Use of novel biomarkers to explore cardiovascular risk and drug effects

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Contents

Chapter 1

Introduction

Novel biomarkers in cardiovascular research

Despite advanced treatment options, cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide with over 17.7 million deaths per year.[1] CVD is a collective term that subsumes a wide range of conditions involving the heart and/or the vascular system. By far the most common underlying cause is atherosclerosis, which can lead to severe clinical manifestations, such as myocardial infarction and ischemic stroke.[2] In the European Union alone, the most common forms of atherosclerotic CVD, coronary artery disease and stroke, cause healthcare costs and production losses of nearly 80 billion EUR each year.[3] Since the second half of the 20th century, the immense economic burden of atherosclerosis has sparked large-scale efforts to improve cardiovascular prevention, leading to the identification of so-called traditional cardiovascular risk factors, such as smoking, diabetes, obesity, hypertension and hyperlipidemia.[4-6] These findings not only provided insights into the pathophysiology of atherosclerosis, but also led to the development of numerous drugs and intervention programs in the following decades.[7-9]

Given their well-established role in atherogenesis, clinical guidelines continue to recommend the use of traditional risk factors as the mainstay of cardiovascular risk assessment and prevention.[10] However, traditional risk factors are absent in up to 20% of all patients with coronary artery disease.[11] Moreover, their relationship with subsequent events in patients with established atherosclerotic CVD is less clear compared to primary prevention settings,[12-14] which may be attributable to more aggressive treatment of risk factors in secondary prevention or differential disease mechanisms for early and advanced stages of atherosclerosis.[15] Yet, current treatment strategies apply a standardized set of therapies to all patients, regardless of individual differences beyond traditional risk profiles. Therefore, personalized medicine has emerged as a therapeutic concept that aims to tailor disease prevention and treatment to a patient's individual characteristics.

This endeavor critically depends on the identification of novel biomarkers for diagnosis, prognosis, treatment selection and drug development. While most common biomarker definitions not only include biological samples, but rather any characteristic related to a biological process,[16] especially serum biomarkers have shown promise for clinical use in cardiovascular prevention, including C-reactive protein, troponins and different hematological parameters.[17,18] Nevertheless, only few risk assessment tools have incorporated such biomarkers (e.g. [19]), and current clinical guidelines do not (yet) recommend their general use in clinical practice.[10] In recent years, a wide range of high-throughput technologies has become available to inexpensively measure biomarkers in large quantities. Consequently, 'omics' approaches, such as genomics, transcriptomics, proteomics and metabolomics, are increasingly being applied to biomarker discovery.

Besides enhancing risk assessment and clinical management, omics profiling offers great potential for drug research. Large-scale biomarker screening may advance our understanding of disease mechanisms, providing knowledge vital for the identification of novel drug targets, but may also contribute to personalized treatment.

In cardiovascular medicine, pharmacogenomics, combined with transcriptional analysis, has yielded insights into the molecular pathways associated with adverse drug effects and interindividual differences in drug efficacy.[20,21] At the same time, in-depth metabolic profiling has become a promising tool for treatment selection, compliance monitoring and for the systemic analysis of drug effects.[22,23]

Thesis outline

This thesis seeks to identify novel biomarkers of atherosclerotic CVD and to advance understanding of the pathophysiological mechanisms underlying CVD progression. Moreover, we demonstrate the utility of high-throughput metabolic profiling for assessing systemic drug effects. **Chapter 2** relates altered monocyte gene expression in childhood obesity to atherosclerotic disease complexity in adult patients. **Chapter 3** investigates the association of loss of chromosome Y with atherosclerotic plaque characteristics and clinical outcome after endarterectomy. **Chapter 4** explores the ability of routinely measured hematological parameters to improve prediction of recurrent vascular events in vascular patients. The remaining chapters report results from metabolic profiling studies. **Chapter 5** presents findings from a prospective study of two angiographic cohorts, in which we identified biomarkers that improve prediction of subsequent cardiovascular events. In **Chapter 6**, we use clinical trial data to explore the effect of metformin on metabolic profiles and to study biomarkers of infarct size and left ventricular ejection fraction after myocardial infarction. In **Chapter 7**, we investigate the longitudinal effect of pravastatin treatment on metabolic profiles, using data from a randomized clinical trial.

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Chapter 2

Monocyte gene expression in childhood obesity is associated with obesity and complexity of atherosclerosis in adults

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Manuscript submitted

Abstract

Childhood obesity coincides with increased numbers of circulating classical CD14++CD16- and intermediate CD14++CD16+ monocytes. Monocytes are key players in the development and exacerbation of atherosclerosis, which prompts the question as to whether the monocytosis in childhood obesity contributes to atherogenesis over the years. Here, we dissected the monocyte gene expression profile in childhood obesity using an Illumina microarray platform on sorted monocytes of 35 obese children and 16 lean controls. Obese children displayed a distinctive monocyte gene expression profile compared to lean controls. Upon validation with quantitative PCR, we studied the association of the top 5 differentially regulated monocyte genes with circulating adipokines and clinical variables in childhood obesity. Finally, a cohort of 351 adults at risk for ischemic cardiovascular disease was used to study the associations of the top 5 differentially regulated monocyte genes with adulthood obesity and complexity of coronary atherosclerosis (SYNTAX score). Downregulation of monocyte IMPDH2 and TMEM134 was observed in childhood obesity, and also associated with obesity in the adult cohort. Moreover, downregulation of TMEM134 was associated with a higher SYNTAX atherosclerosis score. In conclusion, childhood obesity entails monocyte gene expression alterations associated with obesity and enhanced complexity of coronary atherosclerosis in adults.

Introduction

The childhood obesity epidemic has alarming cardiovascular consequences, and thereby limits the worldwide increase in life expectancy.[1,2] Obesity early in life may contribute to the development of cardiovascular disease in several ways. First, childhood obesity tends to result in adulthood obesity, which is an important risk factor for cardiovascular disease, especially when it concerns visceral adiposity.[3,4] Second, childhood and adulthood obesity share independent risk factors for cardiovascular disease, such as a high blood pressure.[5] Furthermore, obesity-induced insulin resistance and hyperglycemia lead to defective insulin signaling in vascular wall lesional cells, which promotes atherosclerosis at the level of the arterial wall.[6] Finally, obesity is associated with low-grade systemic inflammation, which partly results from the adipose tissue production of inflammatory adipokines including chemerin and leptin, and promotes atherogenesis.[7,8]

At a cellular level, monocytes appear to be a pivotal link between obesity and cardiovascular disease. Obesity is accompanied by leukocytosis, particularly of the myeloid lineage.[7,9] Recent studies indicate that adipose tissue derived inflammatory factors such as IL-1β stimulate bone marrow myeloid progenitors, leading to monocytosis in obesity.[10] Next to increased numbers, monocytes show an activated and inflammatory phenotype in obesity. In humans, monocytes fall into three phenotypical categories: classical CD14++CD16-, intermediate CD14++CD16+ and non-classical CD14+CD16++ monocytes.[11] Previously, we have shown that childhood obesity is accompanied by increased numbers and an activated phenotype of the classical CD14++CD16- monocyte subset.[7] These monocytes are equivalent to GR1+Ly6c high monocytes in mice, that differentiate into inflammatory macrophages and foam cells in various atherosclerosis models.[12,13] The increased inflammatory monocyte numbers in childhood obesity may thus contribute to atherogenesis over the years.

The aim of this study was to obtain in-depth understanding of the monocyte gene expression profile in childhood obesity as compared to normal weight controls using micro-array analyses of sorted monocytes. Furthermore, we studied the relation between monocyte gene expression and cardiometabolic risk factors in childhood obesity. Importantly, we focused on cardiometabolic risk markers in childhood obesity that were identified in previous studies,[7,14] including systolic blood pressure, insulin sensitivity (QUICKI), adipokine levels (adiponectin, leptin, chemerin, TNF-R2), and monocyte numbers and CD11b expression. Finally, monocyte gene expression profiles were compared with an established cohort of 351 adults at risk for ischemic cardiovascular disease, to study whether monocyte gene expression profiles in childhood obesity overlap with an atherogenic monocyte phenotype in adults. The adult cohort encompassed several clinical parameters, but we focused on the relation between monocyte gene expression and the SYNergy between percutaneous coronary Intervention with TAXus and cardiac surgery (SYNTAX) atherosclerosis score because it is an established angiographic grading system for evaluating the complexity of coronary atherosclerotic lesions, widely used as a readout for atherosclerotic burden.[15-19]

Table 1: Characteristics of the pediatric study population

	Lean children (n=16)	Obese children (n=35)
Age (years)	10.5 (8.4, 12.9)*	13.9 (10.8, 14.9)*
Boys (number, %)	7 (44)	11 (31)
BMI-SD	0.4 (-0.7, 0.9)**	3.4 (3.1, 3.7)**
QUICKI	0.4 (0.3, 0.4)**	0.3 (0.3, 0.3)**
Systolic blood pressure (SBP, mmHg)	110 (98, 120)**	123 (113, 129)**
HDL-cholesterol (mmol/l)	1.5 (1.3, 1.7)**	1.2 (1.0, 1.4)**
LDL-cholesterol (mmol/l)	2.2 (2.2, 2.4)	2.3 (2.0, 3.0)
Triglycerides (mmol/l)	0.7 (0.5, 0.9)	0.9 (0.7, 1.3)
Adiponectin (µg/ml)	33 (25, 41)	31 (24, 35)
Chemerin (µg/ml)	2.8 (2.5, 3.0)	3.0 (2.6, 3.3)
Leptin (ng/ml)	113 (109, 156)**	329 (274, 462)**
TNF-R2 (ng/ml)	2.5 (2.2, 2.7)**	3.0 (2.7, 3.3)**
Total monocyte number (x 10^9)	0.4 (0.4, 0.5)**	0.6 (0.5, 0.7)**
CD14++CD16- monocyte number (x 10^7)	36.2 (34.3, 43.2)**	52.0 (43.3, 64.7)**
CD14++CD16+ monocyte number (x 10^7)	1.8 (1.2, 2.3)**	4.6 (3.0, 7.0)**
CD14++CD16- CD11b expression (MFI)	5115 (3737, 6180)**	9383 (6252, 13920)**
CD14++CD16+ CD11b expression (MFI)	5645 (4524, 8924)**	10790 (7409, 14270)**

Clinical characteristics and laboratory parameters for lean controls versus obese children. Data is shown as median (interquartile range). *p<0.05, **p<0.01. BMI-SD: standard deviation of body mass index corrected for age and sex, HDL: high-density lipoprotein, LDL: low-density lipoprotein, MFI: median fluorescence intensity, QUICKI: quantitative insulin sensitivity index.

Results

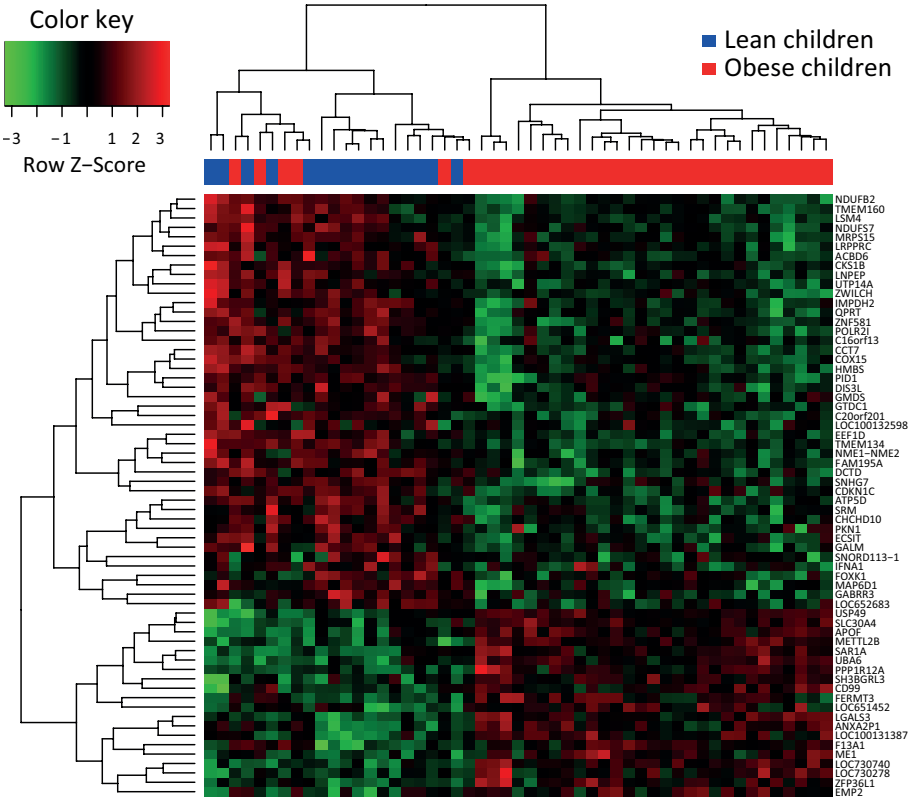
Monocytes in childhood obesity show a distinctive gene expression profile

Obese children exhibited typical clinical and biochemical characteristics with a significantly higher body mass index standard deviation for age and sex (BMI-SD) compared to lean controls (3.4 versus 0.4, p<0.001), a higher systolic blood pressure (BP) (123 mmHg versus 110 mmHg, p=0.005), lower Quantitative insulin sensitivity index (QUICKI) (0.3 versus 0.4, p<0.001), lower high-density lipoprotein (HDL) cholesterol level (1.2mmol/l versus 1.5mmol/l, p=0.004), higher leptin level (329ng/ml versus 113ng/ml, p<0.001) and higher TNF receptor 2 (TNF-R2) level (3.0ng/ml versus 2.5ng/ml, p=0.005) (Table 1). Furthermore, the obese subgroup showed a higher total monocyte number (0.6x109/ml versus 0.4x109/ml, p<0.001), reflecting a higher classical CD14++CD16- monocyte number (52.0x107/ml vs. 36.2x107/ml, p=0.001) and a higher intermediate CD14++CD16+ monocyte number (4.6x107/ml versus 3.3x107/ml, p<0.001). As shown in earlier studies,[7] classical and intermediate monocytes in obese children expressed higher levels of the activation marker and

integrin CD11b than lean control monocytes (CD11b Median Fluorescence Intensity classical monocytes 9383 in obese versus 5115 in lean children, p=0.002; intermediate monocytes 10790 versus 5645, p=0.003). Notably, the obese population showed a higher age compared to the lean controls (13.9 versus 10.5 years), and a lower percentage of boys (31% versus 44%). In order to avoid confounding, all subsequent analyses were corrected for age and sex.

Following microarray multiple testing correction, 67 genes were significantly and differently expressed between the obese and lean participants (Supplemental Table 1 and 2). An unbiased clustering approach revealed a clear separation in monocyte gene expression profiles between lean and obese individuals (Figure 1). The microarray data thus highlighted a distinctive monocyte gene expression profile in obese children.

Figure 1: Heat map and cluster analysis

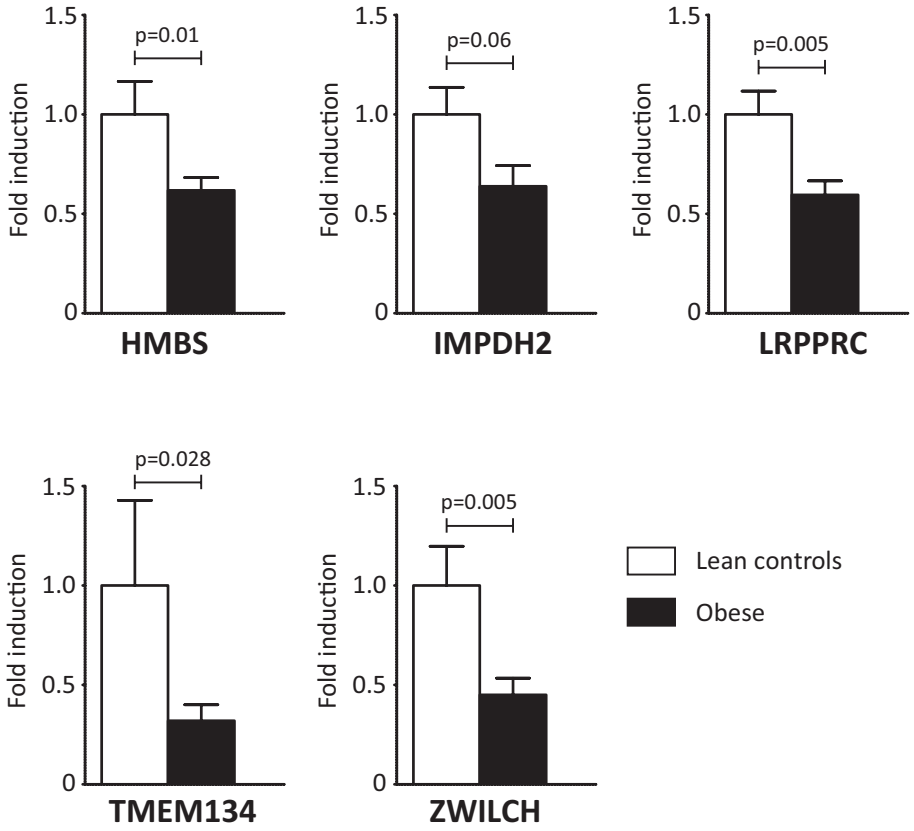


Results of monocyte gene expression analysis in the pediatric study population. The heat map depicts the gene cluster structure as a hierarchical tree with distinct branches and uses row z-score to depict data that deviates above or below the population mean.

Quantitative PCR validation

Quantitative PCR (qPCR) was used to confirm the gene expression results, focusing on the top 20 microarray hits (Supplemental Table 4). qPCR analyses confirmed the observed downregulation of the monocyte genes Hydroxymethylbilane synthase (HMBS) (p=0.01), Leucine Rich Pentatricopeptide Repeat Containing (LRPPRC) (p=0.005), Transmembrane Protein 134 (TMEM134) (p=0.028) and Zwilch Kinetochore Protein (ZWILCH) (p=0.005) in childhood obesity compared to lean controls (Figure 2, Supplemental Table 5). Furthermore, Inosine Monophosphate Dehydrogenase 2 (IMPDH2) showed a trend towards downregulation in obese monocytes (p=0.06). Because of its significant downregulation in monocytes from obese adults (see below), IMPDH2 was included in subsequent analyses as well. Together, these 5 downregulated genes formed the starting point for studying the relation between monocyte gene expression and the clinical phenotype in childhood obesity and adults at risk for ischemic cardiovascular disease.

Figure 2: qPCR validation



qPCR confirms downregulation of 5 monocyte genes in childhood obesity. Graphs show fold induction of the gene of interest, normalized for housekeeping gene expression. Error bars represent SEM. SEM: standard error of mean; qPCR: quantitative polymerase chain reaction.

