Baseline serum phosphatidylcholine plasmalogen concentrations are inversely associated with incident myocardial infarction in patients with mixed peripheral artery disease presentations

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Background and Aims: Despite current best care, patients with peripheral artery disease (PAD) remain at high risk of myocardial infarction and biomarkers to more accurately assess cardiovascular risk are needed. This study assessed the relationship between the serum lipidome and incident myocardial infarction in a cohort of PAD patients.

Methods: 265 PAD patients were followed up for a median of 23 months, during which 18 people suffered a myocardial infarction. Fasting serum concentrations of 332 lipid species were measured via mass spectrometry and their association with incident myocardial infarction was assessed via Cox regression. Secondary analyses investigated prognostic potential of specific lipid species.

Results: Serum concentrations of 10 alkenylphosphatidylcholine (plasmalogen) species and 6 alkyl-phosphatidylcholine species were inversely associated with incident myocardial infarction after adjusting for traditional risk factors and correcting for multiple testing. Use of phosphatidylcholine plasmalogen species PC(P-40:6) concentration added to the ability of traditional risk factors to predict subsequent myocardial infarction risk. Receiver operator characteristic curve analyses demonstrated significant improvement in correctly predicting who suffered a myocardial infarction when serum plasmalogen PC(P-40:6) concentration was added to traditional risk factors.

Conclusions: Serum concentrations of phosphatidylcholine plasmalogens and alkyl-phosphatidylcholines were negatively associated with incident myocardial infarction and have potential to act as novel prognostic markers in at-risk populations.

Key words: Myocardial infarction; plasmalogen; biomarker; peripheral artery disease.

INTRODUCTION

Peripheral artery disease (PAD) is frequently used as an umbrella term encompassing a range of stenosing and aneurysmal disorders of the extra-coronary, and extra-cerebral arteries [1]. Common presentations include lower limb occlusive disease in which blood supply to the leg is restricted due to atherosclerosis and associated thrombosis, and abdominal aortic aneurysm (AAA) usually diagnosed by an infra-renal aortic diameter of ≥30mm. Screening data suggest that AAA affects ~2% of males and ~1% of females aged 65 years and over [2-5]. Lower limb occlusive disease affects ~10% of adults aged above 40 years [6, 7]. PAD represents a cardiovascular risk equivalent to coronary heart disease (CHD), but is underdiagnosed and less intensively managed, possibly due to a primary treatment focus on the presenting complaint [8-13]. The risk of major cardiovascular events such as myocardial infarction (MI) is ~3-fold higher in PAD patients than a healthy age-matched population [6, 14], despite current secondary prevention measures, such as prescription of statins. It is recognised that this residual cardiovascular risk differs significantly between patients, and risk prediction tools such as the SMART score have been proposed to identify sub-groups of patients most likely to benefit from aggressive management [15, 16]. To date, such risk calculators have largely focused on the analysis of routinely monitored clinical parameters. Consequently, the potential for novel circulating markers to improve cardiovascular risk prediction remains largely unknown.

Lipid control remains a major cornerstone in cardiovascular risk management. Currently, dyslipidaemia is assessed by monitoring circulating triglyceride, total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) concentrations. However, each of these lipoprotein fractions contains particles composed of

hundreds of chemically distinct lipids. Mass spectrometry (MS)-based studies have demonstrated that quantifying individual lipoprotein-borne lipid species may provide a more sensitive means to stratify cardiovascular risk than traditional lipid parameters, and may also identify novel therapeutic targets [1, 17-23]. However, most previous studies have focused on patients who present with symptoms of CHD, and the relevance of previously suggested lipidomic markers to patients with PAD presentations remains unclear. The aim of the current study was therefore to investigate the association of circulating concentrations of novel lipids with incident MI in a cohort of patients with mixed PAD presentations, with a view to identifying novel markers with prognostic potential or as potential targets for novel therapies.

MATERIALS AND METHODS

Detailed methods are provided as Supplemental File 1.

Patient recruitment and follow-up: Participants in the current study had been involved in a recent investigation to characterise the serum lipidome in patients with a range of PAD presentations [1]. PAD patients were recruited from out-patient clinics at the Townsville and Mater Hospitals, after providing written informed consent. This study was conducted in accordance with the Declaration of Helsinki with approval from the Human Research Ethics Committees at James Cook University, The Townsville Hospital and the Mater Misercordiae Hospital, Townsville.

Serum analysis: Concentrations of total cholesterol, HDL-C, LDL-C and triglycerides were quantified in fasting serum samples by validated automated assays [24]. Serum very low density lipoprotein cholesterol (VLDL-C) concentrations were estimated as total cholesterol

minus combined HDL-C and LDL-C. C-reactive protein (CRP) concentration was assessed using an enhanced turbidimetric assay. The MS methods used to measure serum lipid concentrations in these patients have been previously discussed, and are detailed in Supplementary File 1 [25].

Recording outcome data: Patients were followed up at out-patient clinics and/or as an in-patient as part of their standard care [26]. The primary outcome measure for this study was hospital admission for MI based on a positive diagnosis by a Royal Australian College of Physicians accredited physician in line with international guidelines [27]. Charts and hospital electronic records of all patients were reviewed to confirm outcome events by a vascular specialist or research nurse. For patients that had not experienced the primary outcome censoring occurred at the date of last in or out-patient review.

Statistical analyses: Differences between patient groups were assessed using the Mann-Whitney U test and Chi-squared test. Correlations were assessed via 2-tailed Spearman rho test. Unadjusted Cox regression was conducted to identify lipids significantly associated with incident MI, followed by secondary Cox regression analyses adjusted for age, gender, smoking, diabetes, hypertension, CHD, AAA and dyslipidemia. P-values from Cox regressions were adjusted using the Benjamini-Hochberg approach with a false discovery rate of 5%, accepting q values <0.05 as significant.

Cox proportional hazards-based models were constructed from panels of: 1) traditional risk factors (age, gender, BMI, smoking, diabetes, hypertension, CHD, AAA, statins, and serum HDL-C, LDL-C, VLDL-C, triglyceride and CRP concentrations) [traditional risk factor models]; 2) Lipid species only [lipidomic models]; and 3) all parameters in the first 2 groups

[combined models]. No violations of the proportional hazards assumption were observed. Multi-model inference analysis assessed the ability of all combinations of ≤6 covariates within each panel to predict MI during follow-up. Subsequent analyses focused on the 25 most highly ranked models for each panel. Coefficients for each covariate were averaged across the models in which they appeared for each panel, and relative covariate importance was assessed according to % incorporation in the top 25 models. Models in each panel were ranked by ascending Akaike's second order information criterion (AICc); those showing increase in AICc ≥2 points compared to the first ranked model were excluded from further analysis. Receiver operator characteristic (ROC) curves were generated for all remaining models, and the area under the curve (AUC) and 95% confidence intervals were calculated. The performance of the models with the highest AUC from each panel was compared using DeLong's test for paired ROC curves. The contribution of each covariate to the performance of the best overall model was assessed in leave-one-out sensitivity analyses investigating the impact of removing single covariates on ROC curve AUC. P-values ≤0.05 were considered to be statistically significant.

RESULTS

Cohort characteristics: Patients with mixed PAD presentations were recruited to the current study (n=265). Demographic characteristics of the cohort are shown in Table 1 and Supplementary Table 1. Median age for the whole cohort was 69 years (inter-quartile range, (IQR) 64-75), ~70% were male and ~86% had a history of ever smoking. The overall prevalence of hypertension, CHD and diabetes were 74%, 46% and ~23% respectively. Approximately 65% of patients were receiving statins and fewer than 10% were prescribed fibrates. 121 of the recruited patients had an AAA and 144 had symptoms of lower limb

atherothrombosis. AAA patients tended to be older, more likely to have a history of CHD, and had higher serum concentrations of creatinine and C-reactive protein than those with lower limb atherothrombosis. Diabetes mellitus was more common in the patients with lower limb atherothrombosis (Supplementary Table 1).

