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High-throughput quantification of circulating metabolites improves prediction of subclinical atherosclerosis

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Aims High-throughput metabolite quantification holds promise for cardiovascular risk assessment. Here, we evaluated whether metabolite quantification by nuclear magnetic resonance (NMR) improves prediction of subclinical atherosclerosis in comparison to conventional lipid testing.

Methods and results Circulating lipids, lipoprotein subclasses, and small molecules were assayed by NMR for 1595 individuals aged 24–39 years from the population-based Cardiovascular Risk in Young Finns Study. Carotid intima–media thickness (IMT), a marker of subclinical atherosclerosis, was measured in 2001 and 2007. Baseline conventional risk factors and systemic metabolites were used to predict 6-year incidence of high IMT (≥90th percentile) or plaque. The best prediction of high intima–media thickness was achieved when total and HDL cholesterol were replaced by NMR-determined LDL cholesterol and medium HDL, docosahexaenoic acid, and tyrosine in prediction models with risk factors from the Framingham risk score. The extended prediction model improved risk stratification beyond established risk factors alone; area under the receiver operating characteristic curve 0.764 vs. 0.737, $P = 0.02$, and net reclassification index 17.6%, $P = 0.0008$. Higher docosahexaenoic acid levels were associated with decreased risk for incident high IMT (odds ratio: 0.74; 95% confidence interval: 0.67–0.98; $P = 0.007$). Tyrosine (1.33; 1.10–1.60; $P = 0.003$) and glutamine (1.38; 1.13–1.68; $P = 0.001$) levels were associated with 6-year incident high IMT independent of lipid measures. Furthermore, these amino acids were cross-sectionally associated with carotid IMT and the presence of angiographically ascertained coronary artery disease in independent populations.

Conclusion High-throughput metabolite quantification, with new systemic biomarkers, improved risk stratification for subclinical atherosclerosis in comparison to conventional lipids and could potentially be useful for early cardiovascular risk assessment.

Keywords Intima–media thickness • Risk factors • Lipoproteins • Tyrosine • Metabolomics

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Introduction

Cardiovascular diseases (CVD) are the leading cause of death worldwide. Current cardiovascular risk assessment rely on traditional risk factors, including blood pressure (BP) and lipid levels; however, these characteristics fail to fully explain cardiovascular risk.[1,2] Novel biomarkers for screening and prognosis of CVD have therefore recently attracted substantial interest.[3,4] Given the metabolic nature of atherosclerosis, circulating metabolites represent a source of biomarkers with potential to augment risk prediction, but the clinical utility remains unknown.[4] Recently, comprehensive metabolic profiling identified five amino acids associated with the risk of future diabetes.[5] In addition, technologies for high-throughput profiling of metabolic status can provide insight into the pathophysiology of atherosclerosis.[6] Metabolite quantification using serum nuclear magnetic resonance (NMR) spectroscopy provides quantitative data on lipoprotein subclasses,[7] as well as a variety of small molecules and lipid constituents.[8] Increased subclinical atherosclerosis, as assessed by carotid intima–media thickness (IMT), is a strong predictor of future cardiovascular events.[9,10] The atherosclerotic processes start early in life and take decades to develop into clinical disease. Identification of novel biomarkers that could help to predict the silent subclinical stage would therefore be valuable for primary prevention.[11] Although the molecular mechanisms may not be identical for the development of subclinical atherosclerosis and cardiovascular endpoints, the identification of biomarkers associated with subclinical atherosclerosis is of clinical importance. Here, we evaluate the associations of systemic metabolites with 6-year incidence of high carotid IMT and/or plaque for risk assessment of accelerated atherosclerosis processes in a population-based cohort of apparently healthy young adults. The aim was to assess whether high-throughput quantification of circulating metabolites by NMR would add to prediction of subclinical atherosclerosis in comparison to conventional lipid testing in prediction models with established non-laboratory risk factors.

Methods

Study sample

The Cardiovascular Risk in Young Finns Study representatively selected 3596 children and adolescents aged 3–18 years in the first survey in 1980.[11] One thousand seven hundred and seventy-three participants attending follow-up surveys in both 2001 and 2007 were eligible for inclusion in the present longitudinal study. An overview of the study design is shown in *Figure 1*. Participants in the present study were representative of the original cohort.[12] All participants underwent physical examination, laboratory assessment of risk factors, and ultrasound assessment of subclinical atherosclerosis at both time points. Participants' smoking status and family history of CVD were ascertained from questionnaire, and body mass index (BMI) and BP were measured. Triglycerides, total cholesterol (total-C), HDL cholesterol (HDL-C), apolipoprotein A1, apolipoprotein B, glucose, and high-sensitivity C-reactive protein were assessed from fasting serum samples by standard assays.[13] Pregnant women ($n = 51$), individuals on lipid-lowering medication ($n = 7$), and individuals missing data ($n = 24$) at baseline (2001) were excluded from analyses. The study complies with the Declaration of Helsinki, all participants gave written informed consent and the study was approved by the local Ethics Committees. For a more comprehensive methods section, refer to Supplementary material online.

