**Title**

Increased levels of circulating fatty acids are associated with protective effects against future cardiovascular events in non-diabetics

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## Abstract

### Aims

Cardiovascular disease (CVD) is a major cause of morbidity and mortality worldwide, particularly in individuals with diabetes. The prediction of CVD risk among diabetics is therefore important in disease management. The aim of the current study was to determine the circulating metabolite profiles associated with the risk of future cardiovascular events, with emphasis on the diabetes status of the participants.

### Methods and Results

Non-targeted metabolomics analysis was performed by high-resolution mass spectrometry in combination with targeted quantification of eicosanoids and endocannabinoids. A total of 375 individuals from the IMPROVE pan-European cohort were included. Following data processing, the three metabolite datasets were concatenated to produce a single dataset of 270 identified metabolites. Factor analysis identified six factors that described 26.6% of the total variability in the given set of predictors. A significant association with cardiovascular events was only observed for one factor following adjustment (p=0.026). From this factor, we identified a free fatty acid signature (n=10 lipids, including saturated, monounsaturated, and polyunsaturated fatty acids) that was associated with lower risk of future cardiovascular events in non-diabetics only (OR=0.65, p=0.03), whereas no association was observed among diabetic individuals. A similar trend was observed in Cox proportional hazard regression, with free fatty acids associating with longer time to incident cardiovascular events in only the non-diabetic group (HR=0.80, p=0.024).

### Conclusions

Increased levels of circulating omega-6 and omega-3 fatty acids are associated with protective effects against future cardiovascular events. However, these effects were only observed in the non-diabetic population, further highlighting the need for stratification in clinical investigations.

**Keywords**

Cardiovascular disease, fatty acids, metabolomics, eicosanoids, endocannabinoids

**Translational Perspective (100 words max)**

Recent reports have identified elevated plasma levels of omega 6 and omega 3 fatty acids as having protective effects for future cardiovascular events. Using a ?3-year? follow-up study of high-risk European subjects, our results corroborate these findings in patients without diabetes, but we report that the observed protective effects were not seen in those with diabetes at baseline. This demonstrates the need to stratify patients by diabetic status in order to present accurate future risk information. Importantly, these data indicate that any recommendations concerning the potential protective effects of dietary fat vary with diabetic status.

## Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide (1), and is especially pronounced amongst individuals with diabetes (2). While multiple potential markers have been proposed for predicting future cardiovascular events, there is significant uncertainty regarding their ability to accurately predict risk (3). Metabolomics has been successfully applied to determine the circulating metabolic profile in an effort to link specific metabolites to the onset of CVD (4-7) and diabetes (8, 9). A recent large prospective study of 3 population-based cohorts employed high-throughput NMR-based metabolomics, in combination with a targeted metabolomics platform, to identify (sets of) biomarkers that improved CVD risk prediction (7). In the current study, we have applied non-targeted high-resolution mass spectrometry to determine the metabolite profiles associated with the risk of future cardiovascular events. These metabolomics studies were complemented with targeted analyses of eicosanoids and endocannabinoids, which have known roles in CVD as well as diabetes (10). We placed particular focus on the effect of diabetic status upon the reported biomarkers within the framework of the SUrrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools (SUMMIT) consortium (11), which aimed to identify and characterize biomarkers for complications of diabetes.

## Methods

Methods including statistical analyses are described in the online data supplement. The study design and participant inclusion and matching criteria are provided in Figure S1.

## Results

The baseline characteristics of the study population according to the presence of diabetes are summarized in Table S1. Among both diabetics and non-diabetics, the cases showed less

favorable anthropometric and metabolic profiles, and used more medication compared to the controls. Case-control differences were accentuated among the individuals with diabetes.

Following data processing, the non-targeted metabolomics profiling yielded 1978 unique features, of which 270 were matched to chemical reference standards by accurate mass and retention time. Of the 270 metabolites, 50 had >25% of the values below the limit of detection. These compounds were excluded to give 220 identified metabolites. The remaining 1708 features were annotated putatively. A total of 104 lipid mediators from the eicosanoid and endocannabinoid platforms were screened, of which 50 compounds were present above the limit of quantification. These lipid mediators are generally present at concentrations too low to be detected by metabolomics, and were therefore quantified using targeted methods. The metabolomics and lipid mediator datasets were concatenated to produce a single dataset of 270 metabolites included in the analyses described below.