Median follow-up time for the cohort was 23.3 (IQR 9.7-44.5) months during which 18 (6.9%) patients suffered an MI requiring hospital admission. The demographic characteristics of patients who did and did not suffer MI are shown in Table 1. Follow-up time was similar for both groups of patients. Patients who suffered an MI had higher C-reactive protein concentrations at recruitment and were more likely to have diabetes mellitus and corresponding prescription of oral hypoglycaemic drugs, than those who did not suffer an MI. Serum LDL-C concentration was significantly higher in the patients who did not suffer MI, although the use of statins and fibrates was similar between those who did and did not suffer an MI. No other differences in risk factors were observed.

The association of serum lipids with incident MI: Serum concentrations of 25 lipid functional classes and subclasses were measured for each patient via MS (Table 2). We previously reported that the median coefficient of variation for lipids measured in this series was 10.4% [1]. Serum concentrations of two lipid classes, total alkylphosphatidylcholine (PC(O)) and total phosphatidylcholine plasmalogen (PC(P)), were significantly inversely associated with incident MI after correcting for multiple testing (hazards ratio for an increase in serum total PC(O) concentration of ~1 standard deviation: 0.43 (95% confidence interval (CI): 0.24-0.74), corrected *p*-value 3.23×10^{-2}; hazard ratio for an increase in serum total PC(P) concentration of ~1 standard deviation: 0.28 (95% CI: 0.13-0.56), corrected *p-value*

$1.03x10^{-2}$). No other serum lipid classes were significantly associated with incident MI (Table 2).

The association of serum concentrations of 17 PC(O) and 12 PC(P) lipid species with incident MI was assessed via Cox regression adjusted for age, gender, smoking, diabetes, hypertension, CHD, AAA presence and dyslipidemia (evidenced by prescription for statins; Table 3). Significant negative associations between 6 (35%) PC(O) species and 10 (83%) PC(P) species with incident MI were observed after correcting for multiple testing (HR ranges for an increase in serum concentration of ~1 standard deviation: 0.37-0.51 and 0.07-0.42 respectively). These PC(P) and PC(O) species were positively correlated with circulating HDL-C and LDL-C concentration although Spearman r correlation coefficients were <0.5 (Supplementary Table 2). Negative correlations between the PC(P) and PC(O) species with VLDL-C and triglyceride concentrations were observed but were not significant for most lipids.

Multimodel inference analysis to identify predictive models for MI: Three panels of Cox regression models were constructed from 1) traditional risk factors only (detailed in materials and methods section), 2) MI associated PC(O) and PC(P) species only (lipidomic models), and 3) traditional risk factors and PC(O) and PC(P) species (combined model). Supplementary Table 3 shows the 25 most highly ranked traditional, lipidomic and combined models.

Serum LDL-C concentration, older age and a history of ever smoking were the most important traditional risk factors for predicting future MI demonstrated by incorporation in 100%, 69% and 42% of the 25 highest ranked traditional risk factor models, respectively. The

best fit traditional risk factor model comprised age and serum LDL-C concentration (AICc 157.38), although this was not considered to be better than the other top 25 models (all increases in AICc values <2 points). In contrast, ROC curve analyses identified a model comprising age, diabetes, LDL and triglycerides to have the strongest predictive ability for MI, evidenced by the highest observed AUC of all traditional risk factor models generated (AUC:0.722 (95% CI: 0.721-0.890; Supplementary Table 3).

Serum PC(P-40:6), PC(O-40:7) and PC(P-38:5) concentrations were the 3 strongest lipidomic predictors for incident MI, featuring in 100%, 86% and 46% of the top ranked models respectively. AICc values suggested that incident MI risk was better modelled by the highest ranked lipidomic model, than the top-ranked traditional risk factor model (AICc of the top ranked lipidomic model: 147.20; difference in AICc of top ranked traditional risk factor and lipidomic models: -10.18). A combination of PC(O-38:5), PC(O-40:7), PC(P-36:5) and PC(P-40:6) generated the highest ROC AUC for the lipidomic models (AUC: 0.805 (95% CI: 0.745-0.925; Supplementary Table 3).

In general, consideration of lipidomic species along with traditional risk factors provided the best ability to model incident MI evidenced by consistently lower AICc scores, and higher ROC AUC values than that achieved for the traditional risk factor, and lipidomic models (Supplementary Table 3). Within the combined models, older age and low serum PC(P-40:6) concentrations were identified as the most powerful predictors of MI evidenced by inclusion in all top 25 models. Of note, combined models ranked 15-25 showed an increase in AICc $\geq$2 points compared to the first ranked model, and were discounted from further analysis (Supplementary Table 3). ROC analyses suggested that the strongest MI predictive ability was achieved when considering a model incorporating age, diabetes and serum

concentrations of LDL-C, triglycerides and the novel lipid PC(P-40:6) (ROC AUC: 0.835; 95% CI: 0.745-0.925).

Comparison of relative model performance: The ROC AUCs of the strongest performing lipidomic and combined models were compared to that of the traditional risk factor model, to assess whether consideration of novel lipid species significantly improved the ability to predict incident MI beyond currently used parameters (Table 4). The ROC AUC of the lipidomic and combined models were both higher than the traditional risk factor model, although a statistically significant improvement in AUC could only be demonstrated for the combined model (p=0.033). No significant difference in ROC AUC was found between the lipidomic and combined models (p=0.371).

Assessing the potential of PC(P-40:6) to predict incident MI: Sensitivity analyses were performed to assess the contribution of each covariate to the performance of the combined model (Supplemental Table 4). Removal of serum PC(P-40:6) significantly reduced model performance (AUC of model excluding PC(P-40:6): 0.722; reduction in AUC after excluding PC(P-40:6): 0.113; p=0.022). Removing any other single covariate did not markedly reduce the AUC of the combined model.

DISCUSSION

The current study identified significant inverse associations of 2 lipid functional classes, the alkyl-phosphatidylcholines (PC(O)) and alkenylphosphatidylcholines (PC(P), plasmalogens)), with incident MI, in a cohort at high risk of cardiovascular events. Specifically serum concentrations of 6 PC(O) and 10 PC(P) lipid species were negatively associated with incident MI after adjusting for traditional risk factors and correcting for

multiple testing. Secondary analyses suggested potential for the identified lipids, particularly PC(P-40:6) to predict incident MI, evidenced by higher AUC for models containing these species, and inclusion of PC(P-40:6) along with more established cardiovascular risk factors in the highest performing predictive model.

A number of previous studies have suggested an association of the PC(O) and PC(P) lipids with cardiovascular events in patients with CHD, and other non-PAD cardiovascular disorders. However, not all studies report the inverse association of these lipids with incident MI [17, 20-22]. Reasons for this remain unclear, although differences in cohort comorbidities and risk factors may contribute to inter-study variations. PC(O) and PC(P) synthesis occurs through the same pathway, suggesting that their association with incident MI may not be metabolically independent [20]. Biosynthesis of the PC(O)s and PC(P)s have been discussed in detail (see refs [20] and [28]), but in brief, both are derived from the precursor, dihydroxyacetone phosphate (DHAP) which is enzymatically modified to produce 1-O-alkyl-2-acyl-glycerol. This intermediary compound can be modified by choline-phosphotransferase to produce the PC(O)s, or give rise to the alkylphosphoethanolamines (PE(O))s in a reaction catalysed by ethanolamine-transferase. Modification of the PE(O)s by Δ1-alkyl desaturase gives rise to the phosphoethanolamine plasmalogens (PE(P)s), which then produce the PC(P)s through either a 2-step reaction catalysed by phospholipase C and choline-phosphotranseferase, or direct conversion catalysed by phasphatidylethanolamine N-methyltransferase.[20] Our data suggest that at least parts of this metabolic pathway may be modified in the PAD patients who subsequently suffered MI, evidenced by parallel reductions in circulating PC(O) and PC(P) concentrations. Moreover, we observed a negative association of circulating PE(P) concentrations with incident MI which further supports this hypothesis, however this relationship was lost when correcting for multiple testing ~~likely due~~

~~to under-powering~~. In contrast, we observed no association of circulating concentrations of PE(O)s, or lysoalkyl-phosphatidylcholines (LPC(O) (a downstream product of PC(O) metabolism) with MI incidence suggesting complex changes in this pathway may be present [20].