Ultrasound assessment

Ultrasound studies were performed using Sequoia 512 ultrasound mainframes (Accustom, CA, USA) with 13.0 MHz linear array transducers. Carotid IMT was measured on the posterior wall of the left common carotid artery. The mean of at least four measurements taken ≈10 mm proximal to the bifurcation was used for carotid IMT. Identical scanning protocols were used in 2001 and 2007. The same single reader blinded to subjects' clinical characteristics and scan sequence manually analysed the digitally stored scans at both time points. Atherosclerotic plaque was defined as a distinct area of the vessel wall protruding into the lumen >50% of the adjacent intima–media layer. Intra-individual reproducibility of ultrasound measurements 3 months after the initial visit was 6.4%.[11]

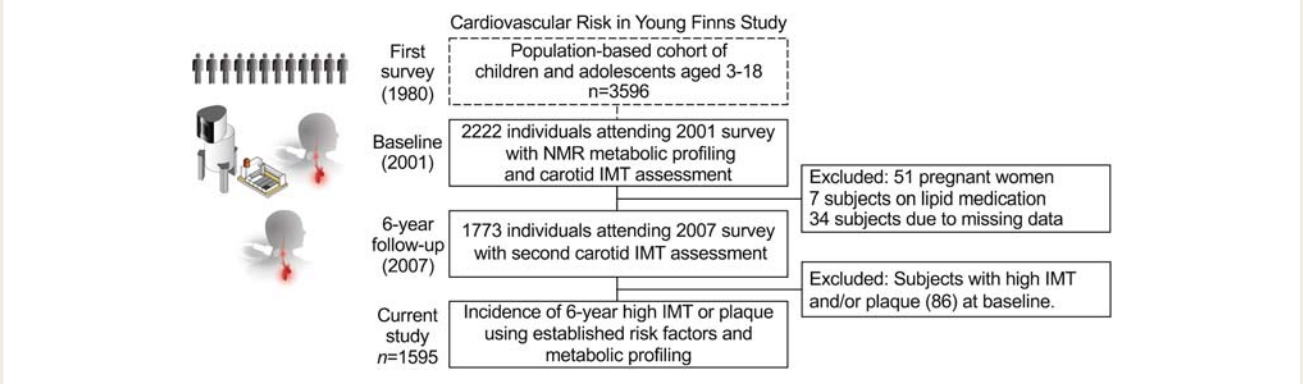

Figure 1 Study flow chart.

Nuclear magnetic resonance spectroscopy and metabolite quantification

Three NMR spectra were recorded from each serum sample on a high-throughput NMR platform.[14] Two spectra were measured from native serum and one from lipid extracts using a standardized protocol (see Supplementary material online). Standard proton NMR spectra were used to quantify 7 lipoprotein major lipid fractions and 14 lipoprotein subclasses in mmol/L and 18 small molecules by regression modelling. An NMR spectrum of serum lipid extracts was recorded to quantify 17 lipid constituent measures.[15] Coefficients of variation and 6-year tracking of the biomarkers highlighted in this study are given in Supplementary material online. Inter-correlations of the assayed metabolites are shown in Supplementary material online, *Figure S1*.

Cross-sectional associations of tyrosine and glutamine with intima–media thickness and coronary artery disease in independent populations

Associations of tyrosine and glutamine with carotid IMT were assessed cross-sectionally in the Health 2000 study, a population-based survey with 1044 Finns of mean age 58 $\pm$ 8 years (range 46–76) who underwent ultrasound sonography.[16] In addition, cross-sectional associations of the amino acids were tested in the subset of individuals from the Cardiovascular Risk in Young Finns Study who only attended the follow-up surveys in either 2001 or 2007 ($n = 830$, mean age 34 $\pm$ 6 years). Methods of the Health 2000 study and clinical characteristics of the individuals studied in the cross-sectional analyses are described in Supplementary material online.

Associations of tyrosine and glutamine were further assessed with angiography-ascertained coronary artery disease (CAD) in the Angiography and Genes Study. This study consisted of 967 patients (mean age 62 $\pm$ 10 years) referred to coronary angiography because of chest pain and clinically suspected CAD.[17] Coronary artery disease diagnosis was defined by at least 50% stenosis of any major coronary artery and severity of CAD by the number of arteries with >50% stenosis. Methods of the Angiography and Genes Study and characteristics of the study population are described in Supplementary material online. Amino acid quantification was conducted with the same analytical platform for all studies.

Statistical analyses

Risk factors and circulating metabolites measured in 2001 were used to predict subclinical atherosclerosis at 6-year follow-up. A dichotomous score representing increased subclinical atherosclerosis was defined as incidence of carotid IMT $\geq$90th percentile (0.750 mm; high IMT) and/or the presence of carotid plaque in 2007.[18] Individuals with high carotid IMT or plaque at baseline ($n = 86$) and missing baseline IMT data ($n = 10$) were excluded.

