-0.006	0.887		-0.01	0.858		-0.042	0.511	
eGFR (mL/min-1.73m ²)	-0.05	0.168		-0.061	0.202		-0.018	0.746	
TBARS (nmol/mL)	0.140	<0.0001 [†]		0.168	<0.0001 [†]		0.102	0.069	
Cholesterol efflux * (%)	-0.704	<0.0001 [†]		-0.611	<0.0001 [†]		-0.839	<0.0001 [†]	

BMI, body mass index; PAD, peripheral artery disease; TIA, transient ischemic attack; NYHA, New York Heart Association; CAD, coronary artery disease; ACS, acute coronary syndrome; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; TBARS, thiobarbituric acid-reactive substances.

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* Cholesterol efflux was measured in 67 subjects with similar apoA1 level (1.4–1.6 mg/dL), among which 24 subjects had DM and 43 subjects had non-DM.
‡
p<0.05

Table 3
Univariate correlates of NT-apoAI/apoAI (mmol/mol) grouped by gender

Spearman's correlation was used to determine correlation of NT-apoAI/apoAI with various clinical parameters grouped by gender.

Variable	Female (n=250)		Male (n=527)	
	r	p	r	p
Age (years)	0.023	0.713	-0.015	0.723
BMI (kg/m ²)	-0.104	0.106	-0.021	0.631
Smoking	0.062	0.337	-0.026	0.548
Hypertension	0.011	0.857	-0.086	0.048 [†]
Dyslipidemia	-0.007	0.916	-0.009	0.830
DM	0.013	0.841	0.121	0.005 [†]
PAD	-0.016	0.807	0.069	0.114
Previous stroke or TIA	0.074	0.246	0.112	0.010 [†]
NYHA functional classification III~IV	0.011	0.860	-0.011	0.798
Renal Failure	-0.006	0.927	0.093	0.042 [†]
ACS	-0.019	0.771	-0.068	0.117
Multi-vessel CAD	0.035	0.586	0.015	0.732
Anti-platelet Drugs	-0.095	0.132	-0.102	0.019 [†]
Lipid lowering drugs	-0.086	0.173	-0.082	0.062
TC (mmol/L)	0.103	0.149	-0.011	0.830
TG (mmol/L)	0.001	0.987	-0.019	0.711
HDL-C (mmol/L)	0.052	0.469	-0.019	0.697
LDL-C (mmol/L)	0.105	0.143	0.016	0.747
CRP (mg/L)	0.081	0.209	0.048	0.273
Glucose (mg/dL)	-0.126	0.095	0.044	0.392
eGFR (mL/min·1.73m ²)	-0.046	0.475	-0.033	0.458
TBARS (nmol/mL)	0.092	0.153	0.159	<0.0001 [†]
Cholesterol efflux* (%)	-0.719	0.001 [†]	-0.610	<0.0001 [†]

BMI, body mass index; PAD, peripheral artery disease; TIA, transient ischemic attack; NYHA, New York Heart Association; CAD, coronary artery disease; ACS, acute coronary syndrome; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; TBARS, thiobarbituric acid-reactive substances.

* Cholesterol efflux was measured in 67 subjects with similar apoAI level (1.4–1.6 mg/dL) (female, n=18; male, n=49).

[†] **p<0.05**

Table 4
Predictors of $\log_{10}$ NT-apoAI/apoAI by multivariate linear regression

Multivariate linear regression analysis was performed by using $\log_{10}$ transformed NT-apoAI/apoAI ratio as the dependent variable, and gender, smoking, hypertension, dyslipidemia, PAD, previous stroke or TIA, ACS, NYHA functional classification III-IV, renal failure, multi-vessel CAD, current aspirin use, current statin use, as well as $\log_{10}$ transformed age, BMI, TG, TC, LDL-C, HDL-C and CRP levels as independent variables.

Variables	p value	B	95% Confidence Interval for B
Age* (years)	0.929	0.026	(-0.552–0.604)
Male gender	0.139	-0.057	(-0.134–0.019)
BMI* (kg/m ²)	0.618	-0.125	(-0.616–0.366)
Smoking	0.941	0.004	(-0.104–0.112)
DM	0.011 [†]	0.097	(0.022–0.172)
Hypertension	0.237	-0.074	(-0.196–0.048)
Dyslipidemia	0.937	-0.003	(-0.081–0.074)
PAD	0.947	-0.003	(-0.093–0.087)
Previous stroke or TIA	0.051	0.100	(0.0001–0.020)
ACS	0.814	-0.009	(-0.082–0.065)
NYHA functional classification III~IV	0.659	-0.017	(-0.093–0.059)
Renal failure	0.545	0.024	(-0.055–0.104)
Multi-vessel CAD	0.827	0.009	(-0.071–0.089)
Aspirin taking	0.067	-0.076	(-0.158–0.005)
Statins taking	0.625	0.023	(-0.071–0.117)
CRP* (mg/L)	0.008 [†]	0.108	(0.028–0.188)
TG* (mmol/L)	0.995	<0.0001	(-0.223–0.221)
TC* (mmol/L)	0.844	0.105	(-0.941–1.150)
LDL-C* (mmol/L)	0.793	0.082	(-0.533–0.698)
HDL-C* (mmol/L)	0.888	-0.024	(-0.363–0.314)
TBARS* (nmol/mL)	<0.0001 [†]	0.179	(0.106–0.252)

BMI, body mass index; DM, diabetes mellitus; PAD, peripheral artery disease; TIA, transient ischemic attack; ACS, acute coronary syndrome; NYHA, New York Heart Association; CAD, coronary artery disease; CRP, C-reactive protein; TG, triglyceride; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TBARS, thiobarbituric acid-reactive substances.