The reason for the negative association of the PC(P) lipids with incident MI is incompletely understood. The PC(P) phospholipids possess a vinyl-ether linked alkyl chain at the *sn-1* position of the glycerol backbone. The *sn-2* position is commonly occupied by an ester-linked polyunsaturated fatty acid (e.g. docosahexanoic acid or arachidonic acid), and the polar head, in this case phosphocholine, is bound to the *sn-3* position [28]. Blood-borne plasmalogens are synthesised by the liver and become incorporated into nascent lipoproteins [29]. The inverse association of serum plasmalogen concentrations with incident MI suggests that these lipids may protect against acute atherothrombotic events. Findings from different studies suggest that lipoprotein-borne PC(P)s may influence MI risk in several ways. Firstly, the vinyl-ether bond at the *sn-1* position of the plasmalogens readily reacts with reactive oxygen species, indicating potential antioxidant functions for these lipids [29-31]. Relevant to the current study, *in vitro* data demonstrate that LDL-C becomes increasingly resistant to copper oxidation following plasmalogen enrichment [32]. Thus, it is possible that the rate of LDL oxidation in patients with low circulating PC(P) concentrations is accelerated as a consequence of weakened antioxidant protection. Secondly, the *in vitro* cholesterol efflux capacity of HDL-C is significantly impaired after specifically depleting plasmalogens [33]. Accordingly, MI susceptibility in patients with low serum PC(P) levels may arise from higher rates of LDL oxidation, increased foam cell formation, and reduced cholesterol efflux. It is therefore possible that therapies aimed at increasing circulating PC(P) concentrations may be beneficial in reducing cardiovascular risk in PAD patients. This hypothesis is supported by

the findings of a recent study which demonstrated reductions in atherosclerotic plaque area and concomitant increases in circulating PC(P) and PC(O) levels in hyperlipidaemic mice receiving batyl alcohol, (a precursor fatty alcohol involved in PC(P) synthesis) [34]. A prior lipidomic study has reported that statin treatment increases the proportion of the plasma lipidome made up by PC(O) and PC(P)s in patients that have metabolic syndrome, suggesting that medications commonly used in PAD patient management may alter PC(O) and PC(P) concentrations [35]. Clinical studies are required to determine whether novel therapies to enhance PC(O) and PC(P) expression, such as batyl alcohol, confer additional benefit beyond conventional treatments.

There are several limitations to the current study. Firstly, the population studied was relatively small, follow up time was relatively short, and not all participants had undergone coronary imaging to rule out asymptomatic CHD. Moreover only 18 patients (~7% of the study population) had an MI. This contrasts with recent data suggesting that MI rates for PAD patients may be as high as 15% over a similar follow-up time to that of the current study [36, 37]. MonteCarlo simulations suggest that 10 outcome events are required for each covariate entered into regression models, although there are suggestions that this may be overly stringent [38]. To overcome hurdles associated with statistical power we applied multi-model inference analyses to identify the most parsimonious models with the greatest predictive power using AICc. *Post-hoc* sample size calculations also suggested that we were able to detect inter-group differences in circulating concentrations of the identified lipid biomarkers with at least 80% power, providing further confidence in the conclusions of this study (Supplementary Table 7). Despite this, several lipid classes which have been linked with MI in other patient series (including phosphoethanolamine plasmalogens) were not identified as potential biomarkers in this patient cohort~~, however, it is unclear whether this is~~

~~the result of type 2 errors, or differences in the pathobiology of PAD and other cardiovascular diseases~~ [39]. In addition, the lack of additional samples from an independent cohort prohibited cross-validation analyses which would provide stronger evidence of the benefit of measuring serum lipid species, particularly PC(P-40:6) in improving MI prediction. However, our findings independently validate those of others which have associated low circulating PC(O) and PC(P) concentrations with increased cardiovascular risk in vascular disease patients. A number of patients in the current study were also receiving statins which could not be ethically withdrawn and instead was adjusted for in the Cox regression analyses. Finally, the absence of some clinical details from our patients such as systolic blood pressure, measurement of high-sensitivity CRP (hsCRP) and detailed carotid imaging data prevented direct comparison of our data with other suggested risk prediction algorithms such as the SMART score [15]. Subsequent studies with larger cohorts are needed to validate the findings presented here and assess whether measurement of the identified lipids improves the performance of previously suggested risk calculators. ~~assess the relevance of our findings to the wider population.~~.

In summary, this study suggests that serum PC(O) and PC(P) concentrations may have potential to act as prognostic biomarkers for MI in PAD patients. Multimodel inference and ROC curve analyses demonstrated that the ability to model incident MI was significantly improved when serum concentrations of the PC(P) and PC(O) species were added to traditional risk factors used in current clinical practice. These findings are in line with previous investigations, suggesting that circulating concentrations of individual lipid species identified by lipidomics may add to the utility of traditional risk factors currently employed in patient management [1, 17]. Notably, the lipid species PC(P-40:6) was suggested to be equally as important as age when assessing MI risk in this cohort. Interestingly, serum PC(P-

40:6) concentration appeared to provide greater power to predict MI than traditional cholesterol parameters evidenced by inclusion in a greater number of classification models, and more marked impact on model performance after exclusion. This suggests that the development of tests to rapidly measure circulating PC(P) concentrations, and the development of adjunct therapies to boost PC(P) expression may be useful in improving PAD patient management. Our findings, however, need to be interpreted with caution, as many participants were receiving statins which may have lessened the association of traditional cholesterol parameters with cardiovascular risk, and also influenced the relationship between the novel lipids and incident MI. Further studies are therefore required to validate the findings of this study and determine their clinical relevance in independent patient cohorts.

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Table 1. Characteristics of patients included in this study

Characteristic	Whole cohort (n=265)	No MI (n=247)	MI (n=18)	*p-value*[a]
Aortic diameter (mm)	27.7 (20-53)	28.0 (20.0 - 53.0)	25.5 (20.0 – 53.8)	0.774
Male	186 (70.2%)	172 (69.6%)	14 (77.8%)	0.466
Age (years)	69.3 (64.0-75.0)	69.0 (64.0 – 75.0)	70.5 (63.5 – 75.8)	0.901
BMI (kg/m^2)	27.7 (24.5-30.6)	27.3 (24.4 – 30.6)	28.9 (27.4 – 32.0)	0.144
Diabetes mellitus	60 (22.6%)	52 (21.1%)	8 (44.4%)	0.022
Ever smoker	227 (85.7%)	213 (86.2%)	14 (77.8%)	0.323
CHD	122 (46.0%)	111 (44.9%)	11 (61.1%)	0.184
Hypertension	196 (74.0%)	182 (73.7%)	14 (77.8%)	0.702
AAA	121 (45.7%)	115 (46.6%)	6 (33.3%)	0.277
Serum concentrations of:				
Creatinine (μM)	83.0 (70.0-100.0)	83.0 (70.0 – 97.0)	94.0 (60.8 – 108.0)	0.418
Triglycerides (mM)	1.7 (1.1-2.4)	1.6 (1.1 – 2.2)	2.2 (1.3 – 2.7)	0.127
Total cholesterol (mM)	4.5 (3.7-5.4)	4.5 (3.7 – 5.4)	4.4 (3.2 – 5.0)	0.102
HDL-C (mM)	1.2 (1.0-1.5)	1.2 (1.0 – 1.5)	1.2 (0.9 – 1.2)	0.456
LDL-C (mM)	2.5 (1.8-3.2)	2.6 (1.8 – 3.3)	2.0 (1.3 – 2.6)	0.023
VLDL-C (mM)	0.7 (0.5-1.0)	0.7 (0.5 – 1.0)	1.0 (0.6 – 1.2)	0.207
CRP (mg/L)	4.0 (2.0-8.0)	3.4 (2.0 – 7.9)	6.0 (4.5 – 8.3)	0.035
Prescription for:				
Aspirin	183 (69.1%)	170 (68.8%)	13 (72.2%)	0.763
CCB	83 (31.3%)	75 (30.4%)	8 (44.4%)	0.214
Beta-blockers	93 (35.1%)	84 (34.0%)	9 (50.0%)	0.170
ACEI	105 (39.6%)	99 (40.1%)	6 (33.3%)	0.572
ARB	48 (18.1%)	42 (17.0%)	6 (33.3%)	0.082
Statins	173 (65.3%)	160 (64.8%)	13 (72.2%)	0.522
Fibrates	6 (2.3%)	6 (2.4%)	0 (0.0%)	0.504
Metformin	36 (13.6%)	31 (12.6%)	5 (27.8%)	0.069
Other oral hypoglycaemic drugs	24 (9.1%)	20 (8.1%)	4 (22.2%)	0.044
Follow up time (months)	23.3 (9.7-44.5)	23.3 (10.8-43.2)	21.1 (6.1-59.7)	0.821

Shown are number (%) for nominal variables and median (inter-quartile range) for continuous variables. Nominal variables are compared between patients who did, or did not suffer MI by Chi-squared test; continuous variables are compared by Mann Whitney U test. AAA = Abdominal aortic aneurysm; BMI = Body mass index; CHD = Coronary heart disease; CCB = Calcium channel blocker; ACEI = Angiotensin converting enzyme inhibitor; ARB = Angiotensin receptor blocker; MI = Myocardial infarction. [a]Comparing patients with and without MI.