Baseline characteristics were compared using two-tailed *t*-tests for normally distributed variables and Kolmogorov–Smirnov tests for variables with skewed distributions. Associations of individual metabolites with 6-year incidence of high IMT or plaque were examined using logistic regression models adjusted for sex, age, systolic BP, BMI, smoking status, and glucose by including these risk factors as covariates in the regression models. To assess the prospective associations of non-lipoprotein metabolite measures beyond established lipid risk factors, regression models for small molecules and serum extract metabolites were further adjusted for total-C, HDL-C, and triglycerides. In lack of additional prospective data on carotid IMT, we tested cross-sectional associations of the novel amino acid biomarkers, tyrosine and glutamine, with carotid IMT using linear regression models adjusted for sex, age, BMI, systolic BP, smoking, glucose, total-C, HDL-C, and triglycerides, in an independent population from the Health 2000 study (see Supplementary material online).[16] Here, variables with skewed distribution were $\log_e$-transformed prior to analyses. In addition, we tested associations of tyrosine and glutamine with the presence and severity of CAD as determined by angiography in the Angiography and Genes Study[17] using regression models adjusted for sex, age, BMI, systolic BP, smoking, total-C, HDL-C, triglycerides, as well as usage of diabetes and lipid-lowering medication.

Evaluation of prediction models

The incremental value of adding circulating metabolite biomarkers to established risk factors for prediction of high IMT was examined based on multivariate logistic regression models. The risk factors composing the Framingham risk score[19] were used for the reference model. Glucose was included as a continuous variable in the models since less than 1% of the study population were diagnosed with diabetes. Because the Framingham risk score has been derived for cardiovascular endpoints, the prediction model was here calibrated to the outcome of 6-year incident high IMT.

Since NMR-based metabolite profiling enables quantification of lipoprotein measures similar to those obtained from standard lipid testing,[7,14] the reference model was compared with an extended model where metabolite measures were allowed to complement or replace conventional lipid measures. The same non-laboratory risk factors (sex, age, systolic BP, and smoking status) were included in both prediction models. Lipid measures and metabolites with significant associations ($P < 0.05$) were included in backward stepwise selection (threshold $P < 0.05$) for model derivation with non-laboratory risk factors forced into the model. Missing metabolite data (0.4%) were imputed using the non-linear iterative partial least-squares algorithm for the extended prediction model.[20]

The ability to discriminate risk was estimated using area under the receiver operating characteristic curve (AUC). Comparisons of AUC between the reference model and the extended model were estimated using the DeLong algorithm.[21] Global fit of the models was compared with log-likelihood ratio χ^2 and Akaike Information Criterion. Calibration of the models within risk deciles was assessed using the Hosmer–Lemeshow (HL) goodness-of-fit, which compares the observed number of events with those predicted from the model.[22] Net reclassification index (NRI) and integrated discrimination index (IDI) were calculated to determine the extent to which risk assessment using the extended model reassigned individuals to risk categories that more correctly reflected whether or not the study subjects developed high IMT during the follow-up.[23,24] Participants were assigned to one of four categories (<5, 5–10, 10–20, and >20%) according to their 6-year risk of incident high IMT/plaque based on the reference model and the extended model. The proportions of participants correctly reclassified to either higher or lower risk categories were compared. Integrated discrimination index represents a continuous variant of NRI and is defined as the difference in mean discrimination slopes between two models.[24]

Risk prediction models were evaluated using 10-fold cross-validation so that prediction of an individual's risk was not influenced by his or her own outcome status. The median of discrimination, reclassification, global fit, and calibration metrics for 100 cross-validation repeats are presented. Results for derivation of the predicted risk in one random half of the study population and comparison of the predictive performance of the models in the other half of the population are presented in Supplementary material online, *Table S7*. Because there is no clinical

consensus on what signifies high IMT, we examined the predictive performance of the models using alternate cut-points to define high IMT; similar results were obtained (see Supplementary material online, *Figure S2*).

The pre-specified hypothesis was improved discrimination and reclassification of subclinical atherosclerosis by NMR-based lipoprotein measures and metabolite biomarkers in comparison to conventional lipids in prediction models with established non-laboratory risk factors. Thus, although many *P*-values are reported, statistical significance was inferred for two-tailed $P < 0.05$. Statistical analyses were performed with MATLAB 7.10 R2010a (MathWorks Inc., Natick, MA, USA).

Results

A total of 1595 individuals had complete ultrasound and lipoprotein lipid data available and 150 developed IMT ≥90th percentile and/or plaque during 6-year follow-up. Baseline characteristics are provided in *Table 1*. Median levels of all quantified metabolites are given in Supplementary material online, *Table S1*. Odds ratios (ORs) for incident high IMT are shown in *Table 2*. Several lipoprotein lipid measures determined by NMR had higher ORs than the conventional lipid measures. Considerable heterogeneity was observed for HDL subclasses, where large HDL had the lowest ORs, which was lower than that of HDL-C.