Table 2: Gene expression and cardiometabolic risk profile in the total pedriatric population

	Genes				
	HMBS	**IMPDH2**	**LRPPRC**	**TMEM134**	**ZWILCH**
Cardiovascular risk factors					
Systolic BP	-2.52 (-18.87, 13.84)	11.46 (-6.47, 29.38)	-0.66 (-21.39, 20.07)	-3.13 (-29.94, 23.67)	-26.43 (-63.45, 10.59)
QUICKI	-0.01 (-0.06, 0.05)	0.03 (-0.02, 0.09)	-0.06 (-0.13, 0.001)	0.06 (-0.02, 0.14)	-0.02 (-0.14, 0.10)
Monocytes					
Total monocyte count	-0.06 (-0.33, 0.20)	-0.25 (-0.54, 0.04)	-0.15 (-0.49, 0.18)	-0.18 (-0.61, 0.24)	-0.51 (-1.11, 0.09)
CD14++CD16- count	-5.08 (-32.18, 22.01)	-25.98 (-55.11, 3.15)	-12.28 (-46.14, 21.58)	-25.81 (-68.19, 16.56)	-54.99 (-114.69, 4.71)
CD14++CD16+ count	-0.26 (-6.86, 6.34)	-2.16 (-9.47, 5.15)	-5.55 (-13.66, 2.56)	2.48 (-7.98, 12.93)	-7.97 (-22.85, 6.91)
CD14++CD16- CD11b MFI	-2442 (-10380, 5495)	-3071 (-12069, 5927)	-10184* (-18305, -2062)	-5021.0 (-18839, 8797)	-19312* (-36097, -2527)
CD14++CD16+ CD11b MFI	362 (-7767, 8491)	-1705 (-1094, 7507)	-6765 (-15456, 1925)	2012 (-12154, 6178)	-8736 (-26844, 9373)
Adipokines					
Adiponectin	13.56 (-1.79, 28.90)	10.41 (-7.67, 28.49)	10.33 (-9.17, 29.83)	20.66 (-3.96, 45.29)	36.01* (1.43, 70.60)
Chemerin	-0.84* (-1.60, -0.09)	-0.25 (-1.13, 0.63)	-0.27 (-1.26, 0.76)	-1.24* (-2.45, -0.03)	-0.14 (-1.96, 1.68)
Leptin	-7.05 (-204.94, 190.85)	106.93 (-110.29, 324.16)	27.60 (-220.06, 275.26)	116.45 (-195.93, 428.84)	311.12 (-130.88, 753.11)
TNF-R2	0.11 (-0.77, 0.99)	1.01* (0.08, 1.94)	0.48 (-0.62, 1.57)	1.02 (-0.34, 2.39)	1.86 (-0.07, 3.79)

The association of the 5 validated genes with cardiometabolic parameters was studied using a linear regression model. Linear regression coefficients (95% CI) for the monocyte genes are shown, adjusted for age, sex and BMI-SD. *p<0.05. BP: systolic blood pressure; CI: confidence interval; MFI: median fluorescence intensity; QUICKI: quantitative insulin sensitivity index.

Monocyte gene expression and clinical variables in childhood

The relation of the 5 validated genes with obesity-associated clinical variables was studied using linear modeling. First, the whole cohort of obese children and lean controls was studied, adjusting linear regression coefficients for age, sex and BMI-SD (Table 2). Downregulation of the monocyte genes HMBS and TMEM134 was associated with an increase of the inflammatory adipokine chemerin (β=-0.84 μg/ml, p=0.03 and β=-1.24 μg/ml, p=0.04, respectively). Furthermore, downregulation of ZWILCH was associated with a decrease in adiponectin levels (β=36.01 μg/ml, p=0.04) and a decrease in IMPDH2 expression was associated with a decrease in TNF-R2 (β=1.01 ng/ml, p=0.04). Finally, downregulation of LRPPRC and ZWILCH was associated with an increased expression of the activation marker CD11b on

classical CD14++CD16- monocytes (β=-10184 MFI, p=0.02 and β=-19312 MFI, p=0.03, respectively).

Second, the relation of monocyte gene expression with clinical variables was studied in the obese subgroup, adjusting linear regression coefficients for age and sex (Table 3). In the obese subgroup, downregulation of IMPDH2 was associated with a decrease in systolic blood pressure (β=20.72mmHg, p=0.04), and downregulation of LRPRC and ZWILCH were associated with an increase in QUICKI (β=-0.08, p=0.04 and β=-0.14, p=0.04). Furthermore, downregulation of LRPPRC was associated with an increase in CD11b expression on classical CD14++CD16- and intermediate

Table 3: Monocyte gene expression and cardiometabolic risk profile in the obese subgroup

	Genes				
	HMBS	**IMPDH2**	**LRPPRC**	**TMEM134**	**ZWILCH**
Cardiovascular risk factors					
Systolic BP	-1.41 (-19.59, 16.78)	20.72* (1.25, 40.19)	11.22 (-15.87, 38.30)	-5.82 (-38.63, 26.99)	-25.87 (-74.00, 22.26)
QUICKI	-0.03 (-0.09, 0.02)	-0.03 (-0.09, 0.04)	-0.08* (-0.16, -0.001)	-0.01 (-0.12, 0.09)	-0.14* (-0.28, -0.001)
Monocytes					
Total monocyte count	0.02 (-0.37, 0.41)	-0.17 (-0.61, 0.28)	-0.05 (-0.64, 0.54)	0.02 (-0.69, 0.72)	-0.35 (-1.39, 0.70)
CD14++CD16- count	4.15 (-5.81, 14.11)	1.09 (-10.44, 12.62)	-3.80 (-18.78, 11.18)	12.66 (-4.75, 30.07)	-6.53 (-33.05, 19.98)
CD14++CD16+ count	6.98 (-33.07, 47.02)	-15.09 (-60.64, 30.46)	1.94 (-57.94, 61.82)	-4.53 (-76.39, 67.34)	-36.60 (-141.66, 68.47)
CD14++CD16- CD11b MFI	3389 (-9927, 16705)	-8455 (-12017, 14902)	-15385* (-29859, -912)	10387 (-18329, 39102)	-24070 (-62508, 14369)
CD14++CD16+ CD11b MFI	2323 (-11681, 16326)	-839 (-14954, 13275)	-19214** (-33658, -4771)	17823 (-11611, 47257)	-23782 (-64228, 16665)
Adipokines					
Adiponectin	16.47 (-0.54, 33.48)	7.72 (-13.85, 29.29)	-0.55 (-27.07, 26.01)	16.56 (-16.04, 49.17)	23.05 (-24.31, 70.40)
Chemerin	-1.09* (-2.14, -0.03)	-0.17 (-1.46, 1.12)	-0.28 (-1.95, 1.40)	-2.68** (-4.45, -0.92)	-1.17 (-4.13, 1.78)
Leptin	580 (-265, 380)	238 (-120, 596)	144 (-333, 621)	377 (-186, 939)	759 (-46, 1563)
TNF-R2	0.16 (-0.99, 1.30)	1.28* (0.07, 2.49)	0.58 (-1.10, 2.26)	1.17 (-0.83, 3.17)	1.19 (-1.80, 4.17)

The association of the 5 validated genes with cardiometabolic parameters was studied using a linear regression model. Linear regression coefficients (95% CI) for the monocyte genes are shown, adjusted for age, sex and BMI-SD. *p<0.05, **p<0.01. BP: systolic blood pressure; CI: confidence interval; MFI: median fluorescence intensity; QUICKI: quantitative insulin sensitivity index.

CD14++CD16+ monocytes (β=-15385, p=0.03, and β=-19214, p=0.01, respectively). Finally, downregulation of HMBS and TMEM134 were associated with increased chemerin levels (β=-1.09 μg/ml, p=0.04 and β=-2.68 μg/ml, p=0.004, respectively), and downregulation of IMPDH2 was associated with decreased TNF-R2 levels (β= 1.28 ng/ml, p=0.04).

In summary, the analyses in the whole group and the obese subgroup show a consistent relation between downregulation of LRPPRC and enhanced monocyte CD11b expression, downregulation of HMBS and TMEM134 and enhanced chemerin levels, and downregulation of IMPDH2 and decreased TNF-R2 levels.

Pathway analysis

Focusing on the functional relevance of the observed gene expression profile, the functional enrichment of the differentially expressed genes in biological processes was assessed using ToppFun, a GO Term enrichment tool. Sixty-four out of 67 genes could be identified by ToppFun and were mapped to pathways involved in biological processes, using a minimum pathway size of 10 genes. These genes were particularly involved in oxidative phosphorylation and distinct metabolic processes (Supplemental Table 3). Previous research has shown that oxidative phosphorylation, in particular oxidative stress, is associated with adulthood obesity and cardiovascular disease in obese individuals.[20] Therefore, we next investigated the association of the validated genes with adult obesity and cardiovascular disease.

Monocyte gene expression and adult obesity and cardiovascular risk

To investigate whether adults at risk show a monocyte gene expression profile similar to obese children, the 5 validated monocyte genes were studied in a cohort of 351 adults at risk for ischemic cardiovascular disease. Clinical characteristics of the adult cohort are provided in Supplemental Table 6.

Downregulation of monocyte IMPDH2 (β=-0.496, p=0.004) and TMEM134 (β=-0.314, p=0.043) was associated with obesity in adults (BMI>30kg/m2), paralleling our findings in children. These relationships remained significant after adjustment for age and sex (Table 4). Next, we tested whether monocyte gene expression was associated with the established SYNTAX coronary atherosclerosis score. The SYNTAX score uses coronary angiography findings to quantify the complexity of coronary atherosclerosis, based on the number of atherosclerotic lesions, their location and their functional impact15. The Syntax score was originally developed to help clinicians select the most appropriate revascularization strategy, but is increasingly being used as a risk stratification tool for adverse ischemic events in patients undergoing percutaneous coronary intervention.[15,21] The SYNTAX score was available for 196 of the 351 adults. Monocyte TMEM134 downregulation was associated with higher SYNTAX scores (β=-0.247, p=0.041) (Table 5). While adjustment of age and sex did not alter this association (β=-0.251, p=0.033), addition of BMI to the model attenuated the associated between TMEM134 expression and SYNTAX scores. In summary, monocyte TMEM134 downregulation is observed in childhood obesity, associated with obesity in adults at risk, and correlates with a higher SYNTAX score in adults at risk in an obesity-dependent fashion.

Table 4: Monocyte gene expression and obesity in the adult cohort

Gene ID	Transcript	Array ID	Model 1 β (95% CI)	Model 2 β (95% CI)
HMBS	ILMN_16358	6060278	-0.213 (-0.505, 0.078)	-0.233 (-0.541, 0.076)
HMBS	ILMN_16358	7320021	-0.194 (-0.496, 0.108)	-0.227 (-0.549, 0.095)
IMPDH2	ILMN_3439	4590026	-0.424* (-0.753, -0.095)	-0.496** (-0.837, -0.156)
LRPPRC	ILMN_23753	6380064	-0.007 (-0.287, 0.272)	-0.013 (-0.304, 0.277)
TMEM134	ILMN_176754	5690711	-0.061 (-0.318, 0.195)	-0.068 (-0.340, 0.203)
TMEM134	ILMN_183533	670671	-0.311* (-0.610, -0.011)	-0.314* (-0.620, -0.009)
ZWILCH	ILMN_166966	7000743	-0.167 (-0.467, 0.133)	-0.168 (-0.485, 0.148)
ZWILCH	ILMN_3475	4850221	-0.247 (-0.556, 0.062)	-0.260 (-0.581, 0.062)

The association of the 5 genes with obesity in the adults was studied using a logistic regression model. Adult obesity was defined as a BMI >30kg/m2 and compared to a normal weight BMI<25kg/m2. Logistic regression coefficients β (95% CI) are shown, both unadjusted (Model 1) and adjusted for age and sex (Model 2). *p<0.05, **p<0.01. BMI: body mass index; CI: confidence interval.

Table 5: Monocyte gene expression and SYNTAX score in the adult cohort

Gene ID	Transcript	Array ID	Model 1 β (95% CI)	Model 2 β (95% CI)	Model 3 β (95% CI)
HMBS	ILMN_16358	6060278	-0.005 (-0.243, 0.232)	0.002 (-0.232, 0.237)	-0.013 (-0.252, 0.227)
HMBS	ILMN_16358	7320021	-0.043 (-0.281, 0.195)	-0.013 (-0.247, 0.221)	-0.030 (-0.268, 0.208)
IMPDH2	ILMN_3439	4590026	-0.133 (-0.370, 0.104)	-0.116 (-0.347, 0.115)	-0.090 (-0.331, 0.150)
LRPPRC	ILMN_23753	6380064	-0.187 (-0.424, 0.049)	-0.188 (-0.419, 0.043)	-0.201 (-0.437, 0.036)
TMEM134	ILMN_176754	5690711	-0.222 (-0.458, 0.014)	-0.211 (-0.443, 0.021)	-0.193 (-0.43, 0.044)
TMEM134	ILMN_183533	670671	-0.247* (-0.483, -0.012)	-0.251* (-0.481, -0.022)	-0.227 (-0.464, 0.011)
ZWILCH	ILMN_166966	7000743	-0.102 (-0.339, 0.136)	-0.171 (-0.404, 0.062)	-0.168 (-0.408, 0.071)
ZWILCH	ILMN_3475	4850221	0.030 (-0.207, 0.268)	0.010 (-0.223, 0.242)	0.048 (-0.197, 0.293)

The association of the 5 validated genes with SYNTAX score (square-root transformed) was studied using linear regression. Linear regression coefficients β (95% CI) are shown. Model 1: unadjusted, Model 2: adjusted for age and sex, Model 3: adjusted for age, sex and BMI. *p<0.05. CI: confidence interval; SYNTAX: SYNergy between percutaneous coronary intervention with TAXus and cardiac surgery.

Discussion

Monocytes are key players in the development and exacerbation of atherosclerosis, both via their role as macrophage foam cell precursors and their role in systemic inflammation.[22] In human studies, increased numbers of classical CD14++CD16- and intermediate CD14++CD16+ monocytes predict cardiovascular events independent of age, sex and classical cardiovascular risk factors.[23,24] Interestingly, childhood obesity also coincides with increased circulating numbers of classical and intermediate monocytes,[7] which prompts the question as to whether the monocytosis in childhood obesity contributes to atherogenesis over the years. The atherogenic role of monocytes in childhood obesity is difficult to study in human models, since longitudinal data are lacking. To the best of our knowledge, this is the first monocyte gene expression study in childhood obesity, and the first endeavor to crosscheck gene expression profiles in an adult cohort at risk. Our study showed a distinctive monocyte gene expression profile in childhood obesity, which correlated with inflammatory adipokine levels and monocyte CD11b expression, the latter of which represents monocyte activation.[7] More importantly, downregulation of monocyte IMPDH2 and TMEM134 was associated with obesity in the adult cohort at risk, and downregulated TMEM134 coincided with a higher SYNTAX score, reflecting an enhanced atherosclerotic burden.[15,21]

Our results stress the relevance of the monocytosis in childhood obesity and raise several interesting questions. First, pathway analysis of differentially regulated monocyte genes in childhood obesity revealed an overrepresentation of oxidative phosphorylation, oxidative stress, and intracellular metabolism pathways, which apparently reflects reprogramming to aerobic glycolysis. While resting immune cells primarily need ATP to meet cellular demands, and use glucose-pyruvate conversion (glycolysis) and oxidative phosphorylation to fulfill these needs, many immune cells in inflammatory microenvironments undergo metabolic reprogramming to aerobic glycolysis in order to engage in cellular growth and proliferation.[25] Aerobic glycolysis involves increased glucose transport, conversion of glucose to lactate, and the use of glycolytic intermediates to provide sufficient biomolecules (amino acids, nucleotides, lipids) for cell growth and proliferation.[25] Interestingly, an upregulation of aerobic glycolysis also coincides with the development of 'trained immunity'.[26] Upon repetitive stimulation with microbial moieties and/or metabolites, monocytes undergo epigenetic reprogramming towards aerobic glycolysis, which enhances the response of the trained monocytes in case of restimulation.[25,27] Recent literature suggests that the development of trained immunity contributes to the development of systemic inflammation and atherosclerosis, and represents an intriguing target for therapeutic intervention.[27]

Second, the role of TMEM134 in monocytes gains traction. In human monocyte studies, the expression of TMEM134 was decreased in classical CD14++CD16- and intermediate CD14++CD16+ monocytes, in contrast to non-classical CD14+CD16++ monocytes.[28] Hence TMEM134 downregulation in childhood obesity and adults with cardiovascular risk may reflect obesity-induced CD14++CD16- and CD14++CD16+ monocytosis. Whether the highly conserved 21.5kDa transmembrane protein TMEM134 plays an active role in monocyte differentiation remains to be elucidated. Notably, the existing studies indicate that TMEM134 affects the prototypical

inflammatory nuclear factor-κB (NF-κB) signaling pathway. TMEM 134 was identified as a binding protein of latent membrane protein 1 (LMP1) and Hepatitis E Virus Open Reading Frame 2 (ORF2), and affected downstream NF-κB signalling via these binding partners.[29,30] Importantly, modulation of downstream NF-κB signalling is considered one of the hallmarks of innate immune programming in chronic inflammation.[31] Therefore, it is tempting to speculate that the observed downregulation of TMEM134 in childhood obesity monocytes is connected to the development of trained immunity, as discussed previously.

Finally, limitations of the current study have to be taken into account. Since our pediatric study population was relatively small, the associations reported in our study are of subtle strength. Due to the small sample size, adjustment of the statistical models for pubertal stage was impossible. The latter has probably not affected our results, as we did adjust our statistical models for age and sex, both highly correlated with pubertal stage. Nonetheless, the possibility of residual confounding cannot be precluded. Second, environmental factors such as freeze-thawing of the monocytes may have influenced gene expression profiles. Though pediatric and adult samples were treated similarly, minor processing differences could impact gene expression profiles. Third, we chose to focus on the 5 qPCR-validated monocyte genes. Thereby, we may have disregarded important monocyte genes that were not included in the qPCR validation. Finally, CD14-positive magnetic bead sorting skewed the analyzed monocyte compartments towards classical CD14++CD16- and intermediate CD14++CD16+ monocytes (Supplemental Figure 1), and partly disregarded the non-classical CD14+CD16++ monocyte subset, which is considered less important for atherosclerosis development.[23,24]

In conclusion, childhood obesity entails monocyte gene expression alterations associated with obesity and enhanced complexity of coronary atherosclerosis in adults. Especially the role of TMEM134 in monocytes gains traction, as downregulation of monocyte TMEM134 was associated with obesity in children and adults, and coincided with a higher SYNTAX atherosclerosis score in adults at risk for ischemic cardiovascular disease.

Methods

Pediatric cohort
Peripheral blood mononuclear cells (PBMC) were studied of 51 children aged 6-16 years (35 obese, 16 lean controls). The cells were derived from a previously published cross-sectional study at the Pediatric Outpatient Department of the Meander Medical Center in Amersfoort, the Netherlands, consisting of 60 obese children and 30 age- and sex-matched lean controls.[7] Because PBMC were available for 35 obese children and 16 lean controls, these children were included in the current study. Importantly, the availability of stored PBMC depended on the amount of blood a patient donated upon inclusion, which varied randomly. Therefore, we believe patients in the current study are a random selection of the previous study.