Table 2: Association of serum classes with MI in 265 PAD patients via univariate Cox regression

Lipid class	Association with MI in PAD patients				
	p-value[a]	Corrected *p-value*[b]	Hazard ratio[c]	95% CI	
				Lower	Upper
Bis(monoacylglycero)phosphate (BMP)	0.949	0.949	0.85	0.01	147.41
Ceramide (Cer)	0.139	0.248	0.56	0.26	1.21
Dihydroceramide (dhCer)	0.117	0.231	0.51	0.22	1.18
Monohexosylceramide (MHC)	0.013	0.092	0.26	0.09	0.75
Dihexosylceramide (DHC)	0.024	0.122	0.44	0.22	0.90
Trihexosylceramide (THC)	0.054	0.149	0.52	0.27	1.01
G_{M3} ganglioside (GM3)	0.052	0.149	0.49	0.24	1.01
Sphingomyelin (SM)	0.015	0.092	0.42	0.21	0.84
Phosphatidylcholine (PC)	0.047	0.149	0.59	0.35	0.99
Alkylphosphatidylcholine (PC(O))	<0.001	**0.032**	0.43	0.24	0.74
Phosphatidylicholine plasmalogen (PC(P))	<0.001	**0.010**	0.28	0.14	0.56
Lysophosphatidylcholine (LPC)	0.069	0.174	0.63	0.38	1.04
Lysoalkylphosphatidylethanolamine (LPC(O))	0.264	0.388	0.72	0.40	1.29
Phosphatidylethanolamine (PE)	0.194	0.324	1.33	0.86	2.06
Alkylphosphatidylethanolamine (PE(O))	0.120	0.231	0.62	0.34	1.13
Phosphatidylethanolamine plasmalogen (PE(P))	0.039	0.149	0.48	0.24	0.96
Lysophosphatidylethanolamine (LPE)	0.585	0.664	0.86	0.50	1.48
Phosphatidylinositol (PI)	0.319	0.443	0.76	0.45	1.30
Lysophosphatidylinositol (LPI)	0.836	0.871	0.95	0.57	1.58
Phosphatidylglycerol (PG)	0.617	0.671	1.12	0.72	1.74
Phosphatidylserine (PS)	0.578	0.664	1.11	0.78	1.57
Cholesterol ester (CE)	0.263	0.388	0.69	0.35	1.33
Free cholesterol (COH)	0.114	0.231	0.52	0.23	1.17
Diacylglycerol (DG)	0.370	0.487	1.23	0.79	1.91
Triacylglycerol (TG)	0.469	0.586	1.12	0.82	1.53

[a] Unadjusted following Cox regression; [b] Benjamini-Hochberg corrected p-value (q-values), bolded text denotes significance; [c] Hazard ratios for a 1 std dev increase in serum concentration.

Table 3. Association of PC(O) and PC(P) species with MI in 265 patients with PAD via Cox regression adjusted for age, gender, smoking, diabetes, hypertension, CHD, AAA and dyslipidaemia (evidenced by statin prescription).

Lipid	Association with MI in PAD patients				
	p-value[a]	Corrected *p-value*[b]	Hazard Ratio[c]	95% CI	
				Lower	Upper
PC(O-32:0)	0.031	0.052	0.48	0.25	0.93
PC(O-32:1)	0.293	0.315	0.69	0.35	1.37
PC(O-32:2)	0.376	0.376	0.19	0.01	7.42
PC(O-34:1)	0.085	0.112	0.55	0.28	1.09
PC(O-34:2)	0.174	0.202	0.63	0.32	1.23
PC(O-34:3)	0.166	0.200	0.62	0.32	1.22
PC(O-34:4)	0.071	0.098	0.48	0.22	1.07
PC(O-35:4)	0.151	0.190	0.58	0.27	1.22
PC(O-36:0)	0.034	0.055	0.02	0.00	0.74
PC(O-36:1)	0.022	**0.045**	0.37	0.16	0.87
PC(O-36:2)	0.027	**0.049**	0.42	0.19	0.91
PC(O-36:3)	0.046	0.071	0.47	0.22	0.99
PC(O-36:4)	0.023	**0.045**	0.51	0.29	0.91
PC(O-36:5)	0.065	0.095	0.17	0.03	1.12
PC(O-38:4)	0.013	**0.044**	0.49	0.28	0.86
PC(O-38:5)	0.016	**0.044**	0.46	0.25	0.87
PC(O-40:7)	0.013	**0.044**	0.40	0.20	0.82
PC(P-32:0)	0.012	**0.044**	0.38	0.18	0.80
PC(P-32:1)	0.214	0.238	0.40	0.09	1.70
PC(P-34:1)	0.014	**0.044**	0.34	0.15	0.81
PC(P-34:2)	0.018	**0.044**	0.39	0.18	0.85
PC(P-34:3)	0.352	0.365	0.08	0.00	16.85
PC(P-36:2)	0.020	**0.045**	0.29	0.10	0.82
PC(P-36:4)	0.010	**0.044**	0.42	0.22	0.81
PC(P-36:5)	0.017	**0.044**	0.17	0.04	0.73
PC(P-38:4)	0.003	**0.022**	0.34	0.17	0.70
PC(P-38:5)	0.002	**0.021**	0.29	0.13	0.64
PC(P-38:6)	0.002	**0.021**	0.22	0.08	0.57
PC(P-40:6)	<0.001	**0.003**	0.07	0.02	0.26

[a] Uncorrected following Cox analysis; [b] Benjamini-Hochberg corrected, bolded text denotes significance; [c] Hazard ratios for an increase in serum concentration of 1 std deviation.

Table 4: Comparing ROC curves of the best traditional risk factor, lipidomic and combined models.

Model	**AUC**	**95% CI**	***p-value***
Traditional risk factor[a]	0.722	0.605-0.840	Reference
Lipidomic[b]	0.805	0.721-0.890	0.249
Combined[c]	0.835	0.745-0.925	0.033

AUC: Area under the ROC curve; p-values obtained following DeLong's test for two paired ROC curves.

[a]Comprising age, diabetes and serum LDL-C and triglyceride concentrations.

[b]Comprising serum PC(O-38:5), PC(O-40:7), PC(P-36:5) and PC(P-40:6) concentrations.

[c]Comprising age, diabetes, and serum concentrations of LDL-C, triglycerides and PC(P-40:6).

Baseline serum phosphatidylcholine plasmalogen concentrations are inversely associated with incident myocardial infarction in patients with mixed peripheral artery disease presentations

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SUPPLEMENTARY MATERIAL.

Supplementary file 1: Detailed materials and methods.

Supplementary Table 1: Conditions for tandem mass spectrometry analysis of lipid species

Supplemental Table 2. Correlations between plasma concentrations of individual lipid species and circulating concentrations of their constituent functional class.

Supplemental Table 3. Characteristics of AAA and atherothrombotic patients included in this study.

Supplemental Table 4. Association of PC(O) and PC(P) species with MI in 265 patients with peripheral artery disease and correlation with circulating lipoprotein fractions

Supplemental Table 5. Multimodel inference comparison of the 25 highest ranked traditional risk factor, lipidomic and combined models for MI prediction, and respective ROC analyses.

Supplemental Table 6: Leave one covariate out sensitivity analysis assessing additive value of measuring serum PC(P-40:6) concentration to predict incident MI in the PAD patient cohort.