Three systemic amino acids were associated with incident high IMT. Most prominent associations were observed for tyrosine and glutamine, with ORs comparable to that of conventional LDL-C. Since these two amino acids have not previously been linked with subclinical atherosclerosis, we further tested the associations cross-sectionally in an independent population and in a subset of individuals from the Cardiovascular Risk in Young Finns Study not eligible for prospective analyses. Both tyrosine and glutamine were found to be associated with carotid IMT in these cross-sectional analyses as shown in *Table 3*. To assess whether tyrosine and glutamine would be linked with clinical manifestations of atherosclerosis, we tested associations of the amino acids with angiography-based diagnosis of CAD in an independent cohort. Here, we found both tyrosine and glutamine to be associated with the presence of CAD ($P = 0.04$). Elevated tyrosine levels were further associated with increased severity of CAD as defined by the number of major coronary arteries with more than 50% stenosis (*Table 3*).

For the serum extract metabolites, esterified cholesterol and polyunsaturated fatty acid levels were significantly associated with 6-year incident high IMT. Docosahexaenoic acid, an ω-3 fatty acid, was inversely associated with incident high IMT, whereas linoleic acid, an essential ω-6 fatty acid, was directly associated with incident high IMT. Results for all assayed metabolites are shown in Supplementary material online, *Table S2*.

Evaluation of prediction models

The predictive ability of the reference model (risk factors from the Framingham risk score) was compared to the extended model (same non-laboratory risk factors but lipid testing complemented by NMR-based metabolite quantification). In derivation of the extended prediction model, the NMR-determined lipid measures LDL-C and medium HDL replaced conventional total-C and HDL-C. Notably, no enzymatically measured lipids or apolipoprotein measures remained in the prediction models when NMR-based lipid measures were included in the model selection. In addition, docosahexaenoic acid and tyrosine were included in the extended prediction model. Comparison of the prediction models in terms of discrimination, reclassification, model fit, and calibration is shown in *Table 4*. The extended model exhibited enhanced risk discrimination and the improvement in AUC was significant ($P = 0.02$). Receiver operating characteristic curves for the prediction models are shown in *Figure 2*. Notably, the extended model displayed a reclassification index of 17.6% ($P = 0.0008$),

Table 1 Baseline characteristics

	IMT <90th percentile (n = 1445)	IMT ≥90th percentile or plaque (n = 150)	*P*-value
Male sex (%)	42 (39–44)	63 (55–71)	<0.0001
Age (years)	31.5 (4.9)	34.3 (4.2)	<0.0001
Body mass index (kg/m^2)	24.6 (4.1)	26.7 (4.9)	<0.0001
Systolic blood pressure (mmHg)	115 (13)	121 (13)	<0.0001
Current smoker (%)	21 (19–24)	22 (15–29)	0.88
Family history of cardiovascular disease (%)	13 (11–15)	19 (12–25)	0.05
Total-C (mmol/L)	5.1 (0.9)	5.5 (1.0)	<0.0001
LDL-C (mmol/L)	3.2 (0.8)	3.7 (0.9)	<0.0001
HDL-C (mmol/L)	1.3 (0.3)	1.2 (0.3)	<0.0001
Triglycerides (mmol/L)	1.1 (0.8–1.5)	1.2 (0.9–1.8)	0.02
Glucose (mmol/L)	5.0 (4.7–5.2)	5.1 (4.9–5.4)	0.0005
C-reactive protein (mmol/L)	0.7 (0.3–1.7)	0.8 (0.3–1.8)	0.63
Carotid intima–media thickness (mm)	0.56 (0.51–0.62)	0.65 (0.58–0.70)	<0.0001

Baseline characteristics according to incident carotid IMT ≥90th percentile or plaque at 6-year follow-up. Values are percentage (95% confidence interval), mean (SD), and median (inter-quartile range), for categorical, normally distributed, and skewed variables, respectively. Characteristics were compared using *t*-tests and Kolmogorov–Smirnov tests.