* after $\log_{10}$ transformation

[†] p<0.05

Table 5
Logistic regression predictors of DM

Logistic regression analysis was performed by using DM as the dependent variable, and age, gender, BMI, smoking, hypertension, dyslipidemia, CRP, TG, TC, LDL-C, HDL-C, TBARS and NT-apoAI/apoAI ratio as independent variables.

Variables	p value	aOR	95.0% C.I. for aOR
Age (years)	<0.0001*	1.051	(1.027–1.075)
Male gender	0.770	0.937	(0.608–1.446)
BMI (kg/m ²)	<0.0001*	1.081	(1.037–1.126)
Smoking	0.009*	2.238	(1.219–4.110)
Hypertension	0.002*	3.401	(1.559–7.418)
Dyslipidemia	0.860	0.962	(0.662–1.486)
CRP (mg/L)	0.456	1.002	(0.996–1.008)
TG (mmol/L)	0.002*	1.680	(1.215–2.323)
TC(mmol/L)	0.019*	0.441	(0.223–0.875)
LDL-C (mmol/L)	0.068	1.943	(0.952–3.966)
HDL-C (mmol/L)	0.585	0.822	(0.408–1.658)
TBARS (nmol/mL)	0.105	1.123	(0.976–1.292)
NT-apoAI/apoAI (mmol/mol)	0.007*	1.005	(1.001–1.008)

BMI, body mass index; CRP, C-reactive protein; TG, triglyceride; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TBARS, thiobarbituric acid-reactive substances; NT-apoAI/apoAI, nitrated apolipoprotein AI/apolipoprotein AI ratio; aOR, adjusted odds ratio; C.I., confidence intervals.

*
p<0.05

Table 6
Group comparisons for different variables between Non-DM and DM groups in 67
selected subjects with similar apoAI level (1.4–1.6 mg/dL)

Values for variables that show normal distribution are given as means $\pm$ SD and evaluated using Student t-test. Values for variables that do not show normal distribution are provided as median (25–75th percentiles) and evaluated using Kruskal Wallis test. Dichotomous and categorical variables are given as percentages and compared using Pearson's χ^2 test.

Variable	Non-DM (n=43)	DM (n=24)	p value
Male (N%)	34 (79.1%)	15 (62.5%)	0.142
Age (years)	65.35 $\pm$ 13.41	68.13 $\pm$ 8.49	0.363
BMI (kg/m ²)	27.49 $\pm$ 5.51	29.72 $\pm$ 5.08	0.126
Smoking (N%)	10 (23.3%)	5 (22.7%)	0.962
Clinical complications or conditions			
Hypertension (N%)	36 (83.7%)	22 (91.7%)	0.360
Dyslipidemia (N%)	24 (57.1%)	18 (75.0%)	0.147
PAD (N%)	8 (18.6%)	5 (20.8%)	0.825
Previous stroke or TIA (N%)	4 (9.5%)	4 (16.7%)	0.392
NYHA functional classification III–IV (N%)	12 (30.8%)	4 (18.2%)	0.283
Multi-vessel CAD (N%)	27 (67.5%)	19 (79.2%)	0.315
ACS (N%)	20 (46.5%)	14 (58.3%)	0.353
Renal Failure (N%)	11 (26.8%)	12 (52.2%)	0.043 [†]
Anti-platelet drugs			
Aspirin (N%)	17 (39.5%)	2 (8.3%)	0.008 [†]
Clopidogrel (N%)	1 (2.3%)	3 (12.5%)	
Aspirin and Clopidogrel (N%)	20 (46.5%)	11 (45.8%)	
Lipid lowering drugs			
Statins (N%)	38 (88.4%)	21 (87.5%)	0.758
Ezetimibe (N%)	3 (7.0%)	1 (4.2%)	
Laboratory parameters			
TC (mmol/L)	4.53 $\pm$ 1.16	4.31 $\pm$ 1.15	0.543
TG (mmol/L)	1.30 (0.90–1.93)	1.10 (0.80–1.95)	0.684
HDL-C (mmol/L)	1.0 (0.8–1.3)	1.1 (0.8–1.3)	0.698
LDL-C (mmol/L)	2.82 $\pm$ 1.11	2.49 $\pm$ 0.96	0.301
CRP (mg/L)	8.9 (5.0–30.7)	7.3 (5.0–26.9)	0.461
Glucose (mg/dL)	106.50 $\pm$ 33.02	142.05 $\pm$ 68.34	0.014 [†]
eGFR (mL/min-1.73m ²)	83.40 $\pm$ 39.55	68.76 $\pm$ 33.61	0.131
ApoAI (mg/mL)	1.50 $\pm$ 0.05	1.47 $\pm$ 0.05	0.036 [†]
TBARS (nmol/mL)	1.86 $\pm$ 1.46	1.62 $\pm$ 1.32	0.547
NT-apoAI (nmol/L)	2164.7 $\pm$ 1242.4	2499.9 $\pm$ 1603.8	0.343
NT-apoAI/apoAI (mmol/mol)	40.30 $\pm$ 23.47	47.52 $\pm$ 30.36	0.282
Cholesterol efflux* (%)	20.77 $\pm$ 3.71	19.15 $\pm$ 3.64	0.09

DM, diabetes mellitus; BMI, body mass index; PAD, peripheral artery disease; TIA, transient ischemic attack; NYHA, New York Heart Association; CAD, coronary artery disease; ACS, acute coronary syndrome; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; TBARS, thiobarbituric acid-reactive substances; ApoAI, apolipoprotein AI; NT-apoAI, nitrated apoAI.

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