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## High-frequency metabolite profiling and the incidence of recurrent cardiac events in patients with post-acute coronary syndrome

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### ABSTRACT

**Purpose:** The aim of this study was to study temporal changes in metabolite profiles in patients with post-acute coronary syndrome (ACS), in particular prior to the development of recurrent ACS (reACS).

**Methods:** BIOMArCS (BIOMarker study to identify the Acute risk of a Coronary Syndrome) is a prospective study including patients admitted for ACS, who underwent high-frequency blood sampling during 1-year follow-up. Within BIOMArCS, we performed a nested case-cohort analysis of 158 patients (28 cases of reACS). We determined 151 metabolites by nuclear magnetic resonance in seven (median) blood samples per patient. Temporal evolution of the metabolites and their relation with reACS was assessed by joint modelling. Results are reported as adjusted (for clinical factors) hazard ratios (aHRs).

**Results:** Median age was 64 (25th–75th percentiles; 56–72) years and 78% were men. After multiple testing correction ( $p < 0.001$ ), high concentrations of extremely large very low density lipoprotein (VLDL) particles (aHR 1.60/SD increase; 95%CI 1.25–2.08), very large VLDL particles (aHR 1.60/SD increase; 95%CI 1.25–2.08) and large VLDL particles (aHR 1.56/SD increase; 95%CI 1.22–2.05) were significantly associated with reACS. Moreover, these longitudinal particle concentrations showed a steady increase over time prior to reACS. Among the other metabolites, no significant associations were observed.

**Conclusion:** Post-ACS patients with persistent high concentrations of extremely large, very large and large VLDL particles have increased risk of reACS within 1 year.

### ARTICLE HISTORY

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### KEYWORDS

Atherosclerosis; repeated measurements; lipids; metabolite kinetics; VLDL



### Introduction

In recent years, the rise of genomics has helped to unravel the human genome and to identify genes that are involved with the development of cardiovascular disease (CVD) (O'Donnell and Nabel 2011). However, CVD is a polygenic and multifactorial disease, which is both influenced by a patient's genetic predisposition and affected by biological and chemical variation downstream of the genetic code. Although genomic research concentrates on the 'static' genotype of a patient, metabolomic research focuses on metabolites, which are the substrates or end-products of all enzymatic processes (Shah and Newgard 2015). Metabolomic research creates a blueprint of a patient's metabolism at a specific time point and, accordingly, captures both the upstream influence of a patient's genotype and downstream variation influencing the metabolism (Shah and Newgard 2015). Eventually, combining knowledge gained through metabolomic research with knowledge

on genetics and clinical risk factors may give rise to novel insights on the pathophysiology of CVD.

### Clinical significance

- Repeatedly measured metabolites carry incremental prognostic information in patients with post-ACS prior to a recurrent ACS event over a single baseline measurement.
- Extremely large VLDL, very large VLDL and large VLDL particle concentrations steadily increase prior to a recurrent ACS in patients with post-ACS.
- Higher extremely large VLDL, very large VLDL and large VLDL particle concentrations in patients with post-ACS are associated with a higher risk of developing reACS within 1 year after initial ACS admission.

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The number of longitudinal studies that have assessed the association between a patient's metabolite profile and development of CVD is increasing (Ruiz-Canela *et al.* 2017). However, these studies relate single baseline measurements to the incidence of CVD events during long-term follow-up (Ruiz-Canela *et al.* 2017). Since the metabolite profile of patients with CVD is not a static given, but will likely be influenced by changes in disease severity over time, repeated metabolite profile measurements might carry incremental prognostic information over a single measurement.

We designed the 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS) to study temporal biomarker changes in patients with post-acute coronary syndrome (ACS). This report describes an analysis of the temporal patterns of 151 metabolites in these patients and the association of the repeatedly measured metabolites with reACS.