Analysis of the scree plot showed that six factors had eigenvalues greater than the average eigenvalue (Figure S2). Accordingly, six factors were retained after the varimax orthogonal rotation for the primary analysis. The six factors described 26.6% of the total variability in the given set of predictors (Table S2 for a description of components for each of the selected factors). Amongst the diabetics, there was no significant association between any of the six factors and cardiovascular events (Table S3). In non-diabetics, two of the factors were significantly associated with cardiovascular events at $p<0.05$. Following adjustment for BMI, hypertension, HDL-cholesterol and anti-platelet medication, only Factor 1 (F1) remained significant ($p=0.026$). F1 contained 39 metabolites (Figure S3), the majority of which were free fatty acids (n=10, Figure 1) or their downstream metabolic products (*e.g.*, eicosanoids [n=19] and endocannabinoids [n=5]). The free fatty acids in F1 included polyunsaturated (PUFAs), monounsaturated (MUFAs) and saturated fatty acids (SAT) (Figure S4). In non-diabetics, high concentrations of free fatty acids were associated with lower odds

to suffer cardiovascular events (OR between 0.27 and 0.80), whereas no association was observed among diabetic individuals (Figure 1). A similar trend was observed in Cox proportional hazard regression for the F1 free fatty acids. F1 associated with longer time to incident cardiovascular events in the non-diabetic group (HR=0.80, p=0.024) (Table S4). All free fatty acids measured were associated with a protective effect in non-diabetic individuals.

In the non-diabetic group, pairwise Spearman rank correlation analysis between the six selected metabolic factors and cIMT measurements identified an association between Factor 2 and all IMT readouts at baseline, as well as baseline ICCAD, which disappeared after adjusting for age, gender and MDS1. Also in non-diabetics, a significant inverse correlation was observed between Factor 3 and progression of Bulb-$IMT_{mean}$ (r=-0.23, p= 0.002), which disappeared after further adjustments for cardiovascular risk factors and medication (β=-0.011, p=0.067). Associations between F1 and cIMT variables were essentially non-significant. However, a significant correlation was found between F1 and ICCAD change over time in non-diabetes, tested in linear regression with adjustment for MDS1, cardiovascular risk factors (hypertension, blood glucose, ever smoking) and medication (lipid-lowering drugs, angiotensin II receptor blockers and anti-platelets) (β=-0.008, p=0.001) (Table S5). To identify the major effects for association with ICCAD change over time among metabolites included in F1, we ran linear regression analysis for each component (Table S6). The strongest association was observed with linoleic acid-derived metabolites, including: 12(13)-epoxy octadecanoic acid (EpOME; β=-0.018, p=0.001), 9- and 13-hydroxyoctadecadienoic acid (9-HODE and 13-HODE; β=-0.017, p=0.002 and β=-0.018, p=0.001, respectively), as well as 13-keto octadecadienoic acid (13-KODE; β=-0.014, p=0.002). Another group of interesting compounds associated with the dynamics of ICCAD were N-stearoyl taurine (β=-0.008, p=0.001) and N-palmitoyl taurine (β=-0.011, p=0.009).

**Discussion**

In the present report, non-targeted metabolomics analyses of plasma from a subset of the IMPROVE cohort identified in non-diabetic subjects a signature of free fatty acids associated with lower risk of future cardiovascular events. These observations corroborate the results recently reported by Würtz et al. (7) in a large prospective discovery cohort of 7,256 individuals, replicated in two other cohorts of 2,622 and 3,563 individuals. The two studies had similar objectives; however, Würtz et al. did not directly examine the effect of diabetes. The IMPROVE study recruited subjects at high-risk of CVD, resulting in a cohort with elderly participants (mean age 64 years) and 30% prevalence of diabetes. Based upon the high prevalence of diabetes, and within the framework of the SUMMIT consortium, our analyses were stratified by diabetes status. We observed that the risk for future cardiovascular events differed significantly between the two strata, and that omega-6 fatty acids were significantly associated with lower risk of future cardiovascular events only in non-diabetics. Würtz et al (7) identified omega-6 fatty acids to be significantly associated with lower risk of future cardiovascular events (HR=0.89) over 15 years of follow-up in a large population with a lower diabetes prevalence (~7.8%), which potentially explains the lack of reported diabetes-related differences.

We also found that circulating levels of MUFAs, herein represented by palmitoleic, oleic, and mead acids, were associated with lower risk of cardiovascular events in non-diabetic individuals. This finding agrees with earlier reports in which dietary MUFAs were shown to directly correlate with the circulating levels (12), and with a favorable lipoprotein profile and thus lower risk of CVD (13). By contrast, Würtz et al reported that increased levels of MUFAs were associated with a slightly higher risk for cardiovascular events (HR=1.17). The protective effect that we observed in relation to increased levels of circulating

fatty acids is not restricted to one type of fatty acid, but includes PUFAs, MUFAs and SATs, with both omega-6 and omega-3 fatty acids.