Table 2 Odds ratios for 6-year incident carotid intima–media thickness ≥90th percentile or plaque

	OR	95% CI	*P*-value
Lipoprotein and lipid measures (laboratory)			
Total cholesterol	1.28	1.08–1.52	0.005
LDL cholesterol (Friedewald)	1.34	1.13–1.59	0.0007
LDL cholesterol (direct measure)	1.19	0.83–1.71	0.35
HDL cholesterol	0.79	0.64–0.97	0.03
Total triglycerides	1.10	0.95–1.29	0.21
Total-C/HDL-C	1.29	1.10–1.52	0.002
Apolipoprotein B	1.37	1.14–1.65	0.0008
Apolipoprotein A-1	0.87	0.71–1.05	0.15
Apolipoprotein B/apolipoprotein A-1	1.33	1.11–1.59	0.002
Major lipoprotein fractions (NMR)			
Total cholesterol	1.35	1.14–1.60	0.0005
IDL cholesterol	1.34	1.13–1.58	0.0006
LDL cholesterol	1.50	1.26–1.77	<0.0001
HDL cholesterol	0.84	0.67–1.06	0.14
Total triglycerides	1.14	0.96–1.36	0.14
VLDL triglycerides	1.12	0.94–1.33	0.2
IDL triglycerides	1.10	0.93–1.31	0.27
Lipoprotein subclasses (NMR)			
Extremely large VLDL	1.03	0.88–1.21	0.69
Very large VLDL	1.08	0.92–1.27	0.34
Large VLDL	1.10	0.93–1.30	0.26
Medium VLDL	1.15	0.96–1.36	0.12
Small VLDL	1.34	1.12–1.60	0.002
Very small VLDL	1.14	0.96–1.36	0.13
IDL	1.28	1.08–1.51	0.004
Large LDL	1.43	1.21–1.69	<0.0001
Medium LDL	1.49	1.26–1.77	<0.0001
Small LDL	1.45	1.21–1.72	<0.0001
Very large HDL	0.87	0.70–1.09	0.23
Large HDL	0.73	0.57–0.94	0.02
Medium HDL	0.80	0.65–0.99	0.04
Small HDL	1.08	0.90–1.29	0.42
Small molecules (NMR)[a]			
Glutamine	1.38	1.13–1.68	0.001
Histidine	1.23	1.02–1.47	0.03
Tyrosine	1.33	1.10–1.60	0.003
Serum extract metabolites (NMR)[a]			
Esterified cholesterol	1.38	1.03–1.85	0.03
ω-6 fatty acids	1.29	1.01–1.65	0.04
ω-3/ω-6 fatty acids	0.81	0.67–0.98	0.03
Linoleic acid	1.32	1.05–1.65	0.02
Docosahexaenoic acid	0.74	0.59–0.92	0.007

Odds ratios (OR) and 95% confidence intervals (CI) for incidence of carotid IMT ≥90th percentile or plaque at follow-up (2007) according to metabolite measures at baseline (2001). Odds ratios were adjusted for sex, baseline age, body mass index, systolic blood pressure, smoking status, and fasting glucose. Small molecules and serum extract metabolite associations were further adjusted for total and HDL cholesterol as well as triglycerides. Values are expressed for 1-SD increase in the predictor variable.
[a]Only metabolites with nominally significant associations are shown. Odds ratios for all assayed metabolites are given in Supplementary material online, *Table S2*.

Table 3 Cross-sectional associations of tyrosine and glutamine with carotid intima–media thickness and coronary artery disease

Carotid IMT[a]	Health 2000 Study			Subset of the Cardiovascular Risk in Young Finns Study not included in prospective analyses		
Metabolite	**β (SE) [μm]**	***P*-value**	***n***	**β (SE) [μm]**	***P*-value**	***n***
Tyrosine	8.9 (4.7)	0.05	1033	6.2 (3.1)	0.02	823
Glutamine	10.1 (4.6)	0.02	1029	7.7 (3.3)	0.009	779
Coronary artery disease[b]	**Presence of coronary artery disease (≥50% stenosis in major coronary arteries) in the Angiography and Genes Study**			**Number of vessels with angiographically ascertained severe stenosis in the Angiography and Genes Study**		
Metabolite	**OR (95% CI)**	***P*-value**	***n***	**β (SE) [number of vessels]**	***P*-value**	***n***
Tyrosine	1.20 (1.01–1.42)	0.04	944	0.097 (0.042)	0.02	944
Glutamine	1.21 (1.01–1.44)	0.04	895	0.025 (0.043)	0.56	895

[a]Linear regression models were adjusted for sex, age, body mass index, systolic blood pressure, smoking status, glucose, total-C, HDL-C, and triglycerides. β-correlation coefficients (standard error) represent the increase in carotid IMT per 1-SD increase in amino acid concentration.
[b]The risk for the presence and severity of coronary artery disease was analysed by logistic and linear regression models adjusted for sex, age, body mass index, systolic blood pressure, smoking status, total-C, HDL-C, triglycerides, and usage of diabetes and lipid-lowering medication. Odds ratios and β-correlations are per 1-SD increase in amino acid concentration.