## Methods

### Study population

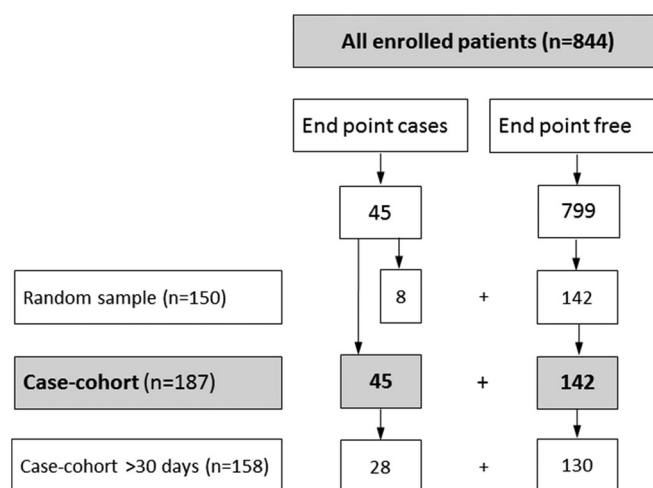
BIOMArCS is a multicentre observational study, conducted during 2008–2015 in the Netherlands. Details concerning the study design have been described elsewhere (Oemrawsingh *et al.* 2016). In brief, BIOMArCS enrolled patients who were admitted for ACS, either with or without ST elevation, and who had at least one additional CVD risk factor. After inclusion, venipuncture was performed at admission, discharge, and subsequently every 2 weeks during the first half-year and every 4 weeks during the second half-year. If logistic circumstances hindered inclusion during hospitalization, patients could be included on the first outpatient visit within 6 weeks after discharge – the absence of early samples was then accepted. Samples were collected non-fasting.

BIOMArCS was approved by the Institutional Review Boards of all enrolling hospitals, and all participating patients provided written informed consent. BIOMArCS is registered in the Netherlands Trial Register as NTR1698 and NTR1106.

### Study design

BIOMArCS enrolled 844 patients, and 45 reached the study endpoint of reACS, defined as a cardiac death, non-fatal myocardial infarction (MI) or unstable angina (UA) requiring urgent coronary revascularization (endpoint cases). For reasons of cost-efficiency, we applied a case-cohort design with respect to the present metabolite analysis (patient selection is shown in Figure 1). A case-cohort design is a pragmatic way to simulate the results, which could have been found in a full cohort (Boersma *et al.* 2019). A random sample of 150 patients was selected from the full cohort (which rendered eight endpoint cases), and was complemented with the remaining 37 endpoint cases outside this random sample. Consequently, the case-cohort sample included all 45 study endpoint cases and 142 endpoint-free patients.

We realized that the metabolites could have been influenced by the index ACS event. We were mainly interested in metabolite patterns after clinical stabilization. Therefore, we restricted our analyses to the 28 study endpoint cases and



**Figure 1.** Patient flow chart. First, a random sample of 150 patients of our full cohort (844) was selected which rendered eight endpoint cases, which was then complemented with all endpoint cases outside the random sample. Consequently, the case-cohort sample included all 45 study endpoint cases and 142 endpoint-free patients. Blood samples were available of 158 patients 30 days post the index event.

130 event-free patients with available blood samples after 30 days following the index event.

### Metabolite analysis

Serum samples were collected and preserved on-site at  $-80^{\circ}\text{C}$ . Subsequently, samples were transported to the Erasmus MC for long-term storage under the same conditions. For the current analysis, serum samples were analysed applying high-throughput automated proton nuclear magnetic resonance (NMR) spectroscopy by Nightingale Health (Soininen *et al.* 2015). In each blood sample, all metabolites were quantified simultaneously, and, subsequently, expressed in absolute concentrations using Nightingale Health's proprietary software (Soininen *et al.* 2015). Details on the applied NMR method are described in the *Supplementary Material*. The NMR method provided the molar concentrations of 151 metabolites, including 14 lipoprotein subclasses and their particle concentrations and lipids compositions, 9 cholesterol metabolites, 2 apolipoproteins, 8 glycerides and phospholipids, 9 fatty acids, 4 glycolysis related metabolites and 9 amino acids.

### Statistical data analysis

Continuous variables are presented as median (25th–75th percentiles). Categorical variables are presented as number (percentage). Differences in continuous data between study endpoint cases and event-free patients were evaluated by Mann–Whitney *U*-tests, whereas categorical variables were evaluated by Pearson Chi-square tests.