Supplemental Table 7: *Post-hoc* sample size analyses calculating the power of the current study to detect inter-group differences in serum concentrations of key lipid biomarkers.

Supplementary file 1: Detailed materials and methods.

Patient recruitment and follow-up: Participants in the current study had been involved in a recent investigation to characterise the serum lipidome in patients with a range of peripheral artery disease (PAD) presentations.[1] Patients were recruited from out-patient clinics at the Townsville and Mater Hospitals, as previously described.[2,3] Patients either had symptomatic lower limb atherothrombosis or an abdominal aortic aneurysm (AAA). Lower limb athero-thrombosis was diagnosed based on an appropriate history of lower limb symptoms, a clinical examination confirming absent or reduced lower limb pulses or a imaging demonstrating stenosis or occlusion of lower limb arteries as previously described.[3] AAA was defined by a maximal infra-renal aortic diameter ≥30mm from imaging assessment as previously described.[4] All patients provided written informed consent at the time of recruitment permitting the use of biological samples and clinical data for research purposes. This study was conducted in accordance with the Declaration of Helsinki and received approval from the Human Research Ethics Committees at James Cook University, The Townsville Hospital and the Mater Misercordiae Hospital, Townsville.

Clinical risk factors and medications: Characteristics collected for each patient included sex, age, history of hypertension, diabetes, coronary heart disease (CHD), smoking and prescribed medications as previously described.[1] Diabetes, dyslipidemia and hypertension were defined by a prior history of diagnosis or treatment of these conditions. Smoking status was defined as having ever or never smoked. CHD diagnosis was based on a history of angina, myocardial infarction or coronary artery revascularisation. Body mass index (BMI) was calculated as weight in kg divided by height in m^2.[3] Current prescriptions for aspirin,

calcium channel blockers, beta-blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers, anti-diabetic agents and lipid modifying drugs were noted.

Serum analysis: Fasting venous blood samples were taken from all patients. After coagulation, blood was centrifuged (4,500*g* for 12 minutes), serum was decanted and stored at -80^{o}C until analysed. Circulating concentrations of total cholesterol, HDL-C, LDL-C and triglycerides were assessed by automated assays as previously described.[5] Serum very low density lipoprotein cholesterol (VLDL-C) concentrations were estimated by subtracting combined HDL-C and LDL-C concentrations from total cholesterol for each patient. Serum C-reactive protein (CRP) concentration was assessed using an enhanced turbidimetric assay as previously described.[5] The mass spectrometry methods used to measure serum concentrations of individual lipid species in these patients have been previously described in detail.[6]

Briefly, samples were randomised then extracted in a single phase extraction with 20 volumes of $CHCl_3$:MeOH (2:1) and 10 µL of an internal standard mix that contained between 50 and 1000 pmol each of 17 non-physiological and stable isotope labelled lipid standards. Following centrifugation, the extracted lipids were dried and reconstituted in water saturated BuOH (50 µL) and then mixed with MeOH (50 µL) prior to analysis by electrospray ionisation tandem mass spectrometry using a Agilent 1200 liquid chromatography system combined with an Applied Biosystems API 4000 Q/TRAP mass spectrometer with a turbo-ionspray source (350^{o}C) and Analyst 1.5 data system. Multiple Reaction Monitoring (MRM) experiments were used for 332 lipid species, which had been previously identified in plasma [6]. Relative lipid amounts were calculated by relating the peak area of each species to the peak area of the corresponding stable isotope or non-physiological internal standard (Table 1). Total lipid classes were calculated from the sum of the individual lipid species within each class.

Liquid chromatography was performed on a Zorbax C18, 1.8 mm, 50 × 2.1 mm column (Agilent Technologies), heated to 50^{o}C. Solvents A and B consisted of tetrahydrofuran:methanol:water in the ratio (20:20:50) and (75:20:5) respectively, both containing 10 mM ammonium formate. All lipid species (5mL injection) were separated under gradient conditions (300 µL/min) 0% B to 38.7% B over 2.0 min, then to 100%B over the next 6.0 min, 2.0 mins at 100% B, a return to 0% B over 0.5 min then 3.5 min at 0% B prior to the next injection.

Recording outcome data: Patients were followed up through attendance at out-patient clinics and/or as an in-patient as part of their standard care as previously described.[3] Patients with lower limb athero-thrombosis were generally reviewed 6 months after initial assessment, and then yearly unless symptoms changed.[2,7,8] Patients with small aneurysms, or large aneurysms that had been repaired were followed up yearly, or at 6 monthly intervals if the aneurysm was nearing a diameter at which intervention was indicated.[9,10] The primary outcome measure for this study was hospital admission for MI based on a positive diagnosis by a Royal Australian College of Physicians accredited physician in line with international guidelines.[11] Charts and hospital electronic records of all patients were reviewed at least once for the identification and date of any outcome events by a vascular specialist or research nurse. Where uncertainty about outcome data was present a discussion occurred between the research nurse and vascular specialist and a consensus was reached. For patients that had not experienced the primary outcome, follow-up was concluded, i.e. censoring occurred, at the date of last in or out-patient review.

Statistical analyses: Differences between groups of patients with AAA or lower limb atherothrombosis, and patients who did and did not suffer MI were assessed using the Mann-

Whitney U test and Chi-squared test. Correlation between variables was assessed using a 2-tailed Spearman rho test.

Initial analyses demonstrated that plasma concentrations of all of the detected lipid species showed highly significant positive correlations with the calculated plasma concentrations for their respective functional class (all p-values <0.001; Supplementary Table 2). Based on the strength of these associations, we reasoned that lipids with biomarker potential would be highlighted in an initial screen determining the association of each functional lipid class with incident MI in unadjusted Cox Regression. This led to secondary analyses whereby the association of lipid species within MI-associated functional classes was assessed using Cox regression models adjusted for age, gender, smoking, diabetes, hypertension, CHD, AAA and dyslipidemia as these are recognised outcome determinants in patients with cardiovascular disease.[3] For all of these analyses, p-values were adjusted for multiple testing using the approach of Benjamini and Hochberg, accepting a false discovery rate of 5%, q values <0.05 were considered significant. Univariate analyses and Cox regression were conducted using SPSS v.21 (IBM).

The potential for serum lipids to improve prediction of MI risk in PAD patients beyond traditional risk factors was assessed via multi-model inference analysis using the R-based MuMIn package.[12] Three types of Cox proportional hazards-based models were constructed from panels comprising: 1) traditional risk factors (age, gender, BMI, smoking, diabetes, hypertension, CHD, AAA, statins, and serum concentrations of HDL-C, LDL-C, VLDL-C, triglycerides and CRP) [risk factor models]; 2) Lipid species only [lipidomic models]; and 3) all parameters in the first 2 groups [combined models]. The ability of all possible combinations of up to 6 covariates within each panel to predict MI incidence was assessed.

The goodness of fit of all generated models was assessed using Akaike's second-order information criterion (AICc). Models were ranked by ascending AICc values with smaller AICc values denoting better model fit. Models showing an increase in AIC $\geq$2 relative to the highest ranked model were considered to have significantly poorer performance. For each covariate group, the relative importance of each variable in predicting MI incidence was assessed by averaging coefficients across the 25 most highly ranked models. The proportionality assumption was assessed for all Cox regression models presented in the current study; no violations were observed.

The performance of the highest ranked traditional risk factor, lipidomic and combined models was compared via receiver operator characteristic (ROC) curves using the R based pROC package as directed by the developers.[13] The biomarker potential of identified serum lipid species was assessed in sensitivity analyses, systematically removing individual covariates from the highest ranked combined model and investigating the impact on the AUC of the ROC curve.