BMI-SD was calculated using the outcomes of the Fifth Dutch Growth Study (2008-2010). Childhood obesity was defined as BMI-SD>2.5, which can be extrapolated toward the international definition of obesity as BMI>30 kg/m^2 for adults.[32,33] Blood pressure was measured using an automated oscillometric method (Dinamap; GE Healthcare, Amersham, UK). Lipid profiles where obtained using standardized laboratory procedures. Written informed consent was obtained from all children and their parents. The study was approved by the Institutional Medical Ethical Review Board of the University Medical Center Utrecht, The Netherlands. All experiments with human biological materials were performed in accordance with the relevant guidelines and regulations.

Adult cohort
CTMM Circulating Cells is a multi-center cohort of four Dutch medical centers that enrolled patients with stable or unstable angina pectoris undergoing coronary angiography, with the aim of identifying cellular biomarkers for the prediction of adverse cardiovascular events. Patients were recruited between March 2009 and September 2011. Details of the study design have been described elsewhere.[34] All participants provided written informed consent. The study was approved by the Institutional Medical Ethical Review Board of the University Medical Center Utrecht, The Netherlands. Data from 351 patients were included in the final analysis after removal of samples with outlying median intensity (Supplemental Table 6). Gene expression profiles were quantile-normalized followed by $\log_2$ transformation. The complexity of coronary atherosclerosis was assessed with coronary angiography using the SYNTAX score system. Two independent observers quantified SYNTAX scores, using SYNTAX score calculator version 2.11. The SYNTAX score is a tool for evaluating the complexity of coronary artery disease, taking into account the number of atherosclerotic lesions, their location and their functional impact.[15] SYNTAX scores were available from 196 of the 351 patients.

Monocytes
In both cohorts, peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque density gradient centrifugation. In the pediatric cohort, flow cytometric phenotyping was performed in earlier studies.[7] Subsequent to isolation, samples were stored in freeze medium (FCS with 10% DMSO, Sigma-Aldrich) until further use.

In order to isolate monocytes, stored samples where thawed and washed in medium comprising of RPMI1640 supplemented with L-glutamate and 25 mM HEPES (Gibco), containing 2% FCS and penicillin/streptomycin (100 U/mL) (Invitrogen). Cells where spun down for 10 min, 1600 rpm at room temperature. PBMCs where then resuspended in MACS buffer, 2%FBS (Biowest), 2%EDTA (VWR chemicals) in PBS (Gibco), and counted using the trypan blue exclusion method (Gibco). Anti-human CD14 magnetic particles where subsequently used to isolate monocytes using the company protocol (BD IMag). The CD14 positive cells were then re-suspended in 500ul of TRIZOL (Life Technologies) and stored at -80°C.

Microarray and data processing
RNA was isolated from the trizol-lysed samples by AROS Applied Biotechnology. Samples of the 35 obese children and 16 healthy control children and the 351 adults underwent the same isolation procedures, and were similarly processed. In short, samples were labeled using the Illumina TotalPrep RNA Amplification Kit and 100ng of total RNA. The IVT product was QC-checked on gel and quantitated using the NanoDrop (Thermo Scientific). 750ng of cDNA was used for the standard Illumina protocol before samples were hybridized on the arrays (Illumina humanHT-12 v3). Arrays were scanned using a Bead Array Reader (Illumina). After inspection of the sample median intensities, samples with a median intensity of <50 were removed. Subsequently, the expression data was quantile-normalised and $\log_2$ transformed using the lumi R package.[35]

qPCR Validation
To validate the 67 differentially expressed genes, qPCR primers were designed for the top 20 hits. Of these primer pairs 17 functioned optimally and were deemed applicable for the validation process (Supplementary Table 4). High quality RNA of 27 obese children and 11 healthy controls was available for the qPCR validation studies. qPCR analysis was performed using SYBR Select Master Mix reagents (Thermo Fischer Scientific) and run using the QuantStudio Flex system (Thermo Fischer Scientific). Data was normalized for housekeeping gene expression of GUSB, 36B4 and B2M, in accordance with international standards.[36]

Statistics
First, demographic characteristics of the study population were presented as numbers and percentages for categorical variables and as means with standard deviation (SD) or medians with interquartile ranges for normal and non-normally distributed continuous variables, respectively. Subsequently, monocyte gene expression profiles of lean and obese children were compared and significant differences between both groups were assessed using Mann Whitney U tests for continuous variables and χ^2 test for binary variables.

Second, monocyte gene expression profiles were compared using the Limma package in R. In short, the Limma package uses empirical Baysian methods for the analysis of gene expression microarray data and is specifically designed for analyzing smaller datasets.[35] For this analysis, the genes functioned as outcome variables (dependent variables) and obesity status as determinant (independent variable). Age and sex

were included in the model as covariates. In addition, to adjust for multiple testing, Benjamini Hochberg (BH) correction was applied. To illustrate the results of the microarray analysis, a heat map was generated using the heatmap.2 function in R. Hierarchical clustering was performed using complete linkage.

Third, linear regression was used to study the relation between obesity status and the gene expression (dependent variable). For this analysis, two models were constructed; a crude model (Model 1) and a model in which age and sex were included as covariates (Model 2) (Supplemental Table 2).

Fourth, the relation between the 5 qPCR validated genes and clinical variables was studied in the whole pediatric cohort (n=51) as well as the obese subgroup (n=35) and the adult cohort (n=351) using linear regression analysis. For the analysis performed in the whole pediatric cohort age, sex and BMI-SD were included as covariates whereas for the analysis in the obese subgroup age and sex were included as covariates. A p-value of <0.05 was considered statistically significant.

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Supplement

Supplemental Table 1: Significantly different genes between obese and lean children in total study population (n=51)

	Log fold change	Crude p-value	FDR-adjusted p-value	β
ACBD6	-0.16	<0.0001	0.03	3.35
ANXA2P1	0.25	<0.0001	0.04	1.75
APOF	0.23	<0.0001	0.03	2.07
ATP5D	-0.16	<0.0001	0.03	2.31
C16orf13	-0.17	<0.0001	0.05	1.33
C20orf201	-0.15	<0.0001	0.04	1.98
CCT7	-0.29	<0.0001	0.03	2.47
CD99	0.29	<0.0001	0.04	1.85
CDKN1C	-0.41	<0.0001	0.05	1.32
CHCHD10	-0.17	<0.0001	0.04	1.86
CKS1B	-0.30	<0.0001	0.03	2.46
COX15	-0.32	<0.0001	0.04	1.57
DCTD	-0.14	<0.0001	0.03	2.88
DIS3L	-0.32	<0.0001	0.04	1.92
ECSIT	-0.21	<0.0001	0.03	3.69
EEF1D	-0.17	<0.0001	0.03	3.04
EMP2	0.14	<0.0001	0.03	2.36
F13A1	0.62	<0.0001	0.03	3.70
FAM195A	-0.35	<0.0001	0.03	2.42
FERMT3	0.11	<0.0001	0.03	3.29
FOXK1	-0.19	<0.0001	0.04	1.89
GABRR3	-0.09	<0.0001	0.03	2.57
GALM	-0.17	<0.0001	0.04	1.47
GMDS	-0.20	<0.0001	0.04	1.57
GTDC1	-0.10	<0.0001	0.04	1.73
HMBS	-0.27	<0.0001	0.03	2.76
IFNA1	-0.09	<0.0001	0.03	2.07
IMPDH2	-0.28	<0.0001	0.02	4.98
LGALS3	0.31	<0.0001	0.03	2.80
LNPEP	-0.12	0.0001	0.05	1.22
LOC100130604	0.36	<0.0001	0.03	2.18
LOC100131387	0.16	<0.0001	0.03	2.19
LOC100132598	-0.08	0.0001	0.05	1.19
LOC651452	0.07	<0.0001	0.03	2.38
LOC652683	-0.10	<0.0001	0.03	2.10
LOC653778	0.40	<0.0001	0.04	1.84
LOC654121	0.20	<0.0001	0.03	2.65
LOC730278	0.31	<0.0001	0.03	2.51
LOC730740	0.16	<0.0001	0.03	3.24
LRPPRC	-0.21	<0.0001	0.03	3.15
LSM4	-0.23	0.0001	0.05	1.20
MAP6D1	-0.33	<0.0001	0.03	2.78
ME1	0.27	<0.0001	0.02	4.37
METTL2B	0.14	<0.0001	0.03	2.86
MRPS15	-0.16	<0.0001	0.05	1.30
NDUFB2	-0.22	<0.0001	0.04	1.63

Supplemental Table 1 (continued)

	Log fold change	Crude p-value	FDR-adjusted p-value	β
NDUFS7	-0.23	<0.0001	0.05	1.28
NME1-NME2	-0.23	<0.0001	0.03	3.42
PID1	-0.44	<0.0001	0.03	2.07
PKN1	-0.15	<0.0001	0.03	2.07
POLR2I	-0.20	0.0001	0.05	1.22
PPP1R12A	0.25	<0.0001	0.04	1.48
QPRT	-0.32	<0.0001	0.00	8.56
SAR1A	0.32	<0.0001	0.04	1.75
SH3BGRL3	0.26	<0.0001	0.03	3.24
SLC30A4	0.19	<0.0001	0.03	2.48
SNHG7	-0.39	<0.0001	0.02	4.61
SNORD113-1	-0.08	<0.0001	0.04	1.73
SRM	-0.17	<0.0001	0.04	1.42
TMEM134	-0.19	<0.0001	0.02	4.54
TMEM160	-0.28	<0.0001	0.04	1.77
UBA6	0.36	<0.0001	0.04	1.53
USP49	0.55	<0.0001	0.04	1.56
UTP14A	-0.16	<0.0001	0.05	1.37
ZFP36L1	0.40	<0.0001	0.04	1.96
ZNF581	-0.17	<0.0001	0.04	1.51
ZWILCH	-0.12	<0.0001	0.03	2.62

Supplemental Table 2: Association between genes and obesity status in total study population (n=51)

	Model 1 β (95% CI)	p value	Model 2 β (95% CI)	p-value
ACBD6	-0.16 (-0.22, -0.10)	<0.0001	-0.16 (-0.22, -0.09)	<0.0001
ANXA2P1	0.26 (0.16, 0.37)	<0.0001	0.25 (0.13, 0.36)	<0.0001
APOF	0.22 (0.12, 0.32)	<0.0001	0.23 (0.12, 0.33)	<0.0001
ATP5D	-0.17 (-0.23, -0.10)	<0.0001	-0.16 (-0.23, -0.09)	<0.0001
C16orf13	-0.15 (-0.23, -0.07)	0.0004	-0.17 (-0.26, -0.09)	<0.0001
C20orf201	-0.16 (-0.22, -0.09)	<0.0001	-0.15 (-0.22, -0.08)	0.0001
CCT7	-0.28 (-0.40, -0.16)	<0.0001	-0.29 (-0.42, -0.16)	<0.0001
CD99	0.26 (0.13, 0.39)	0.0002	0.29 (0.16, 0.43)	<0.0001
CDKN1C	-0.40 (-0.59, -0.22)	<0.0001	-0.41 (-0.61, -0.21)	0.0001
CHCHD10	-0.19 (-0.26, -0.11)	<0.0001	-0.17 (-0.25, -0.09)	<0.0001
CKS1B	-0.28 (-0.41, -0.16)	<0.0001	-0.30 (-0.43, -0.16)	<0.0001
COX15	-0.29 (-0.43, -0.15)	<0.0001	-0.32 (-0.47, -0.17)	0.0001
DCTD	-0.16 (-0.22, -0.11)	<0.0001	-0.14 (-0.20, -0.08)	<0.0001
DIS3L	-0.31 (-0.45, -0.18)	<0.0001	-0.32 (-0.46, -0.17)	<0.0001
ECSIT	-0.21 (-0.29, -0.13)	<0.0001	-0.21 (-0.30, -0.13)	<0.0001
EEF1D	-0.15 (-0.22, -0.08)	<0.0001	-0.17 (-0.24, -0.10)	<0.0001
EMP2	0.12 (0.06, 0.18)	0.0002	0.14 (0.08, 0.20)	<0.0001
F13A1	0.64 (0.41, 0.88)	<0.0001	0.62 (-0.37, 0.88)	<0.0001
FAM195A	-0.34 (-0.48, -0.19)	<0.0001	-0.35 (-0.51, -0.20)	<0.0001
FERMT3	0.11 (0.07, 0.15)	<0.0001	0.11 (0.07, 0.15)	<0.0001
FOXK1	-0.19 (-0.27, -0.11)	<0.0001	-0.19 (-0.28, -0.10)	<0.0001
GABRR3	-0.08 (-0.12, -0.05)	<0.0001	-0.09 (-0.13, -0.05)	<0.0001
GALM	-0.18 (-0.26, -0.11)	<0.0001	-0.17 (-0.26, -0.09)	0.0001
GMDS	-0.18 (-0.27, -0.09)	0.0001	-0.20 (-0.29, -0.10)	<0.0001
GTDC1	-0.10 (-0.14, -0.06)	<0.0001	-0.10 (-0.15, -0.06)	<0.0001
HMBS	-0.25 (-0.36, -0.15)	<0.0001	-0.27 (-0.38, -0.15)	<0.0001
IFNA1	-0.08 (-0.11, -0.04)	0.0003	-0.09 (-0.13, -0.05)	<0.0001
IMPDH2	-0.29 (-0.39, -0.19)	<0.0001	-0.28 (-0.39, -0.18)	<0.0001
LGALS3	0.35 (0.22, 0.48)	<0.0001	0.31 (0.18, 0.44)	<0.0001
LNPEP	-0.13 (-0.18, -0.07)	<0.0001	-0.12 (-0.18, -0.06)	0.0001
LOC100130604	0.38 (0.23, 0.53)	<0.0001	0.36 (0.20, 0.53)	<0.0001
LOC100131387	0.17 (0.11, 0.24)	<0.0001	0.16 (0.09, 0.23)	<0.0001
LOC100132598	-0.08 (-0.11, -0.04)	<0.0001	-0.08 (-0.12, -0.04)	<0.0001
LOC651452	0.06 (0.04, 0.09)	<0.0001	0.07 (0.04, 0.10)	<0.0001
LOC652683	-0.09 (-0.13, -0.05)	<0.0001	-0.10 (-0.14, -0.05)	<0.0001
LOC653778	0.39 (0.21, 0.56)	<0.0001	0.40 (0.21, 0.59)	<0.0001
LOC654121	0.20 (0.13, 0.28)	<0.0001	0.20 (-0.11, 0.28)	<0.0001
LOC730278	0.32 (0.19, 0.45)	<0.0001	0.31 (0.18, 0.45)	<0.0001
LOC730740	0.17 (0.11, 0.23)	<0.0001	0.16 (0.09, 0.23)	<0.0001
LRPPRC	-0.21 (-0.30, -0.13)	<0.0001	-0.21 (-0.30, -0.12)	<0.0001
LSM4	-0.22 (-0.33, -0.12)	<0.0001	-0.23 (-0.34, -0.12)	0.0001
MAP6D1	-0.36 (-0.49, -0.23)	<0.0001	-0.33 (-0.48, -0.19)	<0.0001
ME1	0.27 (0.17, 0.38)	<0.0001	0.27 (0.17, 0.38)	<0.0001
METTL2B	0.13 (0.08, 0.19)	<0.0001	0.14 (0.08, 0.21)	<0.0001
MRPS15	-0.17 (-0.24, -0.10)	<0.0001	-0.16 (-0.24, -0.08)	0.0001
NDUFB2	-0.22 (-0.32, -0.13)	<0.0001	-0.22 (-0.33, -0.12)	<0.0001

Supplemental Table 2 (continued)

	Model 1 β (95% CI)	p value	Model 2 β (95% CI)	p-value
NDUFS7	-0.24 (-0.34, -0.14)	<0.0001	-0.23 (-0.34, -0.12)	0.0001
NME1-NME2	-0.23 (-0.31, -0.14)	<0.0001	-0.23 (-0.32, -0.13)	<0.0001
PID1	-0.43 (-0.62, -0.25)	<0.0001	-0.44 (-0.65, -0.24)	<0.0001
PKN1	-0.14 (-0.20, -0.08)	<0.0001	-0.15 (-0.22, -0.08)	<0.0001
POLR2I	-0.21 (-0.30, -0.12)	<0.0001	-0.20 (-0.30, -0.11)	0.0001
PPP1R12A	0.27 (0.16, 0.38)	<0.0001	0.25 (0.13, 0.37)	0.0001
QPRT	-0.31 (-0.40, -0.22)	<0.0001	-0.32 (-0.42, -0.22)	<0.0001
SAR1A	0.33 (0.19, 0.46)	<0.0001	0.32 (0.17, 0.47)	<0.0001
SH3BGRL3	0.25 (0.14, 0.36)	<0.0001	0.26 (0.15, 0.38)	<0.0001
SLC30A4	0.16 (0.09, 0.24)	<0.0001	0.19 (0.11, 0.27)	<0.0001
SNHG7	-0.37 (-0.51, -0.24)	<0.0001	-0.39 (-0.54, -0.24)	<0.0001
SNORD113-1	-0.07 (-0.10, -0.03)	0.0003	-0.08 (-0.12, -0.05)	<0.0001
SRM	-0.18 (-0.25, -0.10)	<0.0001	-0.17 (-0.25, -0.09)	<0.0001
TMEM134	-0.19 (-0.26, -0.13)	<0.0001	-0.19 (-0.26, -0.12)	<0.0001
TMEM160	-0.28 (-0.40, -0.16)	<0.0001	-0.28 (-0.42, -0.15)	<0.0001
UBA6	0.33 (0.17, 0.49)	0.0002	0.36 (0.19, 0.54)	0.0001
USP49	0.55 (0.31, 0.79)	<0.0001	0.55 (0.29, 0.81)	0.0001
UTP14A	-0.14 (-0.21, -0.07)	0.0002	-0.16 (-0.23, -0.08)	0.0001
ZFP36L1	0.44 (0.27, 0.62)	<0.0001	0.40 (0.22, 0.59)	<0.0001
ZNF581	-0.18 (-0.25, -0.10)	<0.0001	-0.17 (-0.25, -0.09)	<0.0001
ZWILCH	-0.11 (-0.16, -0.06)	<0.0001	-0.12 (-0.17, -0.07)	<0.0001

Model 1: crude; model 2: adjusted for age and sex (reference category: lean). Values are linear regression coefficients (β) with 95% confidence intervals (CI).