Table 4 Comparison of models for the prediction of 6-year incident carotid intima–media thickness ≥90th percentile or plaque based on discrimination, reclassification, model fit, and calibration

Model	AUC	95% CI	P_{AUC}[a]	NRI (%)	P_{NRI}[a]	IDI (%)	P^a_{IDI}	χ^{2b}	P_{χ^2}	AIC	HL	P_{HL}
Reference model: age, sex, systolic BP, smoking status, glucose, total-C, HDL-C	0.737	0.699–0.775	—	—	—	—	—	—	—	918	10.5	0.24
Extended model: non-laboratory risk factors,[c] glucose, LDL-C_{NMR}, medium HDL, docosahexaenoic acid, tyrosine	0.764	0.726–0.802	0.02	17.6	0.0008	2.9	<0.0001	30	<0.0001	892	9.4	0.31

For NRI, participants were assigned to four categories (<5, 5–10, 10–20, and ≥20%) that reflected their 6-year risk of incident high IMT based on each model. Median values of 10-fold cross-validation with 100 repeats are shown. AUC, area under the receiver-operating characteristic curve; CI, confidence interval; NRI, net reclassification index; IDI, integrated discrimination index; AIC, Akaike Information Criterion; HL, Hosmer–Lemeshow statistic.
[a]*P*-values for comparison of the referent model with the extended model.
[b]Log-likelihood ratio χ^2 between the two models.
[c]Non-laboratory risk factors: age, sex, systolic blood pressure, and smoking status.

indicating that a greater number of individuals were reclassified towards more appropriate risk categories than inappropriately reclassified with the prediction model including metabolite biomarkers. The reclassification was most substantial among individuals who did go on to develop high IMT during the 6-year follow-up (NRI = 14.0%) as detailed in *Table 5*. Also improvements in the IDI were observed (IDI = 2.9%; $P = 0.00003$), signifying a significant change in the average predicted risk for the study population. The extended prediction model displayed superior global fit than the reference model (log-likelihood ratio $\chi^2 = 30$) and lower Akaike Information Criterion, supporting the conclusion that the extended model yields improved risk prediction.[25] Calibration was similar for the two models as evident from the HL statistic, which indicates that both models are able to accurately predict the absolute level of risk subsequently observed. Comparable results were obtained when total-C and HDL-C were included in derivation of the extended model (see Supplementary material online, *Table S4*). The predictive performance was also essentially similar using alternate percentile cut-points to define high IMT (see Supplementary material online, *Figure S1*).

Alternative outcomes of subclinical atherosclerosis

Results are presented here for 6-year incident high IMT and/or plaque. Exclusion of individuals at high risk at baseline may potentially cause selection bias; however, all results were similar for prevalent high IMT and/or plaque at follow-up (see Supplementary material online, *Table S5*). In addition, since plaque and high arterial thickening represent different manifestations of subclinical atherosclerosis, these outcomes were also analysed separately (see

Supplementary material online, *Table S5*). The ORs were higher for plaque than for high IMT, reflecting that this outcome is a more specific measure of early atherosclerosis. Of note, NMR-based lipoprotein measures displayed stronger associations than conventional lipids for both outcomes. In addition, tyrosine and glutamine were associated with increased risk for both plaque and high IMT at follow-up, despite the limited statistical power for analysis of carotid plaque. Because accumulation of fatty streaks is a reversible process, it is of interest to identify metabolites associated with short-term progression as well as regression of carotid IMT.[26] Results for high progression (ΔIMT ≥80th percentile), respectively, regression (ΔIMT < 0 μm), in carotid IMT over the 6-year follow-up period are presented in Supplementary material online, *Table S6*. Small very-low-density lipoprotein (VLDL) and LDL measures were associated with both progression and regression of IMT, whereas HDL measures were only associated with IMT progression. Tyrosine and glutamine were suggestively associated in an adverse direction with both extremes of change in carotid IMT. Finally, similar results were found in all analyses when excluding individuals with diabetes and those on anti-hypertensive medication (data not shown).

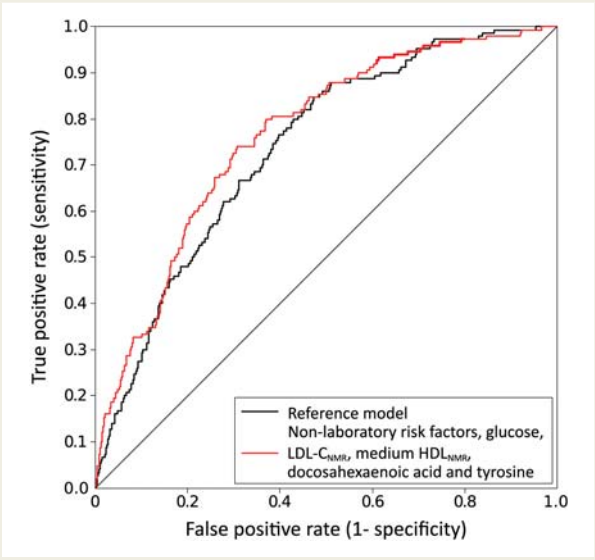

Figure 2 Receiver-operating characteristic curves for 6-year incidence of carotid intima–media thickness ≥90th percentile or plaque for the reference model with risk factors from the Framingham risk score and the extended model with identical non-laboratory measures, but conventional lipid measures replaced by nuclear magnetic resonance-based lipid measures, docosahexaenoic acid and tyrosine.