The linear mixed effects (LME) model was used to describe the evolution of metabolites over time, with adjustment for age, gender, GRACE risk score, diabetes mellitus, history of peripheral arterial disease, statin use and vitamin K antagonist use. Cox proportional hazard regression was used to relate serially measured metabolite level, based on the

LME model, with the incidence of the study endpoint, while adjusting for GRACE risk. The parameters of the LME and Cox models were estimated in a joint model to avoid bias (Rizopoulos 2016). To enable a direct comparison of the relation between different metabolites and the study endpoint, we present adjusted hazard ratios (aHRs) as per one standard deviation (SD) difference.

R statistical software (version 3.4.3) was used for the statistical analyses, in particular the package JMbayes (Rizopoulos 2016). All statistical tests were two-tailed, and  $p$ -values  $<0.001$  were considered statistically significant, to correct for multiple testing. This significance level was determined by matrix spectral decomposition (Li and Ji 2005).

## Results

Median (25th–75th percentiles) age was 63.8 (56.1–71.6) years and 77.8% were men. Study endpoint cases were older than event-free patients, had a higher prevalence of diabetes, history of peripheral arterial disease and vitamin K antagonist usage (Table 1), and had similar characteristics otherwise. For the current analysis, a median (25th–75th percentiles) of 7 (3–10) and 8 (5–9) repeated measurements were available in study endpoint cases and event-free patients, respectively.

In addition, 95% of the 1101 samples were collected in patients on statins. Clinical characteristics did not significantly differ between statin-treated and statin-untreated patients (data not shown). Low-density lipoprotein (LDL) cholesterol was 0.46 (95%CI: 0.061–0.85) mmol/l per SD increase higher in the 55 samples collected in statin-untreated patients ( $p = 0.024$ ).

## Metabolites

Higher concentrations of extremely large VLDL particles (XXL-VLDL-P), very large VLDL-P (XL-VLDL-P) and large VLDL-P (L-VLDL-P) were significantly associated with reACS (aHR 1.60/SD, 95% CI 1.25–2.08; aHR 1.60/SD, 95% CI 1.25–2.08; aHR 1.56/SD, 95% CI 1.22–2.05, respectively) during 1-year follow-up (Figure 2 and Supplemental Table S1). Moreover, the concentrations of these particles steadily increased prior to the reACS (Figure 3).

In addition to the lipoprotein subclass particle concentrations, the lipid composition of each lipoprotein subclass was quantified with NMR (Supplemental Table S2). A lipoprotein particle is composed of phospholipids, cholesterol, cholesterol esters, free cholesterol and triglycerides. Figure 4 shows the aHR's of the lipid components of XXL-VLDL-P, XL-VLDL-P and L-VLDL-P. Overall, the individual lipid components of

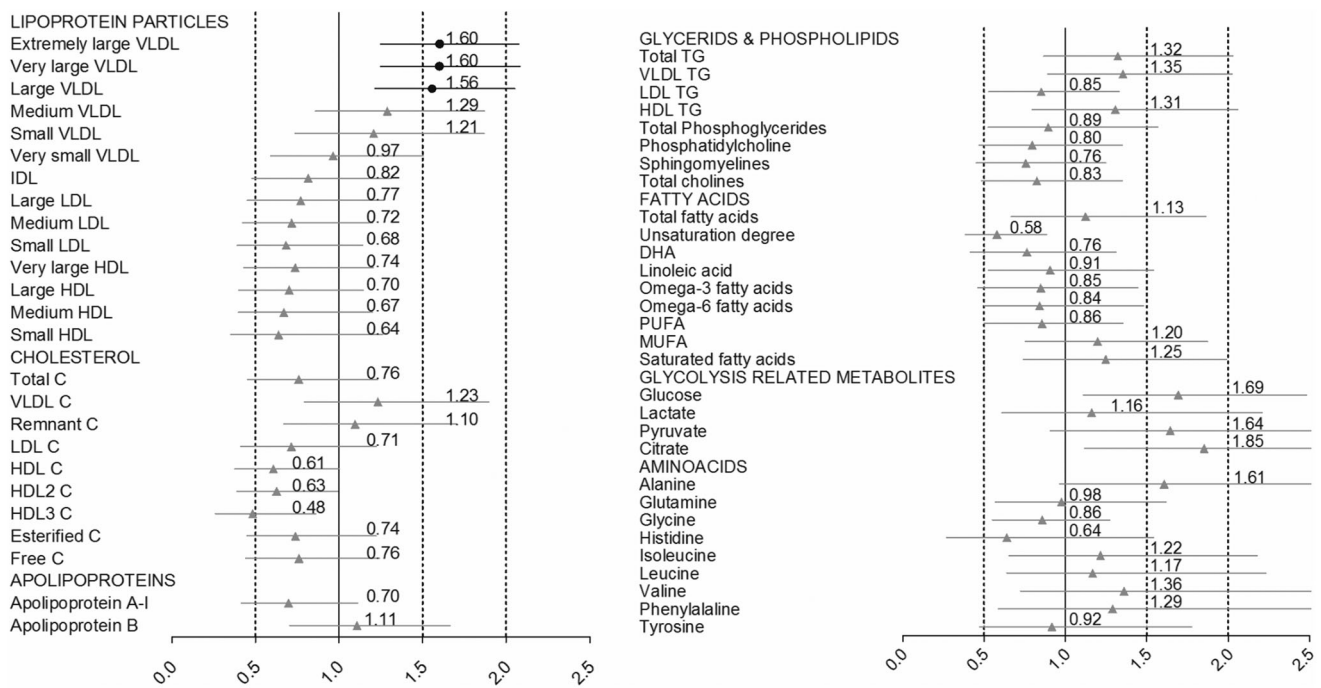
**Table 1.** Baseline clinical characteristics.