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Supplementary Table 1: Conditions for tandem mass spectrometry analysis of lipid species

Lipid class or subclass	No. of species	Internal standard	Pmol[a]	Parent ion	Experiment[b]	Voltage settings[c]			
						DP	EP	CollE	CXP
Dihydroceramide (dhCer)	6	dhCer 8:0	100	$[M+H]^+$	PI, 284.3 *m/z*	90	10	39	18
Ceramide (Cer)	6	Cer 17:0	100	$[M+H]^+$	PI, 264.3 *m/z*	50	10	35	12
Monohexocylceramide (MHC)	6	MHC 16:0 d_3	50	$[M+H]^+$	PI, 264.3 *m/z*	77	10	50	12
Dihexosylceramide (DHC)	6	DHC 16:0 d_3	50	$[M+H]^+$	PI, 264.3 *m/z*	100	10	65	12
Trihexosylceramide (THC)	4	THC 17:0	50	$[M+H]^+$	PI, 264.3 *m/z*	130	10	73	12
G_{M3} ganglioside (GM3)	6	THC 17:0	50	$[M+H]^+$	PI, 264.3 *m/z*	155	10	105	16
Sphingomyelin (SM)	20	SM 12:0	200	$[M+H]^+$	PI, 184.1 *m/z*	65	10	45	11
Phosphatidylcholine (PC)	51	PC 13:0/13:0	100	$[M+H]^+$	PI, 184.1 *m/z*	100	10	45	11
Alkylphosphatidylcholine (PC(O))	17	PC 13:0/13:0	100	$[M+H]^+$	PI, 184.1 *m/z*	100	10	45	11
Alkenylphosphatidylcholine (PC(P))	12	PC 13:0/13:0	100	$[M+H]^+$	PI, 184.1 *m/z*	100	10	45	11
Lysophosphatidylcholine (LPC)	22	LPC 13:0	100	$[M+H]^+$	PI, 184.1 *m/z*	90	10	38	12
Lysoalkylphosphatidylcholine (LPC(O))	9	LPC 13:0	100	$[M+H]^+$	PI, 285.2 *m/z*	90	10	42	5
Phosphatidylethanolamine (PE)	20	PE 17:0/17:0	100	$[M+H]^+$	NL, 141 Da	80	10	31	7
Alkylphosphatidylethanolamine (PE(O))	12	PE 17:0/17:0	100	$[M+H]^+$	NL, 141 Da	80	10	31	7
Alkenylphosphatidylethanolamine (PE(P))	11	PE 17:0/17:0	100	$[M+H]^+$	NL, 141 Da	80	10	31	7
Lysophosphatidylethanolamine (LPE)	6	PE 14:0/0:0	100	$[M+H]^+$	NL, 141 Da	80	10	31	7
Phosphatidylinositol (PI)	13	PE 17:0/17:0	100	$[M+NH_4]^+$	NL, 277 *m/z*	51	10	43	14
Lysophosphatidylinositol (LPI)	4	PE 14:0/0:0	100	$[M+NH_4]^+$	NL, 277 *m/z*	51	10	43	14
Phosphatidylserine (PS)	4	PS 17:0/17:0	100	$[M+H]^+$	NL, 185 Da	86	10	29	16
Phosphatidylglycerol (PG)	1	PG 17:0/17:0	100	$[M+NH_4]^+$	NL, 189 Da	60	10	25	12
Cholesterol ester (CE)	24	CE 18:0 d_6	1000	$[M+NH_4]^+$	PI, 369.3 *m/z*	30	10	20	12
Free cholesterol (COH)	1	COH d_7	1000	$[M+NH_4]^+$	PI, 369.3 *m/z*	55	10	17	12
Diacylglycerol (DG)	26	DG 15:0/15:0	200	$[M+NH_4]^+$	NL, fatty acid	55	10	30	22
Triacylglycerol (TG)	44	TG 17:0/17:0/17:0	100	$[M+NH_4]^+$	NL, fatty acid	95	10	30	12
Bis(monoglycerol)phosphate (BMP)	1	BMP 14:0/14:0	100	$[M+NH_4]^+$	NL, fatty acid dependent	65	10	35	5

[a] Amount of internal standard per sample

[b] PI = precursor ion, NL = neutral loss.

[c] DP = declustering potential (volts); EP = entrance potential (volts); CollE = collision energy (volts); CXP = collision cell exit potential (volts).

Supplementary Table 2: Correlations between plasma concentrations of individual lipid species and circulating concentrations of their constituent functional class.

Lipid class	Number of species	Correlation coefficient*
Bis(monoacylglycero)phosphate (BMP)	1	1.000**
Ceramide (Cer)	6	0.836 (0.789-0.943)
Dihydroceramide (dhCer)	6	0.874 (0.801-0.956)
Monohexosylceramide (MHC)	6	0.950 (0.556-0.970)
Dihexosylceramide (DHC)	6	0.763 (0.619-0.987)
Trihexosylceramide (THC)	4	0.952 (0.873-0.955)
G_{M3} ganglioside (GM3)	6	0.901 (0.864-0.923)
Sphingomyelin (SM)	20	0.820 (0.800-0.859)
Phosphatidylcholine (PC)	51	0.656 (0.470-0.738)
Alkylphosphatidylcholine (PC(O))	17	0.699 (0.608-0.847)
Phosphatidylicholine plasmalogen (PC(P))	12	0.782 (0.692-0.818)
Lysophosphatidylcholine (LPC)	22	0.777 (0.721-0.855)
Lysoalkylphosphatidylethanolamine (LPC(O))	9	0.828 (0.763-0.928)
Phosphatidylethanolamine (PE)	20	0.856 (0.771-0.900)
Alkylphosphatidylethanolamine (PE(O))	12	0.840 (0.716-0.902)
Phosphatidylethanolamine plasmalogen (PE(P))	11	0.783 (0.748-0.857)
Lysophosphatidylethanolamine (LPE)	6	0.848 (0.687-0.865)
Phosphatidylinositol (PI)	13	0.849 (0.765-0.866)
Lysophosphatidylinositol (LPI)	4	0.812 (0.740-0.867)
Phosphatidylglycerol (PG)	1	1.000**
Phosphatidylserine (PS)	4	0.808 (0.670-0.892)
Cholesterol ester (CE)	24	0.767 (0.659-0.832)
Free cholesterol (COH)	1	1.000**
Diacylglycerol (DG)	26	0.837 (0.730-0.875)
Triacylglycerol (TG)	44	0.865 (0.798-0.896)

*Spearman's Rho detailing the correlation between the plasma concentrations of the assessed lipid species and total plasma concentrations of lipids belonging to that functional group. Data are shown as median and inter-quartile range All correlation had p-values <0.001.

** Only 1 lipid species assessed, therefore interquartile range is not applicable.

Supplemental Table 3. Characteristics of AAA and atherothrombotic patients included in this study

Characteristic	AAA patients (n=121)	Lower limb atherothrombosis patients (n=144)	P value*	Combined (n=265)
Aortic diameter (mm)	54.0 (51.0-62.0)	20.0 (18.0-23.0)	<0.001	27.7 (20.0-53.0)
Male	85 (70.2%)	101 (70.1%)	0.985	186 (70.2%)
Age (years)	72.0 (66.0-76.0)	67.2 (61.0-74.0)	<0.001	69.3 (64.0-75.0)
BMI (kg/m^2)	27.7 (25.1-30.4)	27.7 (24.1-31.4)	0.632	27.7 (24.5-30.6)
Diabetes mellitus	16 (13.2%)	44 (30.6%)	0.001	60 (22.6%)
Ever smoker	104 (86.0%)	123 (85.4%)	0.902	227 (85.7%)
CHD	65 (53.7%)	57 (39.6%)	0.021	122 (46.0%)
Hypertension	96 (79.3%)	100 (69.4%)	0.068	196 (74.0%)
MI	6 (5.0%)	12 (8.3%)	0.277	18 (6.8%)
Serum concentrations of:				
Creatinine (μM)	91.0 (75.0-108.5)	79.0 (66.0-91.0)	<0.001	83.0 (70.0-100.0)
Cholesterol (mM)	4.5 (3.7-5.3)	4.5 (3.7-5.5)	0.618	4.5 (3.7-5.4)
Triglycerides (mM)	1.6 (1.2-2.5)	1.7 (1.0-2.3)	0.700	1.6 (1.1-2.3)
HDL-C (mM)	1.1 (0.9-1.4)	1.2 (1.0-1.5)	0.006	1.2 (1.0-1.5)
LDL-C (mM)	2.4 (1.9-3.2)	2.6 (1.7-3.3)	0.822	2.5 (1.8-3.2)
VLDL-C (mM)	0.8 (0.6-1.0)	0.7 (0.5-1.1)	0.679	0.7 (0.5-1.0)
CRP (mg/L)	5.0 (2.9-9.3)	3.0 (1.2-5.0)	<0.001	4.0 (2.0-8.0)
Prescription for:				
Aspirin	79 (65.3%)	104 (72.2%)	0.224	183 (69.1%)
CCB	45 (37.2%)	38 (26.4%)	0.059	83 (31.3%)
Beta-blockers	52 (43.0%)	41 (28.5%)	0.014	93 (35.1%)
ACEI	52 (43.0%)	53 (36.8%)	0.306	105 (39.6%)
ARB	20 (16.5%)	28 (19.4%)	0.539	48 (18.1%)
Statins	84 (69.4%)	89 (61.8%)	0.195	173 (65.3%)
Fibrates	2 (1.7%)	4 (2.8%)	0.540	6 (2.3%)
Metformin	8 (6.6%)	28 (19.4%)	0.002	36 (13.6%)
Other oral hypoglycaemic drugs	8 (6.6%)	16 (11.1%)	0.204	24 (9.1%)