Discussion

In this study of healthy young adults, high-throughput metabolite quantification by NMR improved risk stratification for subclinical atherosclerosis in comparison to conventional lipid testing as evidenced by increased discrimination and improved reclassification. The extended risk prediction model was composed of a combination of lipoprotein lipids (LDL-C$_{NMR}$ and medium HDL), along with the novel biomarkers docosahexaenoic acid and tyrosine in addition to non-laboratory risk factors. While numerous studies have identified biomarkers for CVD, the incremental value of single biomarkers has rarely been shown to improve risk stratification.[4,25] Our data on subclinical atherosclerosis were consistent with these findings; no single metabolite alone improved risk discrimination (data not shown). However, metabolite quantification from multiple pathways enabled by NMR and mass spectrometry appears better able to capture the complex nature of atherosclerosis development,[27] as least in the

Table 5 Reclassification of individuals based on 6-year risk for incidence of carotid intima–media thickness ≥90th percentile or plaque[a]

	Predicted risk	Extended model with LDL-C$_{NMR}$, medium HDL, docosahexaenoic acid, and tyrosine				Reclassified		Net reclassification index	
		0–5%	5–10%	10–20%	≥20%	Up	Down	Value	*P*-value
IMT ≥90th percentile or plaque	Reference model								
	0–5%	10 (58.8%)	7 (41.2%)	0 (0%)	0 (0%)	40 (26.7%)	19 (12.7%)	14.0%	0.006
	5–10%	6 (15.8%)	16 (42.1%)	16 (42.1%)	0 (0%)				
	10–20%	0 (0%)	7 (11.9%)	35 (59.3%)	17 (28.8%)				
	≥20%	0 (0%)	1 (2.8%)	5 (13.9%)	30 (83.3%)				
IMT <90th percentile	Reference model								
	0–5%	537 (89.5%)	62 (10.3%)	1 (0.2%)	0 (0.0%)	201 (13.9%)	253 (17.5%)	3.6%	0.01
	5–10%	127 (30.5%)	204 (49.0%)	81 (19.5%)	4 (0.9%)				
	10–20%	11 (3.6%)	81 (26.8%)	157 (52.0%)	53 (17.5%)				
	≥20%	2 (1.6%)	5 (3.9%)	27 (21.3%)	93 (73.2%)				
Total								17.6%	0.0008

[a]Comparison of models with conventional total-C and HDL-C vs. NMR-based LDL-C, medium HDL, docosahexaenoic acid, and tyrosine. Both models include sex, age, systolic blood pressure, smoking, and glucose.

preclinical stage as investigated here. The improved risk stratification by NMR-based metabolite quantification over conventional risk factors alone suggested in this study features a single experimental platform. The cost of the high-throughput quantification is comparable to that of conventional lipid testing and could potentially represent a cost-effective approach for early cardiovascular risk assessment.

The clinical value of lipoprotein subclass testing remains controversial.[28,29] Although lipoprotein subclass measures by definition are highly correlated with conventional lipid measures[7,15] (cf. see Supplementary material online, *Figure S1*), these more detailed measures provide a better reflection of the underlying biology[30] and appear useful to augment risk assessment in this study. Lipoprotein subclasses, most notably small LDL, have been associated with CVD risk in numerous studies.[31] While small LDL was associated with incidence of 6-year high IMT, we found no evidence of higher atherogenicity for small LDL than for other LDL subclasses or LDL-C (*Table 2*). However, small LDL was included in the prediction model if the conventional lipid measures (total-C and HDL-C) were forced into the extended model (see Supplementary material online, *Table S4*). In addition, we found significant associations for small VLDL and IDL, confirming their suspected role in atherogenesis.[32] Interestingly, NMR-based lipoprotein measures supplanted conventional lipid and apolipoprotein measures in our prediction models, which might suggest that standard lipid testing is not only complemented but could in effect be replaced by NMR. The stronger associations observed for some NMR-based lipoprotein measures, such as total-C and triglycerides, than the corresponding conventional measures can be ascribed to better accuracy in the NMR-based quantification protocol.[33]

The predictive ability of lipoprotein subclasses in relation to carotid IMT has previously been evaluated only in a small cross-sectional study that concluded that subclass testing does not improve identification of subclinical atherosclerosis.[34] In the present study with larger sample size, longitudinal data and a wider metabolite spectrum assayed our results contrast this conclusion. More recently, a large study of healthy females showed that lipoprotein subclass profiling did not improve prediction of cardiovascular endpoints beyond conventional lipids.[7] Although the results in the present study are based on surrogate markers of CVD, the discrepancy may also be attributed to the combination of metabolites assessed here rather than lipoprotein measures alone.