	Overall	Event-free patients	Cases	$p$ Value
No. of patients	158	130	28	
Presentation and initial treatment				
Men	123 (77.8)	102 (78.5)	21 (75.0)	0.88
Age – yr	63.8 (56.1–71.6)	62.3 (55.1–71.0)	68.0 (59.0–76.3)	0.030
Admission diagnosis				0.59
STEMI	69 (43.7)	59 (45.4)	10 (35.7)	
NSTEMI	66 (41.8)	52 (40.0)	14 (50.0)	
UAP	23 (14.6)	19 (14.6)	4 (14.3)	
CAG performed	149 (94.3)	121 (93.1)	28 (100.0)	0.33
PCI performed	124 (84.4)	100 (83.3)	24 (88.9)	0.67
CKMax – U/L	425.0 (179.0–1197.0)	452.5 (196.8–1200.8)	312.0 (135.0–750.5)	0.24
Cardiovascular risk factors				
Smoking				0.89
Current	65 (41.1)	54 (41.5)	11 (39.3)	
Former	48 (30.4)	40 (30.8)	8 (28.6)	
Never	45 (28.5)	36 (27.7)	9 (32.1)	
Diabetes mellitus	32 (20.3)	22 (16.9)	10 (35.7)	0.047
Hypertension	84 (53.2)	70 (53.8)	14 (50.0)	0.87
Hypercholesterolaemia	76 (48.1)	66 (50.8)	10 (35.7)	0.22
Creatinine – $\mu$ mol/L	82.5 (72.3–93.8)	82.0 (73.0–91.8)	86.5 (71.3–95.0)	0.46
Cardiovascular history				
Peripheral arterial disease	15 (9.5)	9 (6.9)	6 (21.4)	0.043
Myocardial infarction	51 (32.3)	42 (32.3)	9 (32.1)	1.00
PCI	47 (29.9)	37 (28.7)	10 (35.7)	0.61
CABG	16 (10.1)	11 (8.5)	5 (17.9)	0.25
Stroke	20 (12.7)	14 (10.8)	6 (21.4)	0.22
Valvular heart disease	5 (3.2)	2 (1.5)	3 (10.7)	0.055
Heart failure	7 (4.4)	4 (3.1)	3 (10.7)	0.20
Medication at first blood sample moment $>7$ days after the index ACS*				
Aspirin	150 (95.5)	122 (94.6)	28 (100.0)	0.45
P2Y12 inhibitor	145 (92.4)	118 (91.5)	27 (96.4)	0.62
Vitamin K antagonist	14 (8.9)	8 (6.2)	6 (21.4)	0.028
Statin	151 (96.2)	125 (96.9)	26 (92.9)	0.64
Beta-blocker	135 (86.0)	108 (83.7)	27 (96.4)	0.15
ACE inhibitor or ARB	131 (83.4)	105 (81.4)	26 (92.9)	0.23

\*The first blood sample  $>7$  days was taken at a median (25th–75th percentiles) of 24 (16–34) days after the index ACS.