Supplemental Table 3: Pathway analysis

Name	ID	Num. of hits	Num. of genes in pathway	Raw p-value	p-value (FDR-adjusted)
oxidative phosphorylation	GO:0006119	5	108	1.58E-05	4.24E-03
purine ribonucleoside monophosphate metabolic process	GO:0009167	7	279	1.63E-05	4.24E-03
purine nucleoside monophosphate metabolic process	GO:0009126	7	280	1.67E-05	4.24E-03
ribonucleoside monophosphate metabolic process	GO:0009161	7	291	2.14E-05	4.24E-03
nucleoside metabolic process	GO:0009116	8	414	2.56E-05	4.24E-03
nucleoside monophosphate metabolic process	GO:0009123	7	304	2.83E-05	4.24E-03
nucleotide metabolic process	GO:0009117	10	694	2.88E-05	4.24E-03
nucleoside phosphate metabolic process	GO:0006753	10	704	3.26E-05	4.24E-03
glycosyl compound metabolic process	GO:1901657	8	436	3.70E-05	4.24E-03
nucleoside biosynthetic process	GO:0009163	5	131	4.02E-05	4.24E-03
nucleotide biosynthetic process	GO:0009165	7	322	4.08E-05	4.24E-03
glycosyl compound biosynthetic process	GO:1901659	5	133	4.32E-05	4.24E-03
nucleoside phosphate biosynthetic process	GO:1901293	7	325	4.33E-05	4.24E-03
organonitrogen compound biosynthetic process	GO:1901566	14	1445	5.17E-05	4.69E-03
purine nucleoside monophosphate biosynthetic process	GO:0009127	4	74	6.50E-05	4.96E-03
purine ribonucleoside monophosphate biosynthetic process	GO:0009168	4	74	6.50E-05	4.96E-03
nucleobase-containing small molecule metabolic process	GO:0055086	10	766	6.63E-05	4.96E-03
ATP metabolic process	GO:0046034	6	242	7.35E-05	5.19E-03
purine ribonucleoside metabolic process	GO:0046128	7	362	8.53E-05	5.71E-03
purine nucleoside metabolic process	GO:0042278	7	365	8.98E-05	5.71E-03
purine ribonucleoside triphosphate metabolic process	GO:0009205	6	263	1.16E-04	6.76E-03
ribonucleoside monophosphate biosynthetic process	GO:0009156	4	86	1.17E-04	6.76E-03
ribonucleoside triphosphate metabolic process	GO:0009199	6	270	1.34E-04	7.12E-03
purine nucleoside triphosphate metabolic process	GO:0009144	6	271	1.37E-04	7.12E-03
ribonucleoside metabolic process	GO:0009119	7	392	1.40E-04	7.12E-03
nucleoside monophosphate biosynthetic process	GO:0009124	4	94	1.65E-04	8.07E-03
nucleoside triphosphate metabolic process	GO:0009141	6	295	2.17E-04	9.91E-03
purine nucleoside biosynthetic process	GO:0042451	4	102	2.26E-04	9.91E-03
purine ribonucleoside biosynthetic process	GO:0046129	4	102	2.26E-04	9.91E-03
ATP biosynthetic process	GO:0006754	3	48	3.74E-04	1.59E-02
ribonucleoside biosynthetic process	GO:0042455	4	121	4.33E-04	1.78E-02
mitochondrial respiratory chain complex I assembly	GO:0032981	3	56	5.90E-04	2.21E-02
NADH dehydrogenase complex assembly	GO:0010257	3	56	5.90E-04	2.21E-02
mitochondrial respiratory chain complex I biogenesis	GO:0097031	3	56	5.90E-04	2.21E-02
purine ribonucleoside triphosphate biosynthetic process	GO:0009206	3	59	6.88E-04	2.50E-02
purine nucleoside triphosphate biosynthetic process	GO:0009145	3	60	7.22E-04	2.55E-02
ribonucleoside triphosphate biosynthetic process	GO:0009201	3	65	9.12E-04	3.11E-02
purine ribonucleotide metabolic process	GO:0009150	7	537	9.28E-04	3.11E-02
generation of precursor metabolites and energy	GO:0006091	6	395	1.01E-03	3.30E-02
ribonucleotide metabolic process	GO:0009259	7	552	1.09E-03	3.46E-02
purine nucleotide metabolic process	GO:0006163	7	556	1.14E-03	3.52E-02
ribose phosphate metabolic process	GO:0019693	7	567	1.27E-03	3.85E-02
mitochondrial respiratory chain complex assembly	GO:0033108	3	77	1.49E-03	4.28E-02
nucleoside triphosphate biosynthetic process	GO:0009142	3	77	1.49E-03	4.28E-02
nicotinamide nucleotide biosynthetic process	GO:0019359	2	20	1.55E-03	4.28E-02

Supplemental Table 3 (continued)

Name	ID	Num. of hits	Num. of genes in pathway	Raw p-value	P-value (FDR-adjusted)
pyridine nucleotide biosynthetic process	GO:0019363	2	20	1.55E-03	4.28E-02
mitochondrion organization	GO:0007005	8	770	1.70E-03	4.60E-02
mitochondrial ATP synthesis coupled electron transport	GO:0042775	3	82	1.79E-03	4.73E-02
purine-containing compound metabolic process	GO:0072521	7	605	1.84E-03	4.73E-02
organophosphate metabolic process	GO:0019637	10	1167	1.86E-03	4.73E-02
cofactor biosynthetic process	GO:0051188	4	181	1.94E-03	4.81E-02
ATP synthesis coupled electron transport	GO:0042773	3	85	1.98E-03	4.81E-02
organophosphate biosynthetic process	GO:0090407	7	614	2.00E-03	4.81E-02
heme biosynthetic process	GO:0006783	2	23	2.05E-03	4.82E-02
organelle inner membrane	GO:0019866	8	564	1.69E-04	3.30E-02

Results of GO Term enrichment analysis. The table lists GO term pathways (>10 genes) involved in biological processes, the number of overlapping genes in the query gene list and each pathway, the size of each pathway, the nominal p value derived from a hypergeometric test and the corresponding FDR adjusted p-value.

Supplemental Table 4: qPCR primer sequences

Gene	Forward	Reverse
36B4	CGGGAAGGCTGTGGTGCTG	GTGAACACAAAGCCCACATTCC
GUSB	CACCAGGGACCATCCAATACC	GCAGTCCAGCGTAGTTGAAAAA
B2M	CCAGCAGAGAATGGAAAGTC	GATGCTGCTTACATGTCTCG
ACBD6	TTGGTGGGCCAGTTATTAGTTC	CCAGTGAAGTAGAGCCCTACC
CHCHD10	CTACCATGGTCTGAGCTCCC	CATTTGTGTCTTGGGTTATCTGTG
CKS1B	CGACGACGAGGAGTTTGAGTATC	TTAGGGACCAGCTTGGCTATGT
COX15	TGGAGTAACTAGGTTGACAGAG	TGGAAATTGCTGGTATCTTTGG
ECSIT	ACAGGAACCTCCATCTCTCAG	GGACTGTTCAGAGCTATGGGC
FERMT3	AGCAGATCAATCGCAAGCAG	ATCCCGTACTTGTCCAGTGT
F13A1	GTGAAGATGATGCTGTGTATCTG	ATGCCATCTTCAAACTGACC
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
IFNA1	GCCTCGCCCTTTGCTTTACT	CTGTGGGTCTCAGGGAGATCA
IMPDH2	GGGCATCATCTCCTCCAGGG	TGCTGCGCTGCAGAATTTCA
LRPPRC	GAGAGATGCCGGAATTGAGC	CTCGGACTTCTCCACCTTCT
NME-1	AAGGAGATCGGCTTGTGGTTT	CTGAGCACAGCTCGTGTAATC
NME-2	CATTGACCTGAAAGACCGAC	ATGATGTTCCTGCCAACCTG
QPRT	GGGCAGCCTTTCTTCGATG	GGAGCCCATACTTCTCCACCA
TMEM134	CAGTTCAGCATTGATGATGCC	TTCTCCAGGTTCTGGTAGCG
USP49	CTCATCCCCTTCTCCCAGAG	TTCCAGGGATAGGTCCCAAA
ZWILCH	TTGGCTGATGGTTTGAGGAC	TGGTATGAAATCACACTACTGCTC

Supplemental Table 5: qPCR validation data

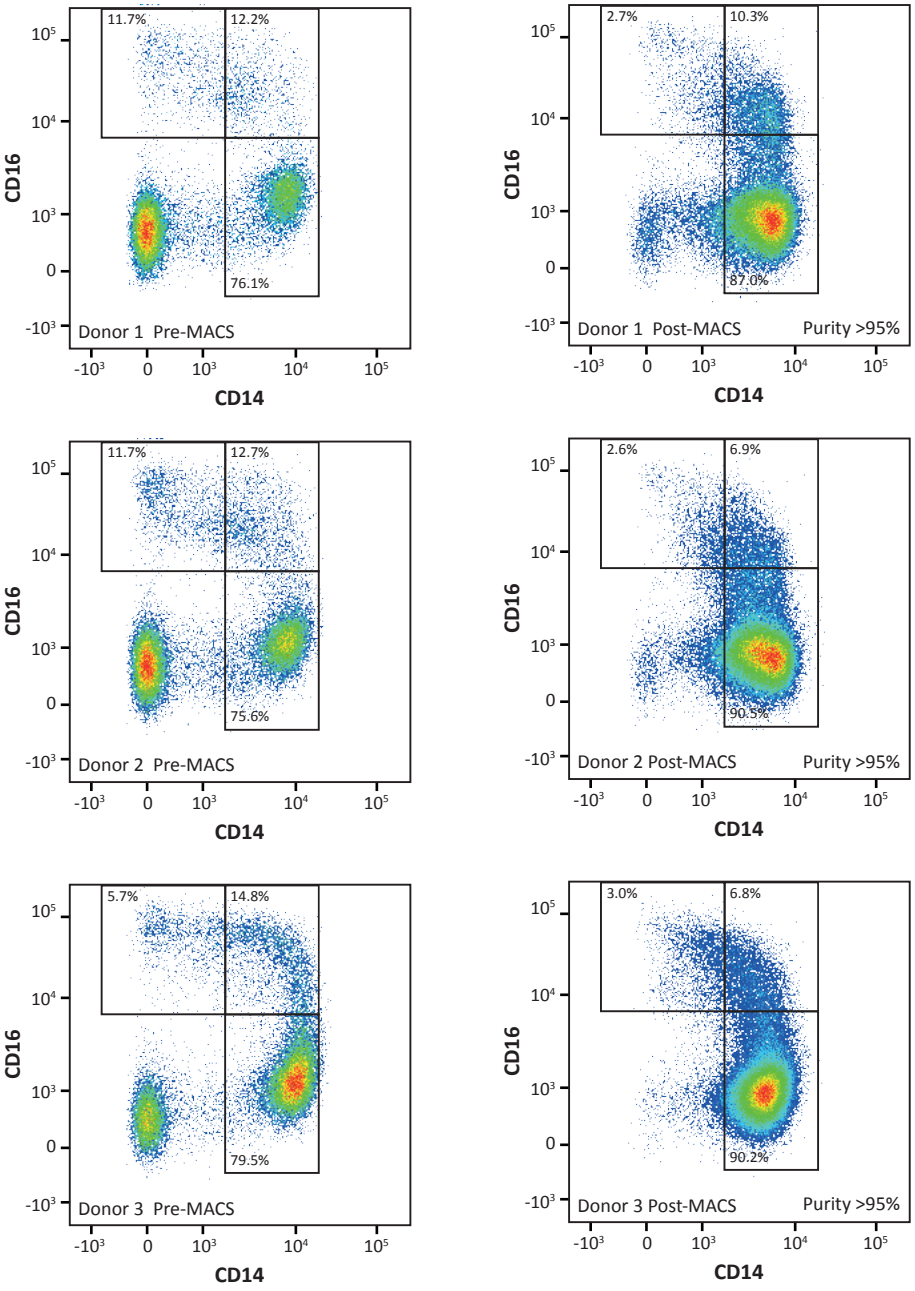
Gene	Fold Induction obese group (95% CI difference in fold induction)	P-value
ACBD6	0.59 (-0.18, 1.0)	0.17
CHCHD10	1.13 (-0.63, 0.37)	0.60
CKS1B	0.85 (-0.19, 0.48)	0.38
COX15	1.13 (-0.97, 0.72)	0.76
ECSIT	1.35 (-0.87, 0.16)	0.17
FERMT3	1.04 (-0.44, 0.36)	0.85
F13A1	1.28 (-0.88, 0.32)	0.35
HMBS	0.62 (0.087, 0.68)	0.01
IFNA1	1.23 (-1.04, 0.59)	0.58
IMPDH2	0.64 (-0.014, 0.74)	0.06
LRPPRC	0.60 (0.14, 0.68)	0.005
NME-1	0.71 (-0.071, 0.65)	0.11
NME-2	0.85 (-0.58, 0.89)	0.68
QPRT	0.73 (-0.39, 0.94)	0.41
TMEM134	0.32 (0.079, 1.28)	0.028
USP49	0.84 (-0.28, 0.60)	0.47
ZWILCH	0.45 (0.18, 0.92)	0.005

Supplemental Table 6: Clinical characteristics of the adult cohort

Variables	
Age, years	62.4±10.1
Male sex	262 (74.6)
BMI, kg/m2	27.4±4.3
Obese (BMI>30)	80 (23.3)
Hypertension	229 (65.2)
Hypercholesterolemia	230 (65.5)
Diabetes mellitus	75 (21.4)
Current smoker	71 (20.2)
Positive family history	151 (43.0)
prior MI	111 (31.6)
prior PCI	130 (37.0)
prior CABG	32 (9.1)
SYNTAX score	13 (6, 22)

Baseline characteristics of the adults with cardiovascular risk (n=351). Discrete variables are given as absolute count (%), continuous variables as mean (SD) or as median (IQR). BMI: body mass index; CABG: coronary artery bypass graft; MI: myocardial infarctions; PCI: percutaneous coronary intervention; SYNTAX: SYNergy between percutaneous coronary intervention with TAXus and cardiac surgery.

Supplemental Figure 1: Magnetic bead sorting



The monocyte subsets of 3 random patients before and after magnetic bead sorting are shown. Purity of the monocyte fraction after magnetic bead sorting was >95%.

Chapter 3

Loss of Y chromosome in blood is associated with major cardiovascular events during follow-up in men after carotid endarterectomy

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Abstract

Background: Recent studies found an immune-regulatory role for Y, and a relation between loss of Y (LOY) in blood cells and a higher risk of cancer and mortality. Given involvement of immune cells in atherosclerosis, we hypothesized that LOY is associated with the severity of atherosclerotic plaque characteristics and outcome in men undergoing carotid endarterectomy (CEA).

Results: LOY was quantified in blood and plaque from raw intensity genotyping data in men within the Athero-Express biobank study. Plaques were dissected, and the culprit lesions used for histology and the measurement of inflammatory proteins. We tested LOY for association with (inflammatory) atherosclerotic plaque phenotypes and cytokines and assessed the association of LOY with secondary events during 3-year follow-up. Out of 366 CEA patients, 61 exhibited some degree of LOY in blood. LOY was also present in atherosclerotic plaque lesions (n=8/242, 3%). LOY in blood was negatively associated with age (β=-0.03/10 yrs; r^2=0.07; p=1.6x10^{-7}), but not with cardiovascular disease severity at baseline. LOY in blood was associated with a larger atheroma size (OR=2.15 [95% CI: 1.06, 4.76]; p=0.04). However, this association was not significant after correction for multiple-testing. LOY was independently associated with secondary major cardiovascular events (HR=2.28 [95% CI: 1.11, 4.67]; p=0.02) in blood when corrected for confounders.

Conclusions: In this hypothesis-generating study, LOY in blood is independently associated with secondary major cardiovascular events in a severely atherosclerotic population. Our data could indicate that LOY affects secondary outcome via other mechanisms than inflammation in the atherosclerotic plaque.

Introduction

Loss of the Y chromosome (loss of Y, LOY) in blood cells was already described in the 60s and affects approximately 15% of the male population of older age.[1] Only recently LOY was associated with a higher risk of (non-haematological) cancer and overall mortality.[2,3] This relationship was speculated to be due to smoking and a disrupted tumor immunosurveillance.[4] Furthermore, LOY was associated with Alzheimer's disease[5] and the occurrence of auto-immune diseases such as primary biliary cirrhosis[6] and auto-immune thyreoiditis[7].

Indeed, the Y chromosome exhibited an immune-regulatory function by acting as a global trans-expression quantitative trait locus in mice.[8] The Y chromosome directly mediated changes in the transcriptome of CD4+ T-cells and macrophages, contributing to altered gene expression and alternative splicing. A role in global immune response was also found in the monocyte and macrophage transcriptome results of males with haplotype I that exhibited a 50% greater risk of myocardial infarction.[9] Comparison of gene expression data between haplotype I and other haplotypes revealed pathways that are related to inflammation and immunity, revealing down-regulation of adaptive immunity and up-regulation of inflammatory response in haplotype I carriers.

Genetic variation on the Y chromosome has been associated with high blood pressure[10] and myocardial infarction[11], independent from traditional cardiovascular risk factors, sex steroids or aggression. Given the global immune-regulatory role of the Y chromosome and the involvement of immune cells in atherosclerosis together with its male predominance, we hypothesized that LOY is associated with more severe atherosclerosis leading to worse outcome in men undergoing carotid endarterectomy (CEA).

Methods

Patient characteristics

The Athero-Express biobank study is an ongoing cohort study that includes atherosclerotic plaques and blood of patients undergoing either carotid endarterectomy (CEA) or femoral endarterectomy in two large tertiary referral hospitals (University Medical Center Utrecht and St Antonius hospital Nieuwegein) in the Netherlands. Clinical data were obtained from medical files and standardized questionnaires. Age was determined as age at surgery. Current smoking was determined as patient-reported smoking in the past year. Hypertension and hypercholesterolaemia were self-reported. Diabetes was considered present in any of the following cases: use of insulin or oral glucose inhibitors, self-reported diabetes mellitus in the patient questionnaire or diabetes mellitus extracted from the medical file. A history of coronary artery was considered present if the patient had suffered a myocardial infarction, or underwent a percutaneous coronary intervention or coronary artery bypass grafting surgery. Peripheral arterial occlusive disease was considered present if the patient either presented with an ankle-brachial index below 0.7, claudication

complaints or underwent percutaneous or surgical intervention for peripheral arterial occlusive disease. Follow-up was obtained by questionnaires sent to the patients by mail 1, 2 and 3 years postoperatively. Major cardiovascular events, including (sudden) cardiovascular death, hemorrhagic or ischemic stroke, myocardial infarction, fatal heart failure or fatal aneurysm rupture, were validated using medical records. The medical ethics boards of both hospitals approved of the study, which is conducted in accordance with the declaration of Helsinki and the subjects gave informed consent.

Sample collection
A detailed description of the sample phenotyping within the Athero-Express study can be found elsewhere.[12] In short, blood was obtained prior to surgery and subsequently stored at -80 degrees. Plaque specimens were immediately processed after removal during surgery. After identification of the area with the largest plaque burden (culprit lesion) the plaque was cut transversely into segments of 5 mm. The culprit lesion was fixed in 4% formaldehyde and subsequently decalcified and embedded in paraffin. Cross-sections were stained for histological examination. Remaining segments were stored at -80 degrees and used for the measurement of inflammatory cytokines and isolation of DNA.