Shown are number (%) for nominal variables and median (inter-quartile range) for continuous variables. Nominal variables are compared by Chi-squared test and continuous variables by Mann Whitney U test. AAA = Abdominal aortic aneurysm; BMI = Body mass index; CHD = Coronary heart disease; CCB = Calcium channel blocker; ACEI = Angiotensin converting enzyme inhibitor; ARB = Angiotensin receptor blocker; MI = Myocardial infarction. * Comparing AAA and atherothrombotic patients.

Supplemental Table 4. Association of PC(O) and PC(P) species with MI in 265 patients with peripheral artery disease and correlation with circulating lipoprotein fractions.

Lipid	Association with MI in PAD patients					Correlation with circulating concentrations of							
	p-value[†]	Corrected p-value[±]	Hazard ratio[‡]	95% CI		HDL-C		LDL-C		VLDL-C**		Triglycerides	
				Lower	Upper	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value
PC(O-32:0)	3.06×10^{-2}	5.22×10^{-2}	0.48	0.25	0.93	--	--	--	--	--	--	--	--
PC(O-32:1)	2.93×10^{-1}	3.15×10^{-1}	0.69	0.35	1.37	--	--	--	--	--	--	--	--
PC(O-32:2)	3.76×10^{-1}	3.76×10^{-1}	0.19	0.01	7.42	--	--	--	--	--	--	--	--
PC(O-34:1)	8.53×10^{-2}	1.12×10^{-1}	0.55	0.28	1.09	--	--	--	--	--	--	--	--
PC(O-34:2)	1.74×10^{-1}	2.02×10^{-1}	0.63	0.32	1.23	--	--	--	--	--	--	--	--
PC(O-34:3)	1.66×10^{-1}	2.00×10^{-1}	0.62	0.32	1.22	--	--	--	--	--	--	--	--
PC(O-34:4)	7.12×10^{-2}	9.84×10^{-2}	0.48	0.22	1.07	--	--	--	--	--	--	--	--
PC(O-35:4)	1.51×10^{-1}	1.90×10^{-1}	0.58	0.27	1.22	--	--	--	--	--	--	--	--
PC(O-36:0)	3.39×10^{-2}	5.45×10^{-2}	0.02	0.00	0.74	--	--	--	--	--	--	--	--
PC(O-36:1)	2.25×10^{-2}	**4.45×10^{-2}**	0.37	0.16	0.87	0.26	<0.001	0.29	<0.001	-0.11	0.072	-0.10	0.122
PC(O-36:2)	2.71×10^{-2}	**4.92×10^{-2}**	0.42	0.19	0.91	0.29	<0.001	0.44	<0.001	-0.09	0.134	-0.09	0.125
PC(O-36:3)	4.63×10^{-2}	7.07×10^{-2}	0.47	0.22	0.99	--	--	--	--	--	--	--	--
PC(O-36:4)	2.30×10^{-2}	**4.45×10^{-2}**	0.51	0.29	0.91	0.15	0.015	0.28	<0.001	-0.13	0.040	-0.12	0.046
PC(O-36:5)	6.53×10^{-2}	9.47×10^{-2}	0.17	0.03	1.12	--	--	--	--	--	--	--	--
PC(O-38:4)	1.35×10^{-2}	**4.38×10^{-2}**	0.49	0.28	0.86	0.22	<0.001	0.35	<0.001	-0.10	0.092	-0.09	0.127
PC(O-38:5)	1.59×10^{-2}	**4.38×10^{-2}**	0.46	0.25	0.87	0.14	0.021	0.26	<0.001	-0.18	0.003	-0.19	0.002
PC(O-40:7)	1.27×10^{-2}	**4.38×10^{-2}**	0.40	0.20	0.82	0.10	0.109	0.16	0.011	-0.07	0.283	-0.05	0.409
PC(P-32:0)	1.16×10^{-2}	**4.38×10^{-2}**	0.38	0.18	0.80	0.29	<0.001	0.44	<0.001	-0.19	0.002	-0.18	0.003
PC(P-32:1)	2.14×10^{-1}	2.38×10^{-1}	0.40	0.09	1.70	--	--	--	--	--	--	--	--
PC(P-34:1)	1.42×10^{-2}	**4.38×10^{-2}**	0.34	0.15	0.81	0.48	<0.001	0.40	<0.001	-0.25	<0.001	-0.26	<0.001
PC(P-34:2)	1.81×10^{-2}	**4.38×10^{-2}**	0.39	0.18	0.85	0.23	<0.001	0.40	<0.001	-0.10	0.117	-0.10	0.090
PC(P-34:3)	3.52×10^{-1}	3.65×10^{-1}	0.08	0.00	16.85	--	--	--	--	--	--	--	--
PC(P-36:2)	2.01×10^{-2}	**4.45×10^{-2}**	0.29	0.10	0.82	0.31	<0.001	0.38	<0.001	-0.11	0.086	-0.12	0.061
PC(P-36:4)	9.57×10^{-3}	**4.38×10^{-2}**	0.42	0.22	0.81	0.16	0.012	0.23	<0.001	-0.10	0.103	-0.10	0.101
PC(P-36:5)	1.69×10^{-2}	**4.38×10^{-2}**	0.17	0.04	0.73	0.19	0.002	0.12	0.045	-0.01	0.924	0.02	0.762
PC(P-38:4)	3.08×10^{-3}	**2.23×10^{-2}**	0.34	0.17	0.70	0.22	<0.001	0.29	<0.001	-0.01	0.929	0.01	0.824
PC(P-38:5)	2.17×10^{-3}	**2.10×10^{-2}**	0.29	0.13	0.64	0.21	0.001	0.22	<0.001	-0.14	0.024	-0.13	0.038
PC(P-38:6)	1.82×10^{-3}	**2.10×10^{-2}**	0.22	0.08	0.57	0.16	0.011	0.18	0.003	-0.10	0.104	-0.08	0.181
PC(P-40:6)	1.02×10^{-4}	**2.96×10^{-3}**	0.07	0.02	0.26	0.21	<0.001	0.20	0.001	-0.07	0.287	-0.05	0.448

[†]Uncorrected p-value from Cox analysis adjusted for age, gender, smoking, diabetes, hypertension, CHD, AAA and dyslipidaemia; [±] Benjamini-Hochberg corrected, bolded text denotes significance; [‡]Hazard ratios for an increase in serum concentration of 1 std deviation; [§]Denotes Spearman rho value. ** VLDL

concentrations estimated by subtracting combined HDL-C and LDL-C from total cholesterol. Correlation with circulating lipoproteins and triglycerides is assessed in MI-associated lipids only.

Supplemental Table 5. Multimodel inference comparison of the 25 highest ranked traditional risk factor, lipidomic and combined models for MI prediction, and respective ROC analyses.