Continuous variables are presented as median (25th–75th percentiles). Categorical variables are presented as number (percentage).

ACE: angiotensin converting enzyme, ARB: angiotensin II receptor blocker, CABG: coronary artery bypass grafting, CKmax: maximum creatinine kinase during the index admission, No: Numero, NSTEMI: non-ST-elevation myocardial infarction, PCI: percutaneous coronary intervention, STEMI: ST-elevation myocardial infarction, Troponinmax: maximum troponin value during the index admission, UAP: unstable angina pectoris, yr: years.



**Figure 2.** Associations of NMR-quantified metabolite profile and reACS. aHR's with 95%CI are presented on an SD scale adjusted for age, gender, GRACE risk score, diabetes mellitus, history of peripheral arterial disease, statin use and vitamin K antagonist use. Black rounds are statistically significant with  $p < 0.001$ ; grey triangles are not. C: cholesterol; DHA: docosahexaenoic acid; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty; TG: triglycerides; VLDL: very low density lipoprotein.

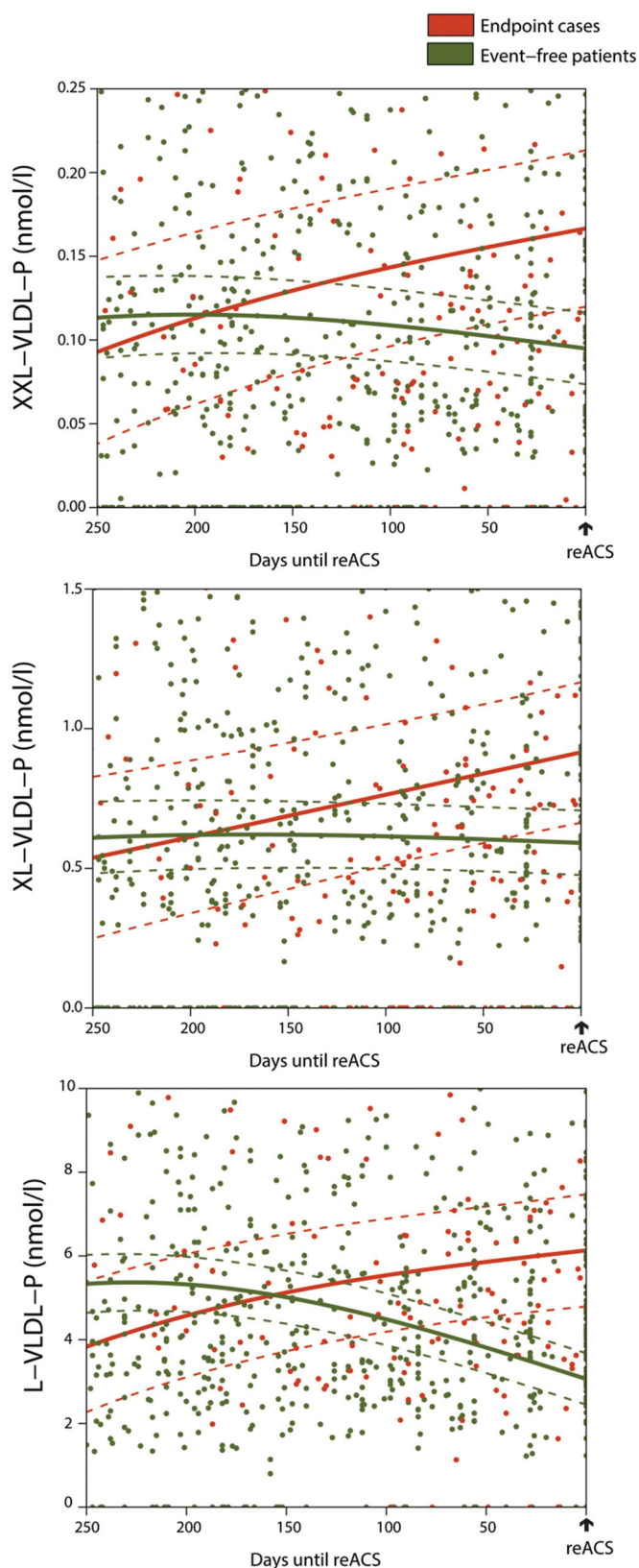
XXL-VLDL-P, XL-VLDL-P and L-VLDL-P were also associated with reACS. However, per lipid component we observed intra-variability (within the particle) and, more importantly, inter-variability (between the particles) in the degree of association with reACS. For instance, in XXL-VLDL the concentration of total cholesterol was associated with reACS with an aHR of 1.58/SD increase (95% CI: 1.18–1.94,  $p < 0.001$ ). In XL-VLDL, the concentration of total cholesterol had an aHR of 1.53/SD increase (95% CI: 1.19–1.97,  $p = 0.006$ ). In L-VLDL, the concentration of total cholesterol had an aHR of 1.34/SD increase (95% CI: 0.89–1.98,  $p = 0.17$ ).