Histological assessment of specimens
Plaque specimens were stained using CD68 (macrophages), α-actin (smooth muscle cells), picro-sirius red (collagen) and CD34 (microvessels). Furthermore the presence of plaque thrombosis was determined, using a combination of luminal thrombi, intraplaque haemorrhage, hematoxylin-eosin staining and Mallory's phosphotungstic acid-hematoxylin staining (fibrin). Either luminal thrombus, intraplaque haemorrhage or both were considered presence of plaque thrombosis. Computerized analyses quantitatively assessed macrophages and smooth muscle cells as percentage of plaque area. Microvessels were identified morphologically and counted in three hotspots and subsequently averaged per slide. Collagen and calcifications were scored semi-quantitatively into no (1), minor (2), moderate (3) or heavy (4) staining at 40x magnification. These categories were grouped into bins (no/minor and moderate/heavy) for the present analyses. The size of the lipid core was assessed using polarized light and cut off at an area of 10% and 40% of the plaque. All histological slides were assessed by the same dedicated technician.

Cytokine measurements of specimens
To determine the effect of LOY on inflammatory phenotypes within the Athero-Express biobank, we analyzed the association between LOY and seven different inflammatory cytokines: IL-6 and TNF-α as pro-inflammatory cytokines, IL-10 as an anti-inflammatory cytokine, RANTES as a marker of T-cell involvement and MCP-1, MCSF and GDF-15 as markers of macrophage involvement. Cytokines were measured by Luminex in plaque lysate (IL-6, TNF- α, IL-10, RANTES, MCP-1, MCSF) or citrate plasma (GDF-15) and normalized to protein content.

Genotyping data and quality control
The methods of the Athero-Express Genomics Study have been described before.[13] Genome-wide SNP genotyping data was collected in 1,858 consecutive CEA patients

using DNA from blood or plaque (when no blood was available) and either the Affymetrix Genome-Wide Human SNP Array 5.0 (AEGS1) or the Affymetrix Axiom GW CEU 1 Array (AEGS2). The quality control pipeline consisted of first excluding samples with low average genotype calling and sex discrepancies based on GCOS4 metrics, and thereafter filtering samples with a call rate >97%, variant call rate >97%, minor allele frequencies >3%, average heterozygosity rate ± 3.0 standard deviations, relatedness (pi-hat >0.20), Hardy-Weinberg Equilibrium ($p < 1.0x10^{-6}$) and based on population stratification (excluding samples >6 standard deviations from the average in 5 iterations during principle component analysis and by visual inspection). After quality control, we kept 1,640 samples for downstream analyses that were imputed using HapMap 2 CEU. For the current study, only the male samples of the AEGS2 (n= 610 total) could be used, as the AEGS1 array does not contain Y chromosomal SNPs.

Determination of loss of Y

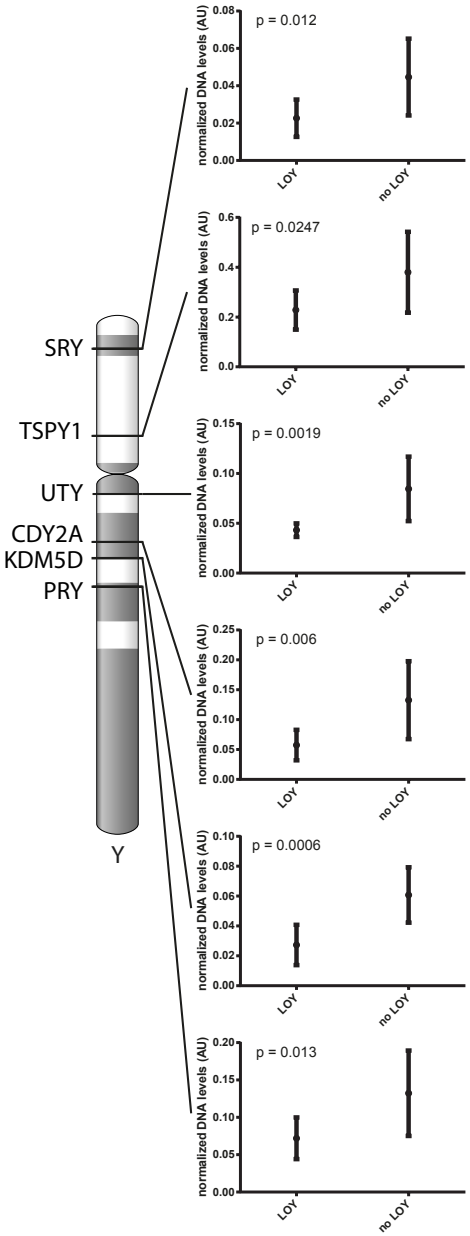
To assess LOY, median $\log_2$ ratios (observed intensity/reference intensity) were computed based on the raw intensity data from the male-specific Y chromosomal probes (mLRRY), excluding PAR1 and PAR2. Two blood samples were excluded due to outlying positive mLRRY values (defined as 1.5 interquartile ranges above the third quartile), leaving 366 blood samples and 242 plaque samples for analysis. We first calculated the peak of each mLRRY histogram using the density function in R for kernel density estimation, as previously described.[2] Next, a noise distribution was derived to compute the cut-off value for LOY. To this end, the positive tail of the kernel density was mirrored over the distribution peak of the kernel density estimates (local median), generating a negative tail. The lower bound of the resulting distribution served as the cut-off value for LOY (Supplemental Figure 1).

As a validation, LOY was assessed by qPCR of six Y chromosomal genes along the Y chromosome in 9 patients that exhibited dichotomous LOY and 8 patients that did not exhibit dichotomous LOY. Presence of one of the genes (TSPY1) was assessed by a commercially available kit (Y-chromosome Detection real-time PCR assay, Primerdesign Ltd). Primer design of the other five primers can be found in Supplemental Table 1. Detected DNA content between patients with and without LOY was compared using t-tests and significant for all genes (Figure 1). Primers were first tested on a female control and all yielded no DNA measurement in that sample.

Replication cohort

Replication of the Cox proportional hazards analysis on secondary cardiovascular events was performed in the AAA-Express.[14] The AAA-Express started as a spin-off of Athero-Express. AAA-Express is a biobank with patients that underwent open aneurysm repair in the UMC Utrecht and St. Antonius Hospital Nieuwegein between 2003 and 2013. Clinical characteristics, genotyping data (using Illumina Human Core Exome chip) and 3-year follow-up data on secondary cardiovascular events was present for 202 blood samples. Patients in Aneurysm Express were genotyped using the Illumina HumanCore Exome chip. Collection of data, including quality control of the SNP data and determination of LOY in this cohort was performed in the same way as in the Athero-Express cohort.

Figure 1: qPCR of Y chromosomal genes



AU: arbitrary units.

Statistical analyses
Binary LOY in blood was associated with baseline characteristics using χ^2 tests and Wilcoxon rank sum tests to determine possible confounders. The data were imputed using single imputation. All variables with a p value <0.1 (age, body mass index (BMI), glomerular filtration rate (GFR), smoking and hypertension) were put into a backstep multivariable model to determine their association with LOY. Remaining significant variables (age and smoking) were put into a multivariable model to assess whether LOY associates with severity of disease characteristics and box-cox transformed plaque phenotypes and inflammatory markers. A Cox proportional hazards model with all covariates that univariably associated with outcome (only age) was used to determine the association between LOY and major cardiovascular events during 3-year follow-up. Cox proportional hazards analysis in AAA included age as a covariate. Meta-analysis of the Athero-Express and AAA-Express cohorts was performed using inverse variance weighting on the models corrected for age. The proportional hazards assumption was assessed using scaled Schoenfeld residuals. Values $p<0.05$ were considered significant. The multiple-testing threshold for plaque characteristics and inflammatory cytokines was set at 0.05/15=0.003. All statistical analyses were carried out using the R computing platform, version 3.0.2.

Results

Loss of Y in blood
We determined median $\log_2$ ratios of Y chromosomal intensity (mLRRY) in 608 patients; in 366 patients we used blood derived DNA. Median Y chromosomal $\log_2$ ratios in these patients were negatively associated with age (β=-0.03/10 yrs, r^2=0.07, p=1.6x10^{-7}, Supplemental Figure 1). Of the 366 patients 61 (17%) exhibited dichotomous loss of the Y chromosome (LOY) in blood defined as mLRRY<-0.075 (Table 1, Figure 1, Supplemental Figure 2). A trend was seen for more smoking, a lower BMI and less hypertension in the LOY group. No other baseline characteristics were found to differ between patients with and without LOY in blood (Table 1).

Loss of Y in plaque
Within 242 patients we determined mLRRY in atherosclerotic plaque tissue. Median $\log_2$ ratios of Y chromosomal probe intensity in plaque were also negatively associated with age (β=-0.02/10 yrs, p=5.02x10^{-8}, Supplemental Figure 1). Of the 242 patients 8 (3%) exhibited dichotomous LOY in plaque defined as median $\log_2$ value of Y chromosomal intensity <-0.129 (Supplemental Figure 2). Because only eight patients suffered from LOY in plaque, we performed our analyses only on patients of whom we had blood-derived DNA.

No loss of chromosome 21
LOY could be a sign of general intensity loss throughout the genome. We therefore determined whether we could find any evidence for loss of chromosome 21. We found a median $\log_2$ ratio of intensity of chromosome 21 probes that was around 0, without any evidence for an association with age (Supplemental Figure 3).

Table 1: Baseline characteristics of patients with and without LOY in blood

	Loss of Y (n=61)	No Loss of Y (n=305)	p-value
Age in years (IQR)	75 (69, 79)	69 (62, 75)	<0.001
BMI (IQR)	24.9 (23.5, 27.0)	25.9 (24.1, 28.4)	0.08
Current smoker, yes (%)	25/60 (42)	88/303 (29)	0.08
Diabetes, yes (%)	10/61 (16)	73/305 (24)	0.26
Hypertension, yes (%)	33/59 (56)	203/296 (69)	0.08
Hypercholesterolemia, yes (%)	31/53 (58)	187/281 (67)	0.33
History of coronary artery disease (%)	19/61 (31)	94/305 (31)	1.00
History of PAOD (%)	12/61 (20)	62/305 (20)	1.00
Use of antiplatelet therapy (%)	56/60 (93)	271/304 (89)	0.45
Use of lipid lowering drugs (%)	44/61 (72)	244/305 (80)	0.23
Bilateral carotid stenosis (%)	17/48 (35)	129/266 (48)	0.13
GFR (MDRD) ml/min/1.73 m2 (IQR)	68.7 (58.6, 82.7)	74.5 (60.4, 87.2)	0.11
LDL in mg/dl (IQR)	105 (86, 127)	94 (70, 124)	0.29
HDL in mg/dl (IQR)	41 (33, 43)	39 (32, 47)	0.52
Total cholesterol in mg/dl (IQR)	174 (148, 186)	162 (135, 200)	0.66
Triglyceride levels in mg/dl (IQR)	98 (80, 148)	123 (89, 177)	0.12
Presenting symptoms (%)			0.27
Asymptomatic	4/60 (7)	42/302 (14)	
TIA	39/60 (65)	172/302 (57)	
Stroke	17/60 (28)	88/302 (29)	

Continues variables are expressed as median (IQR). IQR: inter-quartile range; BMI: body mass index; PAOD: peripheral arterial occlusive disease; GFR: glomerular filtration rate; MDRD: modification of diet in renal disease; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TIA: transient ischaemic attack.

Association with smoking

Previous studies point towards a role of smoking in loss of the Y chromosome. Past smokers and current smokers exhibited a lower mLRRY than never smokers (Supplemental Figure 4). We observed an association between mLRRY and smoking when corrected for age (β=-0.02 for current smokers compared to non-smokers, p= 0.03). In a backward step model, age and smoking were found to be most predictive of LOY (AIC for model with only age and smoking: 307. 25 vs AIC for model with age, smoking, BMI, GFR and hypertension: 310.79). Corrected for age, smoking was associated with dichotomous LOY (OR=2.83 [95% CI: 1.50, 5.35]; p=0.001).

Association with plaque phenotypes

Because dichotomous LOY showed the largest effect on baseline characteristics, this measure was used to investigate the association between LOY and plaque characteristics and secondary cardiovascular outcome. To investigate whether LOY in blood was associated with a more vulnerable plaque phenotype, we assessed the association between dichotomous LOY in blood and seven classical plaque characteristics: amount of calcification, amount of collagen, atheroma size, presence of intraplaque haemorrhage, macrophage and smooth muscle cell content and vessel density within the plaque. Furthermore, we assessed the association between dichotomous LOY in blood and specific inflammatory or anti-inflammatory cytokines within the atherosclerotic plaque. Corrected for age and smoking, dichotomous LOY in blood was nominally associated with a larger than 10% atheroma size (OR=2.15 [1.06, 4.76], p=0.04, Table 2, Supplemental Figure 5).

Association with secondary cardiovascular endpoints

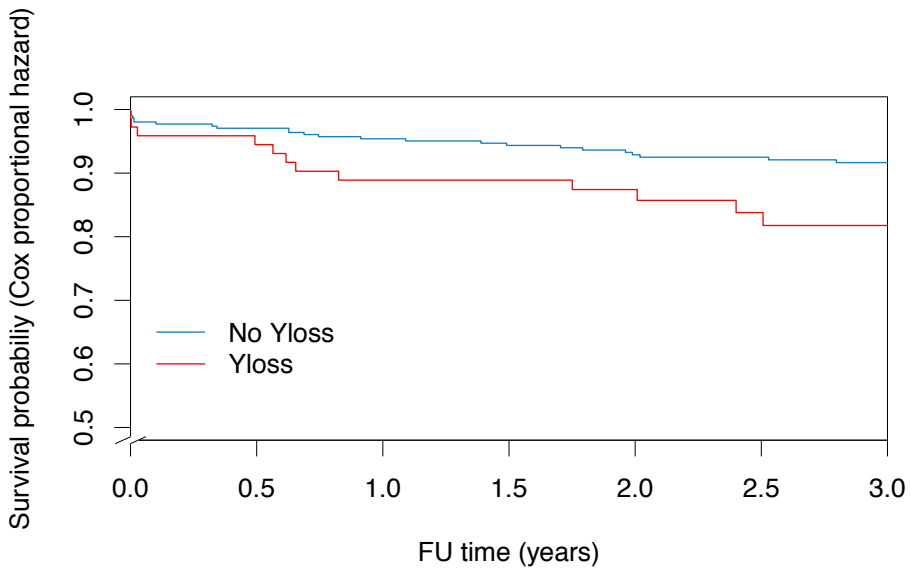
To determine whether dichotomous LOY in blood has an influence on secondary cardiovascular endpoints during follow-up, we used a Cox proportional hazards model correcting for age as this was the only LOY-associated baseline characteristic ($p < 0.1$) that was also associated with major cardiovascular endpoints. During 3 years of follow-up, men with dichotomous LOY in blood had significant more major cardiovascular endpoints (HR=2.28 [95% CI: 1.11,4.67]; p=0.02; Figure 2). We replicated the direction of this effect in the AAA-Express. Of the 202 patients, 29 exhibited LOY. During 3 years of follow-up, men with dichotomous LOY in blood had more major cardiovascular endpoints (HR=1.78 [0.54, 5.85]; p=0.34; Supplemental Figure 6). Meta-analysis of both cohorts confirmed the found effect (HR=2.13 [1.15, 3.94]; p=0.02). Furthermore, we observed the same direction of effect when studying the association of mLRRY in Athero-Express and cardiovascular events during follow-up, corrected for age, although this did not reach statistical significance (HR=0.13 [0.01, 1.33]; p=0.09). The effect was present in both smokers and non-smokers (Supplemental Figure 7). Atheroma size was not associated with major cardiovascular events during follow-up.

Discussion

In this hypothesis-generating study in a population of male carotid endarterectomy patients, loss of the Y chromosome in blood was detectable in both peripheral blood as well as in atherosclerotic lesions. Dichotomous LOY in blood was independently associated with a higher occurrence of major cardiovascular events during a 3-year follow-up period and this effect was replicated in a second cohort of cardiovascular disease patients. However, after correction for multiple testing, no associations were found between dichotomous loss of the Y chromosome and systemic and local (plaque) inflammatory status, suggesting that alternate mechanisms may explain the association between LOY and outcome.

We hypothesized that loss of the Y chromosome as an immunomodulating agent in the male genome would lead to a more severe type of cardiovascular disease by increased inflammation in the vascular wall, leading to a more unstable atherosclerotic plaque phenotype, reflected by a macrophage-rich plaque phenotype with a larger lipid pool, more intraplaque haemorrhage and more inflammatory cytokines. While we found an increase in major cardiovascular events and some preliminary evidence pointing towards a larger lipid pool, we were unable to identify a more inflammatory atherosclerotic plaque in these patients bearing in mind correcting for the testing of 15 different inflammatory phenotypes. One of the reasons could be the different cell-types in which we identified the LOY (blood) and in which we failed to observe an effect (plaque). However, both blood and plaque take part in the systemic inflammatory response in atherosclerotic disease and macrophages in the plaque derive from circulating monocytes. Furthermore, we also identified LOY in the atherosclerotic plaque itself. Interestingly, the amount of patients with LOY in plaque was lower. Although we cannot be sure as to what cell type is responsible for the detectable LOY in plaque, this lower amount of LOY may possibly be due to the fact that the atherosclerotic plaque does not contain as many rapidly dividing cells as compared to peripheral blood. The difference between LOY in plaque and LOY in blood is also reflected by less variation of LOY between the plaque samples. It could also be due to the fact that the plaque is formed by invasion and division of cells over several

Figure 2: Cox proportional hazards model for event-free survival



Model adjusted for age and current smoking; p=0.02.