Metabolite quantification in a high-throughput manner represents a new analytical approach in cardiovascular epidemiology.[5,6,27] Using a population-based cohort of young adults, our results suggest that NMR-based quantification of circulating metabolites is a useful tool for identification of biomarkers for subclinical atherosclerosis. In addition to tyrosine and docosahexaenoic acid, also glutamine exhibited associations with incident high IMT in this study ($P < 0.01$). While glutamine did not remain in the extended prediction model, this amino acid has previously been linked with CAD[27] and could potentially play a role in atherogenesis. Although no prospective validation of the amino acids with 6-year high IMT was available, both tyrosine and glutamine were also cross-sectionally associated with carotid IMT in an independent population (*Table 3*).

Tyrosine is a non-essential amino acid and precursor of thyroid hormones as well as catecholamine neurotransmitters. Tyrosine was recently linked with the risk for the development of type 2 diabetes; however, the pathogenic mechanism behind this association remains elusive.[5] Glutamine is the most abundant amino acid in blood and an important precursor of glucose during fast. In addition, glutamine is known to interact with arginine leading to inhibition of nitric oxide release already at physiological concentrations, which in turn impairs endothelial function.[35] Both tyrosine and glutamine levels were only weakly correlated to conventional lipid measures (see Supplementary material online, *Figure S1*); however, these amino acids have been shown to be part of a principal component which was a strong discriminator between obese and lean individuals and linked with insulin resistance.[36] When adjusting for HOMA-insulin resistance, the association of tyrosine with 6-year incident high IMT was attenuated but remained significant (OR = 1.26; $P = 0.02$), whereas the association of glutamine was essentially unaltered (OR = 1.38; $P = 0.001$). In connection to atherosclerosis, a recent metabolic profiling study on CAD found circulating tyrosine to be a major part of a principal component factor, which was independently associated with CAD.[27] Furthermore, glutamine/glutamate levels were the most significant metabolite discriminator between CAD and non-CAD patients in that study.[27] These results are supported by the present study where both tyrosine and glutamine were associated with diagnosis of CAD in patients referred to angiography due to suspected CAD (*Table 3*). The modest ORs suggest no diagnostic ability of the amino acids; however, the findings nevertheless support a role of tyrosine and glutamine in connection with the presence of CAD. Furthermore, tyrosine was associated with the severity of CAD in terms of the number of coronary arteries with a high degree of stenosis. These results indicate that tyrosine and glutamine levels may not only be markers of preclinical atherosclerosis, but are also be associated with clinical manifestations.

The importance of the dietary mixture of unsaturated fatty acids for cardiovascular prevention is well accepted.[37] The inverse associations of systemic levels of the ω-3 fatty acid docosahexaenoic acid with high IMT support this relation already at the subclinical stage of atherosclerosis where evidence has been inconclusive.[38] The mechanisms responsible for the protective effect of docosahexaenoic acid are not well established, but triglyceride-lowering and anti-inflammatory effects are thought to be implicated.[39] Other mechanisms are, however, likely to be implicated as well as neither triglycerides nor C-reactive protein was associated with subclinical atherosclerosis in this study. Our results suggest that the systemic levels of polyunsaturated fatty acids could have an important role in risk stratification.

The study population represents individuals who might undergo lipid screening; however, the young age of the participants prevented us from studying associations with cardiovascular endpoints. Despite the well-established correlation with clinical outcomes,[9] IMT has a limited ability to capture the complexity of atherosclerotic plaque development and rupture.[10] Nevertheless, biomarkers that predict the silent preclinical stage could have value for primary prevention. We acknowledge that there is no established definition of what constitutes clinically significant high IMT. Using alternative definitions of incident high IMT did

not modify the conclusions drawn (see Supplementary material online, *Figure S1*). The study was conducted in a homogenous population of healthy Finnish adults and care must be taken before generalizing to other populations. The biomarkers identified in the present study yield novel insights into the molecular aetiology of atherosclerosis; however, further validation in prospective settings and with cardiovascular endpoints is warranted before used for risk assessment strategies. NMR spectroscopy represents a low-cost means for high-throughput profiling of metabolites; however, the inherent low sensitivity of the analytical platform limits quantification to highly abundant metabolites. In this respect, the wider metabolite coverage achieved by mass spectrometry holds further promise both for risk assessment and discovery of pathways linked to CVD processes.[40,41] On the other hand, NMR compares favourably to mass spectrometry in terms of ease of sample preparation, automation, reproducibility, and the possibility for lipoprotein subclass profiling, and the two analytical methods can therefore be regarded as complementary.[42]

In summary, NMR-based quantification of circulating metabolites improved risk stratification of subclinical atherosclerosis in comparison with conventional lipid risk factors. We also identified systemic levels of docosahexaenoic acid, glutamine, and tyrosine as predictors of carotid IMT. More accurate risk assessment at an early phase of atherosclerosis development obtainable with these biomarkers has potential to benefit individualized treatment strategies and prevention of cardiovascular events.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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