Among the other assessed metabolite groups, no significant associations were observed between metabolite concentration and reACS.

## Discussion

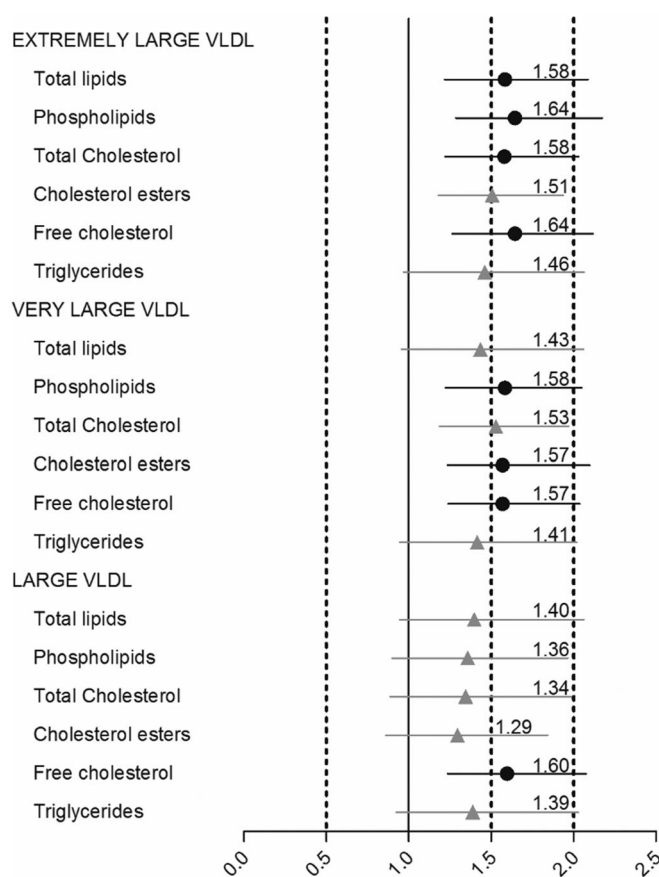
This study assessed the association between repeatedly measured metabolite profiles and the incidence of reACS during one year follow-up in patients with post-ACS. Patients who experienced reACS had steadily increasing concentrations of XXL-VLDL-P, XL-VLDL-P and L-VLDL-P during 1-year of follow-up until the moment of the endpoint event. No significant associations were observed between longitudinal serum concentrations of cholesterol metabolites, apolipoproteins, glycerides and phospholipids, fatty acids, glycolysis related metabolites or amino acids and reACS. Hence, serial blood sampling may benefit the prognostic accuracy of lipoprotein particle concentrations over a single baseline measurement. In a larger study cohort with more patients developing cardiac outcome, one should assess the frequency of sampling needed for accurate prognostication using lipoprotein particle concentrations.