Table 2: Associations of LOY with measures of (inflammatory) plaque phenotypes

Plaque phenotype	Beta of LOY (95% CI)	Odds Ratio of LOY (95% CI)	p-value
Atheroma size (>10%)	NA	2.15 (1.06, 4.76)	0.04
Atheroma size (>40%)	NA	1.84 (0.98, 3.41)	0.05
Calcification (major)	NA	0.86 (0.47, 1.58)	0.62
Collagen (major)	NA	0.82 (0.39, 1.64)	0.59
Intraplaque haemorrhage (present)	NA	0.87 (0.48, 1.58)	0.65
Macrophage (increase of plaque area)	0.19 (-0.19, 0.57)	NA	0.33
Smooth muscle cells (increase of plaque area)	0.05 (-0.33 , 0.42)	NA	0.81
Vessel density (increase per field)	-0.005 (-0.05, 0.04)	NA	0.84
IL-6 in plaque (per pg/mL plaque lysate)	-0.37 (-1.81, 1.08)	NA	0.61
IL-10 in plaque (per pg/mL plaque lysate)	-0.45 (-1.56, 0.67)	NA	0.41
TNF-α in plaque (per pg/mL plaque lysate)	-0.32 (-1.33, 0.69)	NA	0.52
MCSF in plaque (per pg/ug plaque lysate)	0.17 (-0.34, 0.68)	NA	0.51
RANTES in plaque (per pg/ug plaque lysate)	-0.23 (-0.88, 0.43)	NA	0.50
MCP-1 in plaque (per pg/ug plaque lysate)	0.14 (-0.18, 0.46)	NA	0.39
GDF-15 in plasma (per SD pg/mL plasma)	0.11 (-0.11, 0.34)	NA	0.33

Models were corrected for age and current smoking. Continuous variables are box-cox transformed. CI: confidence interval; IL: interleukin, MCP-1: monocyte chemotactic protein; MCSF: macrophage colony-stimulating factor; RANTES: regulated on activation, normal T cell expressed and secreted; TNF: tumor necrosis factor.

decades, during which the Y chromosome is possibly not yet lost. In agreement, from experimental atherosclerosis studies it has been established that plaque macrophages mostly derive from local proliferation rather than continuous infiltration.[15]

There are a few other possible explanations for the fact that we did not find any other association with plaque phenotype or inflammation. Firstly, LOY could be so detrimental to the male body that all patients suffering from it die before they develop an operable form of atherosclerosis and thereby simply do not end up in our study. Secondly, LOY could influence atherosclerosis in an earlier phase of the disease, for example affecting disease progression. Patients in the Athero-Express biobank suffer from severe end-stage disease and are, because of the operative guidelines, equally affected. Furthermore, a limitation of the current study is that it is limited in power to

detect small but biologically relevant differences because of a relatively small sample size. With an event probability of 12%, to obtain 80% power for observing a hazard ratio of 2.0, one needs 1006 samples and we had only 366 (power of 29%).

A recent study found a relation between LOY in blood and both (non-hematological) cancer and overall mortality in healthy men from the longitudinal ULSAM cohort aged 71-84 years.[2] However, not all increased mortality risk during over 40 years of follow-up could be attributed to malignant diseases. This leaves the question of what is causing the other deaths unanswered. In a follow-up study, LOY was also associated with smoking, a risk factor for both cancer and death. Smoking, however, is also a major risk factor for cardiovascular disease. This increased risk is due to several factors, including inflammation but for example also coagulation, endothelial dysfunction and adverse lipid profiles.[16] In our data, smoking was also significantly associated with mLRRY and with dichotomous LOY when corrected for age. Uncorrected, the absence of a significant association between smoking and dichotomous LOY may be explained by a lack of power (to obtain 80% power for observing a difference between 42% and 29%, one needs 580 samples (of which 20% LOY cases) and we had only 366). In a sensitivity analysis, we observed an effect in both smokers and non-smokers. In summary, we found preliminary evidence to support the hypothesis that the association between LOY and mortality is through a higher risk of major cardiovascular events and that this association cannot be solely explained by smoking as a risk factor.

The mechanism by which the Y chromosome is lost remains elusive. A recent genome-wide approach identified TCL1A that is associated to haematological malignancies as a genetic susceptibility locus for LOY at chromosome 14.[17] It might be that loss of the Y chromosome reflects general genomic instability of which the small and last to be replicated Y chromosome is the first victim. Rapidly dividing cells might not take their time to replicate its telomeres and this may lead eventually to loss of the entire chromosome. However, previous experiments blasting the Y chromosome apart have shown that it might be replicated and passed on to daughter cells, even when shattered into pieces even smaller than its original size.[18] Atherosclerosis might also accelerate genomic instability due to the formation of reactive oxygen species. However, we did not find a large proportion of LOY in the atherosclerotic plaque itself.

In our hypothesis-generating study, we found first preliminary evidence that LOY is independently associated with the occurrence of secondary major cardiovascular events in male patients after CEA. We replicated this effect in a cohort of male patients undergoing surgical aneurysm repair. More research is needed in a large sample of patients developing cardiovascular disease, preferably a cohort study that recorded cardiovascular disease incidence, to definitively answer the question how LOY is associated with adverse cardiovascular events and specify which events are most likely to be the cause of this association, whether or not smoking is the causative factor and whether or not LOY is also associated with incidence or progression of cardiovascular disease.

Supplemental materials are avialble online.

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Chapter 4

Routinely measured hematological parameters and prediction of recurrent vascular events in patients with clinically manifest vascular disease

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Manuscript in preparation

Abstract

Background: The predictive value of traditional risk factors for vascular outcomes in patients with manifest vascular disease is limited, underscoring the need for novel biomarkers to improve risk stratification. Since hematological parameters are routinely assessed in clinical practice, they are readily available candidates. We therefore assessed the incremental value of hematological parameters for prediction of recurrent vascular events.

Results: We used data from 3,922 vascular patients, enrolled in the Second Manifestation of ARTerial Disease (SMART) study from January 2005 onwards. Measurements of hematological parameters were extracted from the Utrecht Patient Oriented Database (UPOD). We first investigated the association of 22 hematological parameters with recurrent vascular risk, using Cox proportional hazards models. After adjustment for all variables included in the SMART risk score (SRS), lymphocyte %, neutrophil count, neutrophil % and red cell distribution width (RDW) were significantly associated with vascular outcome. We next tested whether these four parameters improved risk prediction compared with the SRS. When individually added to the SRS, lymphocyte % improved prediction of recurrent vascular events (continuous net reclassification improvement (NRI): 17.4% [95% CI: 2.1, 32.1%]; improvement in c-statistic: 0.011 [0.000, 0.022]). The combination of lymphocyte % and neutrophil count improved continuous reclassification by 22.2% [3.2, 33.4%] and discrimination (c-statistic) by 0.011 [95% CI: 0.000. 0.022]. However, lymphocyte % alone and combined with neutrophil count only modestly increased risk estimates for patients with an event during follow-up. Lymphocyte % and RWD yielded a continuous NRI of 18.7% [3.3, 31.9%], but mainly increased risk estimates for events in the higher risk range. The discrimination improvement for this model was 0.016 [0.004, 0.028].

Conclusion: Several hematological parameters were independently associated with recurrent vascular events. Lymphocyte % alone and in combination with other parameters enhanced discrimination and continuous reclassification compared to the SRS. However, the incremental value of these risk prediction models was limited for patients who experienced a recurrent event.

Introduction

The most common underlying cause of cardiovascular disease is atherosclerosis, leading to over 13 million deaths per year worldwide.[1] The implementation of preventive therapies critically depends on the reliable identification of individuals at risk. In clinical practice, vascular risk assessment is primarily based on risk factors, such as smoking, hypertension, diabetes, obesity and hyperlipidemia.[2] While a large body of evidence has underpinned the significance of such traditional risk factors in primary prevention,[3-5] their predictive value for recurrent risk in patients with established vascular disease is less clear.[6-8] Thus, novel risk factors are needed to improve risk stratification in secondary prevention and to establish the pathophysiological processes underlying recurrent vascular risk.

The SMART risk score (SRS) has been specifically developed to predict recurrent vascular events in patients with established atherosclerotic vascular disease.[9] This score not only includes traditional risk factors, but also vascular disease history, renal function and high-sensitive C-reactive protein (hs-CRP), an inflammatory marker associated with vascular risk.[10] Besides hs-CRP, several other biomarkers have been linked to prognosis of vascular disease, including N-terminal pro-type brain natriuretic peptide, troponins, ST2 and growth-differentiation factor-15.[6,11] A recent study identified different routinely-measured hematological parameters that predict outcomes in patients with coronary artery disease.[12] Because these parameters are measured by most hematology analyzers, they are readily available for use in clinical practice without the need to rely on expensive equipment. Despite their potential clinical utility, no study has yet assessed whether hematological parameters improve prediction of recurrent events beyond established secondary risk factors used in the SRS. Combining data from the Second Manifestation of ARTerial Disease (SMART) study and the Utrecht Patient Oriented Database (UPOD), we investigated the incremental value of routinely measured hematological parameters for the prediction of recurrent vascular events. We first investigated the association of 22 hematological parameters with recurrent vascular risk and then assessed parameters independently associated with recurrent events improved risk prediction compared to the SRS.

Methods

Study population

We conducted this study in patients with a clinical manifestation of atherosclerotic vascular disease (cerebrovascular disease, coronary artery disease, peripheral artery disease or abdominal aortic aneurysm) enrolled in the SMART study. Details on disease definitions and recruitment procedures have been published previously.[9,13] Briefly, the SMART study enrolled patients aged 18-79 who were referred to the University Medical Center Utrecht for clinical manifestations of atherosclerotic vascular disease or the treatment of vascular risk factors. Because complete hematological parameters were not available before 2005, we restricted our analysis to a subset of patients enrolled from January 2005 onwards. At baseline, patients were

requested to fill in a questionnaire on medical history, symptoms of vascular disease and vascular risk factors. During follow-up, questionnaires were sent to patients or their general practitioner twice a year to obtain information on their health status. Moreover, hospital discharge letters were collected to verify vascular events. All events were adjudicated by three members of the Endpoint Committee. The outcome of interest was a composite endpoint of vascular death, ischemic or hemorrhagic stroke or myocardial infarction, as previously described in more detail.[9] All patients provided written informed consent. The SMART study was approved by the Ethics Committee of the University Medical Center Utrecht.

Hematological parameters

We enriched the SMART cohort with 22 routinely measured hematological parameters, using UPOD, which comprises clinically relevant data from all patients admitted to the University Medical Center Utrecht, including laboratory measurements. Hematological parameters were quantified with the Abbott Cell-Dyn system, which is based on the multi-angle polarized scatter separation technique. Further details on the quantification of hematological parameters in UPOD have recently been published elsewhere.[12]

Statistical analysis

As for the derivation of the SRS, we truncated all continuous variables, including all hematological parameters, at the 1st and the 99th percentile to reduce the impact of outliers.[9] Using single imputation by additive regression, we imputed missing values for all variables included in the SRS (total n=126; 0.2%). The variable with the highest percentage of missing values was hs-CRP (n=75; 1.9%). To facilitate comparison between different hematological parameters, all values were scaled to SD units prior to analysis.

We first evaluated the association of each of the 22 hematological parameters with recurrent vascular events, using Cox proportional hazards modeling adjusted for all SRS variables [age, sex, diabetes mellitus, current smoking, systolic blood pressure, total cholesterol, high-density lipoprotein (HDL) cholesterol, hs-CRP, estimated glomerular filtration rate (eGFR), years since first vascular event, history of cerebrovascular disease, history of coronary artery disease, history of abdominal aortic aneurysm, history of peripheral artery disease]. Analogous to the SRS, hs-CRP was $\log_e$-transformed and quadratic terms were added for age and eGFR.[9] Since none of hematological parameters showed a skewness >2, $\log_e$-transformation was not applied. Hematological parameters were entered as quadratic polynomials if the addition of a quadratic term improved model fit ($p<0.05$), as indicated by the likelihood ratio test. Accordingly, we added a quadratic term for hematocrit. The proportional hazards assumption was tested for each model using scaled Schoenfeld residuals. Associations of hematological parameters with outcome were adjusted for multiple testing. Since several of the 22 parameters were highly correlated (Supplemental Figure 1), we estimated the effective number of independent tests for multiple testing correction using principal component analysis. The first 11 principal components explained over 95% of the variance in the hematology data, yielding a significance threshold of 0.05/11=0.0045.

We next evaluated the additive predictive value of hematological parameters significantly associated with outcome by comparing biomarker models to a reference model in terms of discrimination and reclassification. The reference model was constructed by fitting the SRS variables to our dataset. The biomarker models included the SRS variables and one of the hematological parameters significantly associated with recurrent event risk. We additionally assessed the performance of prediction models that included combinations of hematological parameters. To evaluate discrimination, we calculated Harrell's c for each model and compared c-statistics between each biomarker model and the reference model as proposed by Kang et al.[14], using the compareC R package (https://cran.rproject.org/web/packages/compareC/index.html). Reclassification was assessed by continuous net reclassification improvement (NRI), as implemented in the nricens R package (https://cran.r-project.org/web/packages/nricens/index.html), which computes NRI for censored survival data. Confidence intervals for NRI were computed by bootstrapping. To obtain robust reclassification indices, we assessed continuous NRI at 7 years, given a median follow-up of 4.6 years (IQR: 2.5-6.9 years). 7 years also corresponds to the follow-up period for which the SRS was initially calibrated before risk estimates were extrapolated to 10-year risk predictions.[9] Due to the absence of established categories for the 7-year risk of recurrent vascular events, we did not assess categorical NRI.

Results

In total, 3,922 patients with manifest vascular disease enrolled in the SMART cohort were included in this study. Baseline characteristics of the study population are summarized in Table 1. During a median follow-up of 4.6 years (IQR: 2.5-6.9 years), 310 recurrent vascular events occurred. In contrast to Dorresteijn et al.,[9] we only included patients recruited from 2005 onwards. Compared to this study, we observed lower event rates (1.7% vs. 2.6%), most likely reflecting improved secondary prevention therapies. In line with this, the proportion of patients treated with statins was higher in our study. Table 2 shows baseline values of all 22 hematological parameters stratified by event status.

First, we studied the association of hematological parameters with secondary vascular outcomes. Supplemental Table 1 displays adjusted and unadjusted effect estimates for all hematological parameters. Since most hematological parameters are directly or indirectly related to immunological processes, we assessed whether these associations were independent of hs-CRP. The addition of hs-CRP particularly attenuated effect estimates for white blood cell count, neutrophil count, monocyte count and neutrophil % (Supplemental Figure 2). Four parameters remained significantly associated with vascular events after adjustment for the SRS variables (Figure 1). Lymphocyte % showed a negative association with the outcome (HR in SD units: 0.80 [95% CI: 0.71, 0.91]), whereas neutrophil count (HR in SD units: 1.19 [1.06, 1.33]), neutrophil % (HR in SD units: 1.22 [1.08, 1.37]), and RDW (HR in SD units: 1.16 [1.05, 1.28]) were positively associated with recurrent events.

Table 1: Baseline characteristics

	All (N=3922)	No vascular event (N=3612)	Vacular event (N=310)
Age, years	61 (54, 68)	61 (54, 67)	64 (56, 71)
Male sex	2850 (73)	2610 (72)	240 (77)
Type of vascular disease			
Cerebrovascular disease	1125 (29)	1032 (29)	93 (30)
Coronary artery disease	2588 (66)	2373 (66)	215 (69)
Peripheral artery disease	531 (14)	481 (13)	50 (16)
Abdominal aortic aneurysm	236 (6)	213 (6)	23 (7)
Years since first vascular event			
less than 1 year	2283 (60)	2140 (61)	143 (48)
1-2 years	389 (10)	363 (10)	26 (9)
over 2 years	1110 (29)	980 (28)	130 (44)
Current smoking	1060 (27)	954 (27)	106 (34)
Diabetes mellitus	704 (18)	628 (17)	76 (25)
Systolic blood pressure, mm Hg	136 (124, 149)	135 (124, 149)	140 (129, 155)
Diastolic blood pressure, mm Hg	80 (73, 88)	80 (74, 88)	81 (73, 90)
eGFR, ml/min/1.73 m2	77 (66, 88)	77 (67, 88)	70 (60, 84)
Total cholesterol, mmol/l	4.3 (3.7, 5.1)	4.3 (3.7, 5.1)	4.3 (3.7, 5.1)
LDL cholesterol, mmol/l	2.4 (1.9, 3.0)	2.4 (1.9, 3.0)	2.4 (1.9, 3.1)
HDL cholesterol, mmol/l	1.2 (1.0, 1.4)	1.2 (1.0, 1.4)	1.1 (1.0, 1.4)
Triglycerides, mmol/l	1.2 (0.9, 1.8)	1.2 (0.9, 1.8)	1.3 (0.9, 1.9)
hs-CRP, mg/l	1.7 (0.8, 3.8)	1.6 (0.8, 3.6)	2.7 (1.3, 6.5)
Medication			
Lipid-lowering drugs	3140 (80)	2888 (80)	252 (81)
Blood pressure-lowering drugs	3086 (79)	2829 (78)	257 (83)
Glucose-lowering drugs	560 (14)	497 (14)	63 (20)
Antithrombotic drugs	3493 (89)	3206 (89)	287 (93)

Discrete variables are expressed as count (%), continuous variables as median (IQR). Type of vascular disease is not mutually exclusive as patients may have experienced several manifestations of vascular disease. eGFR: estimated glomerular filtration rate (see [9]); HDL: high-density lipoprotein; hs-CRP: high-sensitivity C-reactive protein; IQR: inter-quartile range; LDL: low-density lipoprotein.

We next added each of the four hematological parameters that was independently associated with recurrent event risk to a reference model comprising all SRS variables to assess discrimination and continuous reclassification (Table 3). We observed the largest continuous reclassification improvement for lymphocyte %. For events, this parameter improved continuous reclassification by 13.6%, for non-events by 3.8%, yielding a continuous NRI of 17.4% [95% CI: 2.1, 32.1%]. Additionally, lymphocyte % improved discrimination (c-statistic) by 0.0110 [95% CI: 0.0004, 0.0216]. We also tested whether lymphocyte % combined with other parameters further improved the predictive performance of the SRS. Neutrophil % was not included into a multi-biomarker model because this parameter was highly correlated with lymphocyte % (r=-0.92). Lymphocyte % and neutrophil count improved continuous net reclassification by 22.2% [3.2, 33.4%]. The discrimination improvement was 0.0112 [0.0004, 0.220], which was comparable to that achieved by lymphocyte % alone.

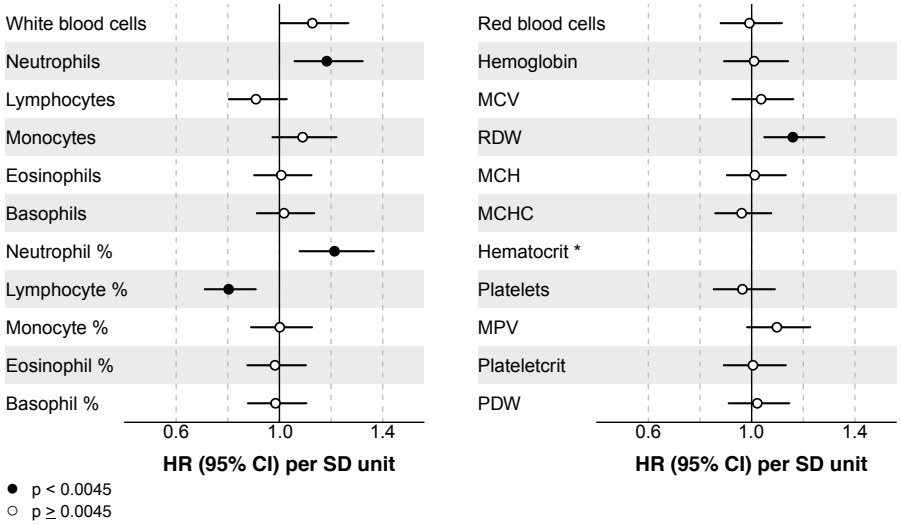
For lymphocyte % and RWD combined, the continuous NRI was 18.7% [3.3, 31.9%], the discrimination improvement was 0.016 [0.004, 0.028]. With a continuous NRI of 17.2% [4.1, 32.8%], all three parameters yielded a lower reclassification improvement than the combination of lymphocyte % and neutrophil count. The discrimination improvement for this model was 0.016 [0.004, 0.028]. Figure 2 illustrates the change in predicted risk for different biomarker models, stratified by event status. While lymphocyte % alone and the combination of lymphocyte % and neutrophil count showed the largest continuous reclassification improvement (Table 3) for events, risk estimates increased only modestly in patients who experienced an event. Lymphocyte % and RDW combined predominantly increased risk estimates for events in the higher risk range.