Although not a primary goal, we also analyzed the relationships between metabolomics factors and carotid artery ultrasound measurements taken in the participants at the baseline and in progression over 3 years of follow-up. Measurement of cIMT is a non-invasive way of assessing atherosclerosis, shown to be strongly associated with asymptomatic early stage of the process (14). In the original IMPROVE study, the progression of the maximum IMT detected after 15 months in the whole carotid tree, regardless of location (Fastest-$IMT_{(max\text{-}progr)}$), was significantly associated with the risk of subsequent vascular events, whereas none of the other cIMT measures showed predictive value (15). In the present study, the only significant association found was between F1 and lower change over time in ICCAD in non-diabetics. ICCAD measured in plaque free areas is assumed to reflect carotid expansion due to atherosclerosis and correlates with several vascular risk factors. Interestingly, the protective associations between F1 and change in ICCAD were driven by metabolic products of linoleic acid (12[13]-EpOME and 9[10]-EpOME, 9-HODE, 13-HODE and 13-KODE) and taurine derivatives (N-stearoyl taurine and N-palmitoyl taurine). The data on linoleic acid (and its derivatives) are unclear, but a recent meta-analysis reported a suggestive relationship between dietary linoleic acid and diabetes as well as CVD (16). Taurine, an abundant amino acid-like compound distributed throughout human tissues, has a long list of biological activity, including atheroprotective, anti-inflammatory and anti-obesity effects (17, 18). Taurine has also been studied in relation to cardiovascular prevention and obesity, although the effects of taurine ingestion in humans remain unclear (17, 18). The metabolic effects of palmitic and stearic conjugates have not been well studied and are unclear in the current context.

All participants of the present study were Europeans, which, to some extent, precludes generalization of the observations to other populations. Another limitation is the relatively small size, particularly of the diabetes subset, and lack of a replication cohort. However, the data confirms previous similar results in larger studies (7), and moreover generates the hypothesis that those findings are not applicable to entire populations. The lack of protective effects observed for any of the measured fatty acids with respect to occurrence of cardiovascular events among diabetic participants, calls for further studies into the increased CVD risk in these patients.

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**Disclosures**

None.

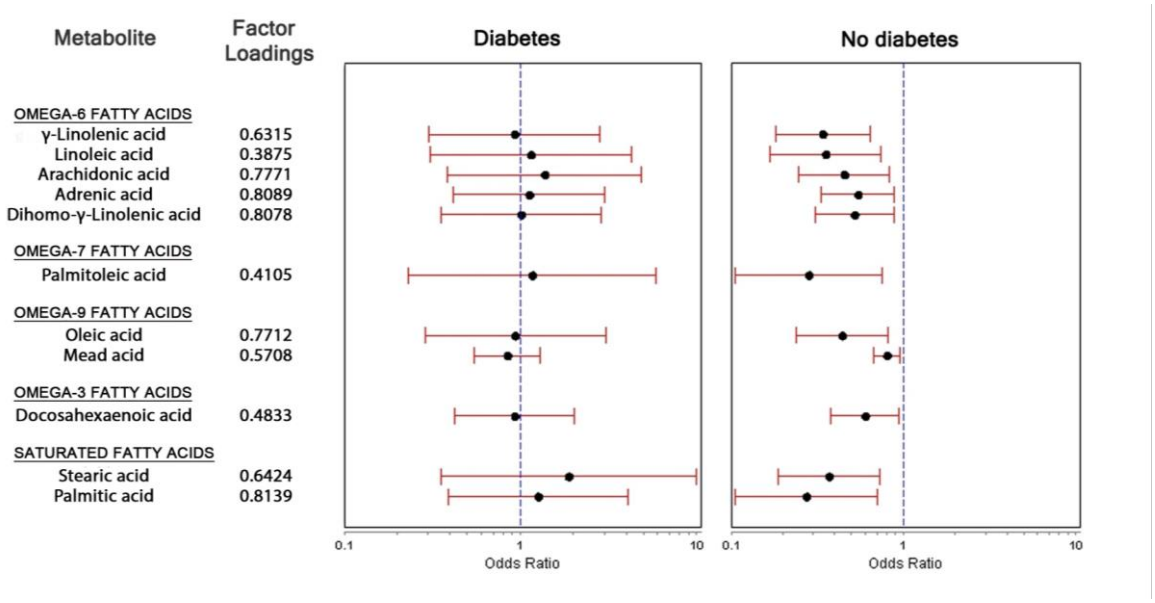


**Figure 1**. **Association of the free fatty acids within Factor 1 with incidence of cardiovascular events stratified by diabetes status.** Factor analysis of the n=270 metabolites from the concatenated metabolomics, eicosanoid and endocannabinoid datasets identified six factors that described 26.6% of the total variability in the given set of predictors (Table S2). Following adjustment for BMI, hypertension, HDL cholesterol and anti-platelet medication, only Factor 1 (F1) remained significant (p=0.026). F1 contained 39 metabolites (Figure S3), the majority of which were free fatty acids (n=10) or their downstream metabolic products (*e.g.*, eicosanoids [n=19], endocannabinoids [n=5]). Stratification by diabetic status showed that, high concentrations of free fatty acids were only associated with lower odds to suffer cardiovascular events in non-diabetics (OR between 0.27 and 0.80). No association was observed among diabetic individuals. Linoleic acid was manually added given its relevance to CVD, even though the loadings were below the <0.4 cutoff.

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