Our study predominantly consisted of statin-treated patients. Previously, Wurtz *et al.* (2016) showed in a combined analysis of population-based cohorts, that statin-use lowered most of their NMR-quantified metabolite concentrations. In particular, statins effectively lowered multiple lipoprotein concentrations in addition to LDL cholesterol. In our study, despite statin use, XXL-VLDL-P, XL-VLDL-P and L-VLDL-P concentrations were significantly higher in patients who experienced a reACS, whereas total VLDL cholesterol was not. Since recent years, studies are advocating the added value of lipoprotein particle concentrations to lipoprotein cholesterol concentrations for clinical prognosis in patients with CVD (Rosenson and Underberg 2013). Moreover, in 2011, the American National Lipid Association Expert panel has advised to study the use of lipoprotein particle concentrations to enhance treatment management, to address the residual risk of statin-treated patients with CVD for adverse outcome (Davidson *et al.* 2011). Subsequently, several studies have found that LDL particle concentration is a better predictor of adverse outcome than LDL cholesterol in patients with CVD on lipid-lowering treatment (Rosenson and Underberg 2013). One can argue that the latter finding might also be true for VLDL. It has been previously described that elevated VLDL cholesterol levels are an independent predictor of adverse outcome in the general population and in patients with CVD, and it has been suggested that VLDL cholesterol in combination with LDL cholesterol may be a better determinant of adverse outcome than LDL cholesterol alone (Sacks *et al.* 2000, Liu *et al.* 2006, Ren *et al.* 2010). In our study, we found that the larger VLDL particle concentrations were associated with reACS, whereas total VLDL cholesterol was not. Hence, further research should establish if VLDL particle



**Figure 3.** Longitudinal trajectory of XXL-VLDL-P, XL-VLDL-P and L-VLDL-P prior to reACS. nmol: nanomol; l: litre; L-VLDL-P: large VLDL particles; reACS: repeated acute coronary syndrome; XXL-VLDL-P: extremely large VLDL particles; XL-VLDL-P: very large VLDL particles.

concentrations provide incremental prognostic information to LDL particle concentrations in statin-treated patients with CVD to address their risk of developing adverse outcome.



**Figure 4.** Associations of NMR-quantified components of extremely large, very large and large VLDL particles and reACS. aHR's with 95%CI are presented on an SD scale adjusted for age, gender, GRACE risk score, diabetes mellitus, history of peripheral arterial disease, statin use and vitamin K antagonist use. Black rounds are statistically significant with  $p < 0.001$ ; grey triangles are not. VLDL: very low density lipoprotein.

Although not significant, plasma glucose appeared to correlate with reACS in our study. Previously, it has been demonstrated that hyperglycaemia induces overproduction of larger VLDL particles (Adiels *et al.* 2005). Thus, potentially, the patients with post-ACS who experienced reACS had a certain grade of hyperglycaemia which may have induced the overproduction of larger VLDL particles and subsequent pathological atherogenesis.

Currently, results obtained by metabolite profiling are difficult to compare across various study populations, due to lack of a uniform way to quantify metabolites and otherwise heterogeneous study methods (Ruiz-Canela *et al.* 2017). Although NMR is a cost-effective tool to obtain detailed knowledge on metabolites (Rankin *et al.* 2014), the sensitivity of this technique is limited compared with other metabolite profiling techniques such as mass spectrometry. Still, also mass spectrometry has downsides, including automation of the technique and the fact that it cannot detect lipoproteins (Rankin *et al.* 2014). Therefore, in our view, NMR suits purposes of epidemiological studies including ours, whereas mass spectrometry is more suited for detailed metabolite discovery. Eventually, the field of CVD metabolite research should focus on developing uniform study methods, as well as profiling techniques to obtain more reliable and

comparable results. Under such conditions, the knowledge that will be gained through metabolite profiling might enable a precision-medicine approach to CVD treatment.

### Limitations

This study used 1101 serial blood samples to assess the time course of NMR-quantified metabolites and their longitudinal association with incident ACS. Nonetheless, as only 28 study endpoint cases were available, we cannot exclude the possibility that our study was underpowered. In addition, freezing and thawing of serum samples could have influenced the metabolite measurements. However, our samples were kept frozen at  $-80^{\circ}\text{C}$  throughout complete storage and transportation of the samples up until quantification. Lastly, because of the exploratory character of our study, we could not provide a mechanical interpretation of our findings.

### Conclusion

Post-ACS patients with persistent high concentrations of extremely large, very large and large VLDL particles have increased risk of reACS within 1 year.

### Disclosure statement

No potential conflict of interest was reported by the author(s).

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