Table 2: Overview of hematological parameters

	Unit	No vascular event	Vacular event
White blood cells	10^9/l	6.6 (5.5, 7.9)	7.2 (5.9, 8.7)
Neutrophils	10^9/l	3.8 (3.0, 4.7)	4.2 (3.5, 5.4)
Lymphocytes	10^9/l	1.9 (1.5, 2.4)	1.9 (1.5, 2.3)
Monocytes	10^9/l	0.54 (0.44, 0.67)	0.58 (0.49, 0.70)
Eosinophils	10^9/l	0.19 (0.12, 0.28)	0.21 (0.15, 0.28)
Basophiles	10^9/l	0.04 (0.02, 0.06)	0.04 (0.03, 0.06)
Neutrophil %	%	57.9 (52.1, 63.7)	60.3 (55.1, 66.2)
Lymphocyte %	%	29.4 (24.4, 34.7)	26.2 (21.8, 32.0)
Monocyte %	%	8.2 (6.9, 9.7)	8.2 (6.8, 9.8)
Eosinophil %	%	2.9 (1.9, 4.2)	3.0 (2.1, 4.1)
Basophile %	%	0.61 (0.39, 0.88)	0.58 (0.36, 0.78)
Red blood cells	10^{12}/l	4.7 (4.4, 5.0)	4.6 (4.2, 4.9)
Hemoglobin	mmol/l	8.8 (8.3, 9.3)	8.8 (8.2, 9.3)
MCV	fl	89.8 (87.1, 92.5)	89.9 (86.9, 92.8)
RDW	%	12.1 (11.7, 12.7)	12.3 (11.8, 13.3)
MCH	amol	1898 (1824, 1971)	1896 (1815, 1982)
MCHC	mmol/l	21.1 (20.7, 21.5)	21.1 (20.5, 21.5)
Hematocrit	l/l	41.7 (39.3, 44.1)	41.7 (38.6, 44.4)
Platelets	109/l	237 (202, 280)	235 (203, 276)
MPV	fl	7.7 (7.2, 8.4)	7.9 (7.3, 8.6)
Plateletcrit	ml/l	0.19 (0.17, 0.22)	0.20 (0.17, 0.23)
PDW	10(GSD)	16.1 (15.8, 16.6)	16.2 (15.8, 16.6)

Median (IQR) values stratified by event status. GSD: geometric standard difference; IQR: inter-quartile range; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV: mean platelet volume; PDW: platelet distribution width; RDW: red cell distribution width.

Figure 1: Adjusted HRs for recurrent vascular events



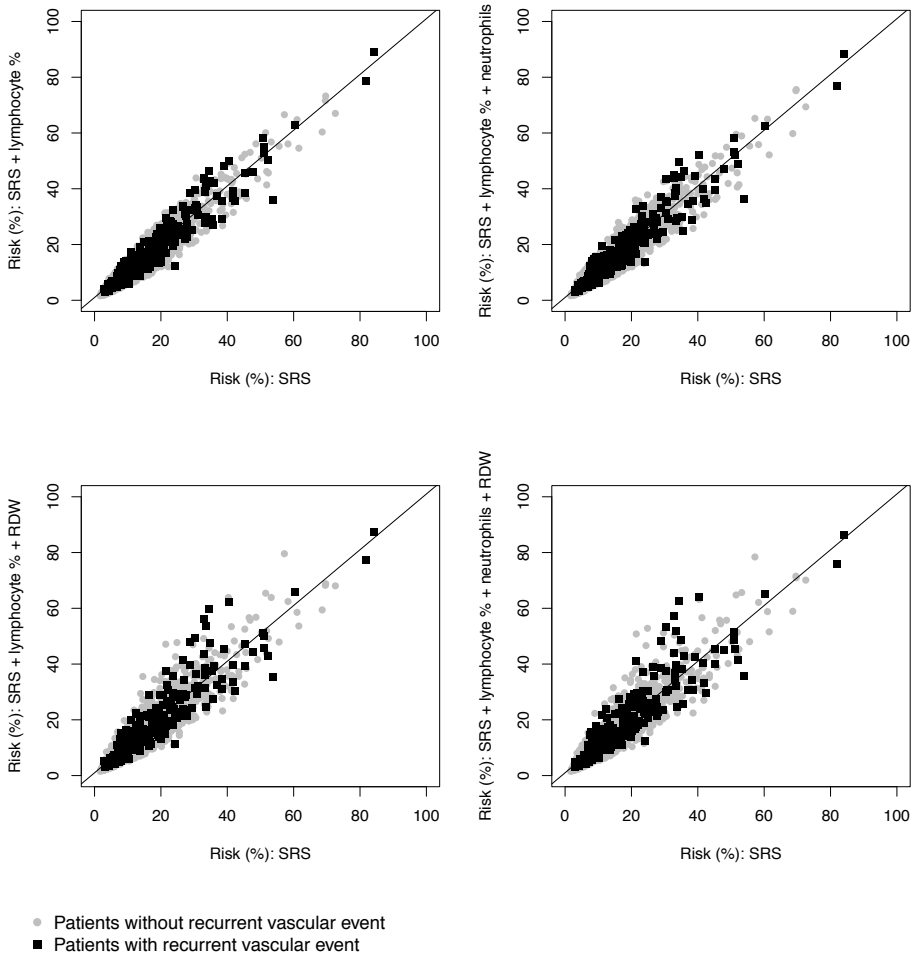
Each of the 22 hematological parameters was analyzed separately. HRs are given in given per SD-unit adjusted for SRS variables. *A quadratic term was added for hematocrit. Likelihood-ratio test for quadratic polynomial after adjustement for all SRS variables: $\chi 2(df=2)=6.2$; p=0.045. CI: confidence interval; HR: hazard ratio; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV: mean platelet volume; PDW: platelet distribution width; RDW: red cell distribution width; SD: standard deviation; SRS: SMART risk score.

Table 3: Predictive performance of hematological parameters

		Reclassification improvement %		
	Change in c-statistic (95% CI)	with event	without event	Net (95% CI)
Neutrophils	0.006 (-0.002, 0.014)	-9.1	15.6	6.5 (-6.0, 22.7)
Neutrophil %	0.008 (-0.002, 0.018)	7.2	6.7	13.9 (-0.3, 27.7)
Lymphocyte %	0.011 (0.000, 0.022)	13.6	3.8	17.4 (2.1, 32.1)
RDW	0.007 (-0.001, 0.015)	-11.3	25.0	13.6 (-1.9, 26.4)
Lymphocyte % + neutrophils	0.011 (0.000, 0.022)	14.8	7.4	22.2 (3.2, 33.4)
Lymphocyte % + RDW	0.016 (0.004, 0.028)	9.0	9.7	18.7 (3.3, 31.9)
Lymphocyte % + neutrophils + RDW	0.016 (0.004, 0.028)	5.1	12.0	17.2 (4.1, 32.8)

First, hematological parameters significantly associated with outcome were individually added to a model containing all SRS variables. For each biomarker model (SRS + hematological parameter), we evaluated improvement in discrimination (c-statistic) and reclassification (continuous NRI) compared to the reference model (SRS). We then assessed the predictive performance of multi-biomarker models comprising combinations of lymphocyte % and other hematological parameters. NRI: net reclassification improvement; RDW: red cell distribution width; SRS: SMART risk score.

Figure 2: Predicted risk of reference model vs. biomarker models



Predicted 7-year risks for refernce model (SRS) vs. selected biomarker models (SRS + hematological parameters) stratified by event status. Patients who did not experience a recurrent vascular event during 7-years of follow up (gray circles) were correctly reclassified if there predicted risk was lower after the addition of hematological parameters to the SRS (below the black line). Patients who experienced an event (black squares) were correctly reclassified if there predicted risk was higher after the addition of hematological parameters to the SRS (above the black line). RDW: red cell distribution width; SRS: SMART risk score.

Discussion

In this study, we evaluated the incremental predictive value of routinely measured hematological parameters for the prediction of recurrent vascular events in patients with established vascular disease. We first investigated the association of 22 parameters with recurrent event risk. Out of the four parameters significantly associated with outcome, lymphocyte % showed the largest continuous NRI when individually added to the SRS. Overall, the combination of lymphocyte % and neutrophil count yielded the largest continuous NRI compared to the SRS.

Lymphocytes have been implicated in the modulation of inflammatory processes at distinct stages of atherogenesis.[15] Numerous observational studies in patients with coronary artery disease have reported associations of low absolute and relative lymphocyte levels with poor cardiovascular outcomes.[12,16-21] However, some studies found no link between absolute lymphocyte count and all-cause mortality in pre-existing coronary artery disease.[22-24] Consistent with a role of low lymphocyte levels in vascular disease progression, lymphocyte apoptosis is enhanced in myocardial infarction, but not in stable angina, indicating that low lymphocyte levels may specifically reflect inflammatory processes in advanced atherosclerosis (e.g. plaque rupture).[25] In our study, however, lymphocyte % rather than absolute lymphocyte count was associated with recurrent vascular events. Accordingly, lymphocyte levels were comparable between patients with and without a recurrent event during follow-up – unlike concentrations of other white blood cell types, such as neutrophils and monocytes (Table 2). Low lymphocyte % may thus reflect increased levels of other white blood cell types in patients at risk.

Besides lymphocyte %, both absolute and relative neutrophil count were independently associated with recurrent vascular risk without improving risk prediction when individually added to the SRS. The combination of lymphocyte % and absolute neutrophil count showed the largest increase in continuous reclassification of all models tested, but only moderately increased risk estimates for events. The neutrophil to lymphocyte ratio has been widely studied as a marker of cardiovascular risk, suggesting that neutrophil levels are associated with poor prognosis of coronary and peripheral artery disease.[26] There is mounting evidence that neutrophils play an important role in early and advanced atherosclerosis by exacerbating endothelial dysfunction, recruiting monocytes to atherosclerotic lesions, promoting foam cell formation and by destabilizing atherosclerotic plaques.[27]

RDW was also independently associated with clinical outcome. Several studies have linked increased RDW to poor outcomes in patients with coronary artery disease[12,28,29], stroke[30] or peripheral artery disease[31]. RDW is a measure of the variation in erythrocyte volume. The mechanisms by which RDW relates to cardiovascular risk are unknown. Severe inflammation is associated with inhibition of erythrocyte maturation, which results in anisocytosis, suggesting that RDW reflects enhanced inflammation in atherosclerosis, potentially relevant to disease progression.[32] However, RDW did not improve risk prediction and, when combined with lymphocyte %, yielded a continuous NRI comparable to that achieved by lymphocyte % alone. Moreover, RDW

and lymphocyte % predominantly increased risk estimates for events in the higher risk range. Since patients with a high SRS would already be eligible for increased surveillance and more extensive treatment, the added value of RDW for clinical risk prediction is limited.

In the unadjusted analysis, total white blood cell count and monocyte count were strongly associated with recurrent events. However, adjustment for all SRS variables attenuated effect estimates for both parameters, especially due to the inflammatory marker hs-CRP (Supplementary Figure 2). In vitro findings suggest that CRP interacts with monocytes to enhance inflammation in acute coronary syndrome.[33] Thus, hs-CRP and monocytes may share a common pathophysiological pathway, whereas other hematological parameters may reflect inflammatory processes that do not, or to a lesser extent, involve CRP. Overall, our findings lend further support to the inflammatory hypothesis of atherothrombosis and add to recent clinical trial data suggesting that anti-inflammatory therapy reduces cardiovascular risk in secondary prevention.[34]

Hematological parameters are routinely measured in many hospitals and do not require expensive equipment for analysis, underscoring their clinical potential. In our study, lymphocyte % alone and combined with other hematological parameters yielded the largest continuous NRI. However, these models only marginally improved discrimination and absolute risk estimates for events. Thus, it remains uncertain whether the addition of hematological parameters to established risk assessment tools would influence clinical decision making in secondary prevention. Moreover, the ability of hematological parameters to predict recurrent vascular risk may vary between different manifestations of vascular disease, such as myocardial infarction and ischemic stroke. Since hematological parameters were not available from all SMART patients, the sample size of our study population was limited because. Therefore, we could not perform stratified analyses for different vascular disease groups. Overall, further research is required to corroborate our findings in other cohorts and to establish the predictive value of hematological parameters for different manifestations of vascular disease.

In conclusion, we identified several hematological parameters that were independently associated recurrent vascular event in patients with vascular disease. When added to a model comprising all SRS variables, lymphocyte % alone and in combination with other hematological parameters, especially with neutrophil count, improved risk prediction, but only modestly increased risk estimates for patients who experienced an event.

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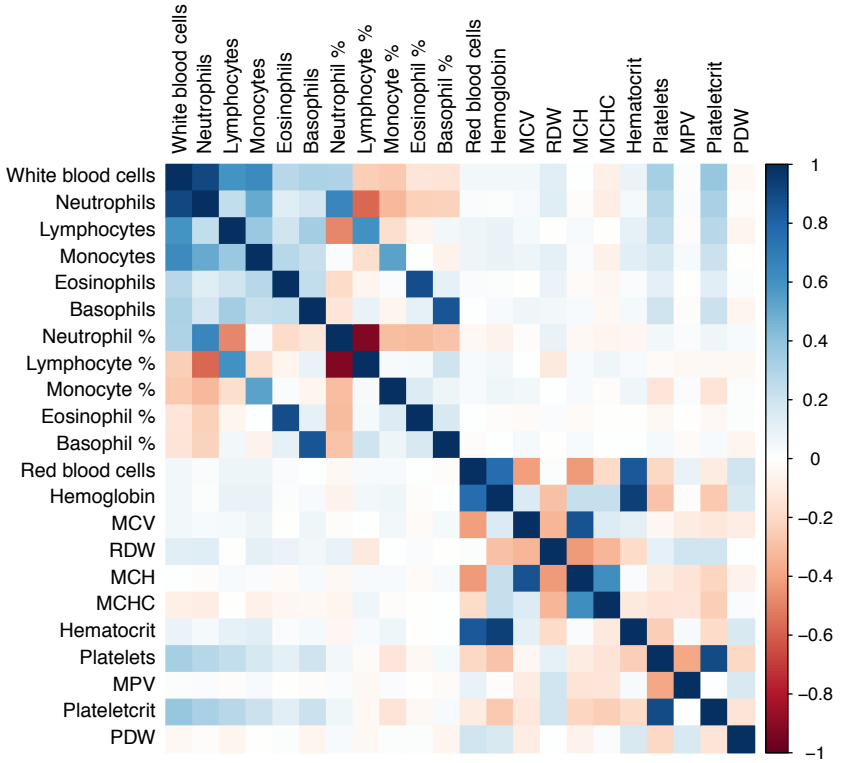
Supplement

Supplemental Table 1: Unadjusted and adjusted HRs

	Unadjusted		Adjusted for hsCRP		Ajusted for SRS variables	
	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
White blood cells	1.32 (1.20, 1.45)	<0.0001	1.18 (1.07, 1.32)	0.0018	1.13 (1.00, 1.27)	0.044
Neutrophils	1.38 (1.25, 1.51)	<0.0001	1.25 (1.12, 1.38)	<0.0001	1.18 (1.06, 1.32)	0.0031
Lymphocytes	0.95 (0.85, 1.07)	0.39	0.92 (0.82, 1.03)	0.15	0.91 (0.80, 1.03)	0.13
Monocytes	1.29 (1.17, 1.43)	<0.0001	1.18 (1.06, 1.31)	0.0026	1.09 (0.97, 1.22)	0.14
Eosinophils	1.13 (1.02, 1.25)	0.024	1.08 (0.97, 1.19)	0.17	1.01 (0.90, 1.12)	0.90
Basophils	1.05 (0.95, 1.17)	0.34	1.02 (0.92, 1.14)	0.70	1.02 (0.91, 1.13)	0.75
Neutrophil %	1.39 (1.24, 1.56)	<0.0001	1.28 (1.14, 1.43)	<0.0001	1.21 (1.08, 1.37)	0.0014
Lymphocyte %	0.68 (0.61, 0.77)	<0.0001	0.75 (0.66, 0.84)	<0.0001	0.80 (0.71, 0.91)	0.00048
Monocyte %	1.03 (0.92, 1.15)	0.66	1.04 (0.93, 1.16)	0.50	1.00 (0.89, 1.13)	0.98
Eosinophil %	1.01 (0.90, 1.13)	0.82	1.02 (0.91, 1.14)	0.70	0.98 (0.88, 1.10)	0.77
Basophil %	0.92 (0.83, 1.04)	0.17	0.96 (0.86, 1.08)	0.48	0.98 (0.88, 1.10)	0.79
Red blood cells	0.92 (0.82, 1.03)	0.13	0.98 (0.87, 1.09)	0.68	0.99 (0.88, 1.12)	0.89
Hemoglobin	0.94 (0.84, 1.05)	0.28	1.03 (0.92, 1.15)	0.66	1.01 (0.89, 1.14)	0.88
MCV	1.08 (0.97, 1.21)	0.17	1.09 (0.98, 1.21)	0.13	1.04 (0.93, 1.16)	0.53
RDW	1.30 (1.19, 1.42)	<0.0001	1.21 (1.10, 1.33)	0.00011	1.16 (1.05, 1.28)	0.0039
MCH	1.04 (0.93, 1.16)	0.53	1.06 (0.95, 1.19)	0.26	1.01 (0.90, 1.13)	0.84
MCHC	0.94 (0.84, 1.05)	0.26	0.98 (0.88, 1.10)	0.74	0.96 (0.86, 1.08)	0.49
Hematocrit*						
linear term	1.01 (0.91, 1.12)	0.88	1.07 (0.97, 1.19)	0.19	1.05 (0.94, 1.18)	0.38
quadratic term	1.13 (1.06, 1.22)	0.00032	1.11 (1.00, 1.01)	0.0027	1.09 (1.02, 1.18)	0.014
Platelets	0.99 (0.89, 1.11)	0.89	0.90 (0.80, 1.01)	0.064	0.96 (0.85, 1.09)	0.56
MPV	1.12 (1.01, 1.25)	0.04	1.12 (1.00, 1.24)	0.049	1.10 (0.98, 1.23)	0.10
Plateletcrit	1.03 (0.92, 1.15)	0.57	0.93 (0.83, 1.04)	0.22	1.01 (0.89, 1.13)	0.93
PDW	1.05 (0.94, 1.18)	0.38	1.07 (0.95, 1.19)	0.27	1.02 (0.91, 1.15)	0.71

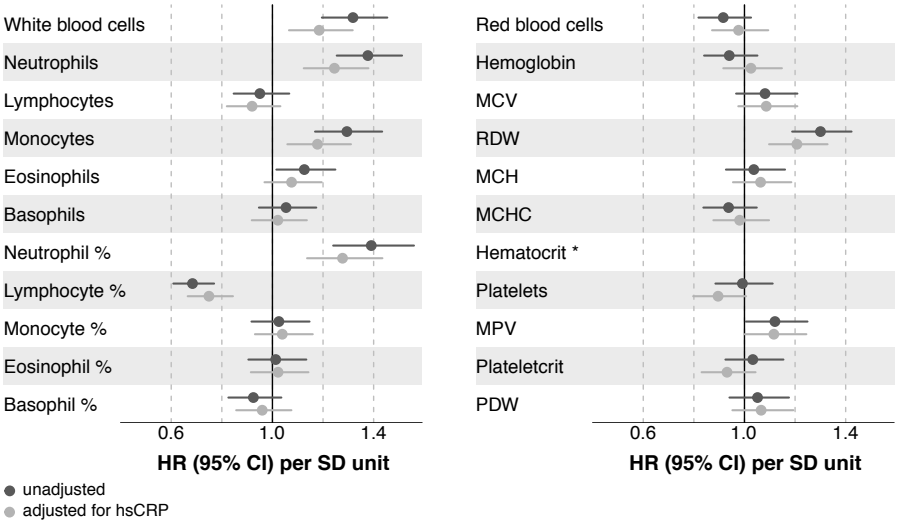
Each of the 22 hematological parameters was analyzed separately. HRs for recurrent vascular events are shown unadjusted, adjusted for $\log_e$(hs-CRP) and adjusted for all SRS variables. HRs are given per SD-unit. *A quadratic term was added for hematocrit. Likelihood-ratio test for quadratic polynomial after adjustement for all SRS variables: $\chi 2(df=2)=6.2$; p=0.045. CI: confidence interval; HR: hazard ratio; hs-CRP: high-sensitive C-reactive protein; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV: mean platelet volume; PDW: platelet distribution width; RDW: red cell distribution width; SD: standard deviation; SRS: SMART risk score.