Model Rank	Traditional risk factor model				Lipidomic model				Combined model			
	Included features	**AICc**	**Delta**	**AUC (95% CI)**	**Included features**	**AICc**	**Delta**	**AUC (95% CI)**	**Included features**	**AICc**	**Delta**	**AUC (95% CI)**
1	1/7	157.38	0.00	0.658 (0.532-0.785)	16/22/24	147.20	0.00	0.795 (0.705-0.885)	1/7/8/9/24	138.09	0.00	0.831 (0.742-0.920)
2	1/4/7	157.75	0.37	0.700 (0.574-0.825)	15/16/24	148.01	0.82	0.799 (0.706-0.893)	1/7/9/24	138.43	0.34	0.822 (0.724-0.920)
3	7/8	157.76	0.38	0.674 (0.539-0.809)	12/16/24	148.14	0.94	0.786 (0.683-0.890)	1/7/9/16/24	138.92	0.83	0.823 (0.725-0.920)
4	1/7/8	157.91	0.53	0.671 (0.537-0.806)	12/16/22/24	148.23	1.03	0.793 (0.702-0.883)	1/7/8/10/24	139.06	0.97	0.828 (0.737-0.920)
5	1/7/9	157.97	0.59	0.681 (0.564-0.797)	14/16/24	148.33	1.14	0.791 (0.691-0.892)	1/7/8/16/24	139.45	1.36	0.821 (0.724-0.917)
6	7	158.11	0.73	0.660 (0.534-0.786)	24	148.41	1.21	0.780 (0.677-0.883)	1/3/7/9/24	139.53	1.45	0.820 (0.722-0.918)
7	1/4/7/8	158.20	0.82	0.701 (0.571-0.832)	11/16/22/24	148.43	1.23	0.797 (0.708-0.886)	1/7/10/24	139.71	1.62	0.818 (0.719-0.917)
8	1/6/7	158.27	0.89	0.663 (0.531-0.795)	16/17/22/24	148.47	1.28	0.796 (0.706-0.886)	1/4/8/9/24	139.72	1.63	0.835 (0.754-0.916)
9	4/7/8	158.29	0.91	0.700 (0.569-0.830)	16/24	148.55	1.36	0.784 (0.682-0.887)	1/7/10/16/24	139.77	1.68	0.822 (0.724-0.920)
10	1/2/7	158.45	1.07	0.671 (0.556-0.787)	13/16/24	148.62	1.42	0.793 (0.694-0.892)	1/7/8/24	139.82	1.74	0.816 (0.720-0.913)
11	1/3/7	158.51	1.13	0.674 (0.547-0.802)	11/16/24	148.64	1.44	0.786 (0.682-0.890)	**1/4/7/9/24**	**139.85**	**1.76**	**0.835 (0.745-0.925)**
12	1/3/4/7	158.58	1.20	0.701 (0.571-0.831)	15/16/22/24	148.69	1.49	0.802 (0.714-0.889)	1/7/9/24/25	139.86	1.77	0.822 (0.721-0.923)
13	1/7/10	158.60	1.22	0.679 (0.562-0.797)	16/18/22/24	148.77	1.57	0.785 (0.685-0.884)	1/7/9/23/24	139.91	1.82	0.822 (0.723-0.922)
14	**1/4/7/9**	**158.67**	**1.29**	**0.722 (0.605-0.840)**	14/16/22/24	148.79	1.60	0.796 (0.706-0.886)	1/4/8/16/24	140.03	1.94	0.819 (0.729-0.909)
15	1/7/8/9	158.70	1.32	0.699 (0.580-0.818)	12/24	148.87	1.68	0.778 (0.673-0.882)	1/4/9/16/24	140.09	2.00	-
16	3/7/8	158.73	1.35	0.678 (0.541-0.814)	16/19/22/24	148.95	1.75	0.792 (0.702-0.882)	1/7/9/24/29	140.10	2.01	-
17	3/7	158.76	1.38	0.671 (0.542-0.800)	16/22/23/24	149.05	1.85	0.792 (0.696-0.889)	1/4/9/24	140.17	2.08	-
18	2/7/8	158.76	1.38	0.685 (0.564-0.806)	**15/16/21/24**	**149.06**	**1.87**	**0.805 (0.721-0.890)**	1/8/9/24	140.20	2.11	-
19	1/2/7/8	158.81	1.43	0.685 (0.564-0.807)	16/17/24	149.07	1.88	0.788 (0.688-0.889)	1/7/9/10/24	140.22	2.13	-
20	4/7	158.90	1.51	0.698 (0.571-0.824)	11/15/16/24	149.14	1.94	0.797 (0.702-0.892)	1/7/9/21/24	140.35	2.26	-
21	1/5/7/8	158.90	1.52	0.676 (0.550-0.802)	13/16/22/24	149.15	1.95	0.792 (0.701-0.884)	1/7/9/24/28	140.35	2.26	-
22	1/4/6/7	158.97	1.59	0.702 (0.569-0.834)	12/16/21/24	149.16	1.96	0.791 (0.694-0.888)	1/5/7/9/24	140.38	2.29	-
23	1/6/7/8	158.98	1.60	0.679 (0.541-0.818)	11/24	149.16	1.96	0.779 (0.672-0.885)	1/7/9/22/24	140.38	2.29	-
24	1/5/7	159.00	1.62	0.662 (0.542-0.783)	19/24	149.16	1.97	0.778 (0.673-0.882)	1/7/9/24/27	140.40	2.31	-
25	3/4/7/8	159.06	1.68	0.700 (0.566-0.834)	16/20/22/24	149.17	1.98	0.792 (0.701-0.884)	1/7/9/24/26	140.40	2.31	-

AICc: Akaike's second order information criterion; Delta: difference in AICc between each model and the highest ranked model in each group (shaded cells = delta >2); AUC (95% CI): Area under the ROC curve and 95% confidence intervals (only calculated for models with delta values ≤2). Models with the highest AUC for each series were considered to have the best performance (shown in bold text). Note actual AUC for combined model 8: 0.8349; AUC for combined model 11: 0.8351; thus combined model 11 selected was as the best performing.

Key of covariates: 1) Age; **2)** BMI; **3)** Serum CRP concentration; **4)** Diabetes; **5)** Sex; **6)** Serum HDL-C concentration; **7)** Serum LDL-C concentration; **8)** Smoking; **9)** Serum triglyceride concentration; **10)** Serum VLDL-C concentration; **11)** Serum PC(O-36:1) concentration; **12)** PC(O-36:2) concentration; **13)** Serum PC(O-36:4) concentration; **14)** Serum PC(O-38:4) concentration; **15)** Serum PC(O-38:5) concentration; **16)** Serum PC(O-40:7) concentration; **17)** Serum PC(P-32:0) concentration; **18)** Serum PC(P-34:1) concentration; **19)** Serum PC(P-36:2) concentration; **20)** Serum PC(P-36:4) concentration; **21)** Serum PC(P-

36:5) concentration; **22)** Serum PC(P-38:5) concentration; **23)** Serum PC(P-38:6) concentration; **24)** Serum PC(P-40:6) concentration; **25)** AAA; **26)** Hypertension; **27)** Coronary heart disease; **28)** Serum PC(P-34:2) concentration; **29)** Statin prescription.

Supplemental Table 6: Leave one covariate out sensitivity analysis assessing additive value of measuring serum PC(P-40:6) concentration to predict incident MI in the PAD patient cohort.

Covariate removed	AUC	95% CI	p-value*
None (complete model)†	0.835	0.745-0.925	Reference
Age	0.830	0.736-0.923	0.701
Diabetes	0.822	0.724-0.920	0.408
Serum LDL-C concentration	0.827	0.740-0.913	0.508
Serum triglyceride concentration	0.812	0.716-0.909	0.173
Serum PC(P-40:6) concentration	0.722	0.605-0.840	0.033

*P value obtained after comparing the AUC of the ROC curve of the covariate removed model to the complete model (DeLong's test for two paired ROC curves).

† Complete model includes age, diabetes and serum concentrations of LDL-C, triglycerides and PC(P-40:6)

Supplemental Table 7: *Post-hoc* sample size analyses calculating the power of the current study to detect inter-group differences in serum concentrations of key lipid biomarkers.

Lipid assessed	Mean plasma concentration ±std deviation		Effect size*	Power*
	No MI (n=247)	MI (n=18)		
Total PC(O) (nMol/mL)	36.3 ±9.6	29.8 (±8.8)	0.71	0.82
Total PC(P) (nMol/mL)	24.4 ±7.2	18.6 (±4.6)	0.96	0.97
PC(P-40:6) (pMol/mL)	565.1 ±331.2	342.5 ±135.0	0.88	0.95

*Calculated with G*Power v 3.1.9.2. All tests use a 2-tailed alpha of 0.05.