Supplemental Figure 1: Correlation heat map of hematological parameters



Correlation heat map of hematological parameters, Correlations were assessed Pearson's correlation coefficient. MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV: mean platelet volume; PDW: platelet distribution width; RDW: red cell distribution width.

Supplemental Figure 2: Unadjusted and CRP-adjusted HRs



HRs for recurrent vascular events are shown unadjusted and adjusted for $\log_e$(hs-CRP). HRs are given in given per SD-unit. *A quadratic term was added for hematocrit. CI: confidence interval; HR: hazard ratio; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV: mean platelet volume; PDW: platelet distribution width; RDW: red cell distribution width; SD: standard deviation.

Chapter 5

Use of metabolic profiling to predict subsequent cardiovascular events in patients with coronary artery disease

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Abstract

Background: Traditional cardiovascular risk factors are limited in their ability to predict subsequent cardiovascular events in patients with established coronary artery disease (CAD). Therefore, novel biomarkers are required to improve prediction of subsequent events and to advance understanding of the pathological mechanisms driving CAD progression.

Methods: Using high-throughput metabolomics, we assessed associations of 72 lipid and metabolite measures with the 3-year risk of subsequent cardiovascular events in 1,511 patients with angiographically documented CAD from the Utrecht Coronary Biobank (UCORBIO) and the Angiography and Genes Study (ANGES). To evaluate the predictive capacity of metabolic measures, we derived risk scores with and without biomarkers in UCORBIO and tested their performance in ANGES.

Results: After adjustment for demographic and clinical variables, triglycerides in intermediate-density lipoproteins (IDL-TG; HR per SD: 1.45 [95% CI: 1.23, 1.70]) and low-density lipoproteins (LDL-TG; HR=1.39 [1.18, 1.63]), phenylalanine (HR=1.32 [1.15, 1.52]), creatinine (1.28 [1.05, 1.45]) and the concentration of small high-density lipoprotein particles (S-HDL; HR=0.77 [0.66, 0.89]) were significantly associated with subsequent cardiovascular events. The combination of LDL-TG, phenylalanine and S-HDL improved both discrimination and reclassification.

Conclusions: Using metabolic profiling in two angiographic cohorts, we identified LDL-TG, phenylalanine and S-HDL as biomarkers for subsequent cardiovascular events in patients with established CAD. Our findings provide novel insights into the pathophysiology of CAD progression and may help identify novel targets for drug development in secondary prevention.

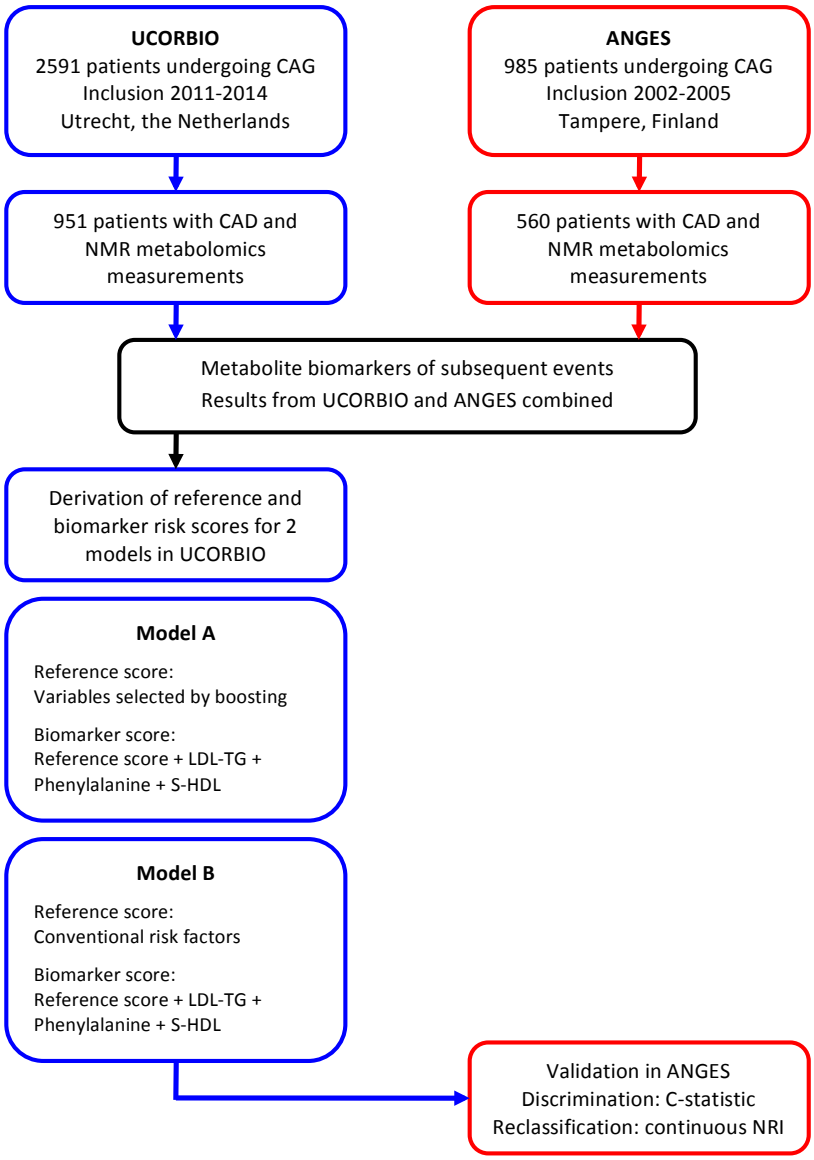
Introduction

With 1.7 million deaths and a prevalence of over 30 million cases, coronary artery disease (CAD) is the leading cause of mortality and morbidity in Europe.[1] Medical costs of coronary artery disease are projected to rise dramatically within the next decades,[2] highlighting the need to cost-effectively identify patients at risk who would benefit the most from expensive therapies. Risk stratification is particularly important in secondary prevention because patients with established CAD are at increased risk of suffering subsequent cardiovascular events and require life-long treatment and monitoring.

While international guidelines for secondary prevention[3,4] promote the use of risk scores[5,6] specifically developed to predict in-hospital and short-term outcome, the assessment and management of intermediate and long-term risk mainly relies on the same risk factors as for primary prevention,[7] even though the assumption that these risk factors equally well predict subsequent events has been questioned, following observations indicating that overweight[8], smoking[9,10] and high blood pressure[11,12] are not associated with poorer clinical outcome in cardiovascular patients. In line with this, biomarkers are better predictors of secondary cardiovascular events than traditional cardiovascular risk factors.[13] Moreover, the absence of traditional risk factors is not associated with a positive prognosis after myocardial infarction.[14] Such findings can be attributed to differential disease mechanisms for early and advanced stages of CAD[15] or aggressive treatment of traditional risk factors in secondary prevention, which may attenuate risk estimates for subsequent cardiovascular events.[13] Given the poor predictive value of traditional risk factors in secondary prevention, novel risk markers are required to improve prognosis of CAD and to advance understanding of the pathological pathways underlying disease progression.

With the advent of high-throughput nuclear magnetic resonance (NMR) and other metabolomics technologies, metabolic profiling has emerged as a powerful tool for biomarker assessment. Consequently, in-depth metabolic analysis is increasingly being used in cardiovascular epidemiology to study biomarkers associated with hypertension[16], subclinical atherosclerosis[17], left ventricular function[18] and cardiovascular events in initially healthy individuals[19]. In this study, we used a NMR-based metabolomics platform to assess associations of 72 lipid and metabolite measures with the 3-year risk of subsequent cardiovascular events in two European cohorts, which included a total of 1,511 patients with angiographically documented CAD: the Utrecht Coronary Biobank (UCORBIO) and the Angiography and Genes Study (ANGES). To evaluate the predictive capacity of lipid and metabolite measures in comparison with demographic and clinical variables, we derived risk scores with and without metabolic biomarkers in UCORBIO and tested their performance in ANGES (Figure 1).

Figure 1: Illustration of the study design



Results for 72 lipid and metabolite measures were analyzed separately in UCORBIO and ANGES and combined using inverse variance weighting. Subsequently, risk prediction scores with and without biomarkers were derived in UCORBIO and tested in ANGES, using two prediction models. Model A was based on demographic and clinical variables selected by boosting: age, hypertension, multivessel disease, myocardial infarction at inclusion, history of cerebrovascular accident, history of CABG, use of renin inhibitors and use of diuretics. Model B was based on traditional risk factors: age, sex, BMI, hypertension, diabetes mellitus, current smoking status, total cholesterol and HDL cholesterol. LDL-TG, S-HDL and phenylalanine were added to each model to obtain biomarker risk scores. CABG: coronary artery bypass grafting; CAG: coronary angiography; HDL-C: high-density lipoprotein cholesterol; LDL-TG: triglycerides in low-density lipoprotein; S-HDL: small high-density lipoprotein particle concentration NMR: nuclear magnetic resonance; NRI: net reclassification index.

Materials and methods

Study populations

We used data from UCORBIO (Utrecht, the Netherlands) and ANGES (Tampere, Finland), two prospective angiographic cohort studies. Baseline characteristics are shown in Table 1. From these two cohorts, we included patients with angiographically documented CAD (≥50% stenosis of at least one coronary artery). The major endpoint was the first occurrence of a cardiovascular event after recruitment, including cardiovascular death, non-fatal myocardial infarction or ischemic stroke. UCORBIO enrolled 2,591 patients undergoing coronary angiography for any indication at the University Medical Center Utrecht (the Netherlands) between October 2011 and December 2014 (www.clinicaltrials.gov; identifier: NCT02304744). 951 out of 1,198 patients, who were selected for metabolic profiling, (1) provided blood samples <30

Figure 1: Illustration of the study design

	UCORBIO (N=951)	ANGES (N=560)
Age, years	65.1 ± 10.9	64.3 ± 9.1
Male	724 (76.1)	430 (76.8)
BMI, kg/m2	27.4 ± 4.4	28.0 ± 4.1
Diabetes mellitus	231 (24.3)	189 (33.8)
Current smoker	230 (26.9)	74 (13.2)
Myocardial infarction (inclusion)	329 (34.6)	57 (10.2)
Multivessel disease	505 (53.1)	369 (65.9)
Hypertension	554 (59)	340 (60.9)
Medical history		
Myocardial infarction	528 (56.2)	246 (45.5)
Cerebrovascular accident	107 (11.3)	102 (19.3)
PCI	283 (29.8)	79 (14.2)
CABG	123 (12.9)	56 (15.8)
Lipid measures, mmol/l		
Total cholesterol	4.2 ± 1.2	4.2 ± 1.0
HDL-C	1.2 ± 0.3	1.2 ± 0.3
Triglycerides	1.4 (1.0, 1.9)	1.5 (1.1, 1.8)
Medication use		
Statins	584 (61.6)	432 (77.1)
P2Y12 inhibitors	278 (29.3)	38 (6.8)
Beta blockers	544 (57.4)	489 (87.3)
Renin inhibitors	485 (51.2)	211 (37.7)
Diuretics	281 (29.7)	139 (24.8)

Discrete data are expressed as absolute count (%), continuous data as mean ± SD or median (interquartile range). BMI: body mass index; CABG: coronary artery bypass grafting; HDL-C: high-density lipoprotein cholesterol; PCI: percutaneous coronary intervention; SD: standard deviation.

days after hospital admission (2) showed >50% stenosis, (3) were able to complete at least one follow-up questionnaire (recruitment before February, 2014) and were thus included in the analysis. ANGES recruited patients who were scheduled for coronary angiography at Tampere University Hospital (Finland) between September 2002 and July 2005. 560 out of 985 patients from ANGES, who were selected for metabolic profiling, showed >50% stenosis and were included in this study.

In UCORBIO, patient characteristics and procedural details were assessed at inclusion. During follow-up, patients completed questionnaires once a year to obtain information on hospitalizations and subsequent cardiovascular events. General practitioners and hospitals were contacted to confirm reported cardiovascular events. All patients gave written informed consent prior to inclusion. The study was approved by the Ethics Committee of the University Medical Center Utrecht (reference number 11-183) and was conducted in accordance with the Declaration of Helsinki. In ANGES, study nurses collected patient characteristics using a questionnaire (for details see [20]). Follow-up data were collected from Pirkanmaa Hospital District medical records (diagnoses and procedural codes) and from Statistics Finland death records (cause of death). The study was approved by the Ethics Committee of Tampere University Hospital. All patients gave written informed consent, and the study conforms to the Declaration of Helsinki.

Metabolic profiling
EDTA serum samples were stored at -80 °C prior to analysis. A high-throughput ^{1}H NMR metabolomics platform[21] was used to quantify 72 lipid and metabolite measures: 56 lipid-related measures (including concentrations of 14 lipoprotein subclasses), 8 amino acids, 4 glycolysis related metabolites and 4 other metabolites.

Statistical analysis
Metabolic measures with skewness >2 were $\log_e$-transformed. To facilitate comparison of effect estimates across metabolic measures, all concentrations were scaled to standard deviation (SD) units within each cohort. In total, 0.5% of all metabolic measures and covariate values in UCORBIO and 1.9% in ANGES were missing. These values were imputed for each cohort separately based on all covariates, metabolic measures and (time to) events using single imputation by additive regression. Results for imputed and non-imputed metabolic measures are shown in Supplemental Table 1. To build a clinical prediction model for subsequent cardiovascular events, we selected informative variables in UCORBIO, using a boosting method for Cox proportional hazards models implemented in the CoxBoost R package (https://cran.r-project.org/web/packages/CoxBoost/index.html). The optimal step size number was determined by 10-fold cross-validation. The following variables were considered: age, sex, BMI, diabetes mellitus, hypertension, current smoking status, multivessel disease, myocardial infarction at inclusion (indication for angiography), history of myocardial infarction, history of cerebrovascular accident, history of percutaneous coronary intervention (PCI), history of coronary artery bypass grafting (CABG), total serum cholesterol, HDL cholesterol, use of statins, use of renin inhibitors, use of diuretics, use of P2Y12-inhibitors and use of beta-blockers. Eight variables were selected to be included in the clinical prediction model: age, hypertension, multivessel disease,

myocardial infarction at inclusion, history of cerebrovascular accident, history of CABG, use of renin inhibitors and use of diuretics.

These variables served as covariates in Cox proportional hazards models that were fit for each metabolic measure using time to first subsequent cardiovascular event as the outcome measure. Associations were analyzed separately for each cohort and meta-analyzed using inverse-variance weighting. Given the longer follow-up period in ANGES (maximum: 13 years) as compared to UCORBIO (maximum: 3 years), follow-up data were truncated at 3 years in ANGES. The proportional hazards assumption was tested by assessing the correlations between scaled Schoenfeld residuals and follow-up time. In addition, we compared associations of metabolic measures with subsequent events over time in ANGES. Since several metabolic measures were highly correlated, we corrected for multiple testing by adjusting the nominal level of significance for the number of independent tests, which was estimated separately for each cohort according to the method of Li and Ji[22]. The number of independent tests was estimated at 25 in UCORBIO and 27 in ANGES. The maximum estimate was selected for multiple-testing correction, rendering an adjusted significance level of 0.05/27=0.0019.

To assess the ability of metabolic measures to improve risk prediction beyond clinical and demographic variables as well as traditional risk factors, we evaluated risk scores with and without metabolic markers derived in UCORBIO and tested their performance in ANGES. To construct biomarker risk scores, we added all possible combinations of metabolic measures, significantly associated with subsequent events, to the previously selected clinical covariates, including age, hypertension, multivessel disease, myocardial infarction at inclusion, history of cerebrovascular accident, history of CABG, use of renin inhibitors and use of diuretics (Model A). The biomarker combination resulting in the best fit in terms of Akaike Information Criterion was included in the risk scores. To further assess the predictive value of metabolic measures, we constructed another risk prediction model based on traditional cardiovascular risk factors, including age, sex, BMI, hypertension, diabetes mellitus, current smoking status, total cholesterol and HDL cholesterol (Model B).

To obtain weights for the reference risk scores without metabolic biomarkers, we first derived linear predictors for Model A and Model B in UCORBIO. We next added the three selected metabolic measures to each model and again derived linear predictors in UCORBIO to compute weights for the biomarker risk scores. All continuous variables were scaled to SD units for model derivation and validation. We next calculated 3-year absolute risk estimates in ANGES based on the linear predictors of the reference and biomarker risk scores derived from UCORBIO. Baseline hazard estimates were refit in ANGES to account for potential regional differences in experimental conditions between both cohorts.

The predictive performance of the three metabolic measures was assessed by comparing the reference score of Model A and Model B to the corresponding biomarker score. To assess discrimination, we computed c-statistics for censored data using jackknife estimation.[23] Based on the 3-year risk of subsequent cardiovascular events, we also

evaluated the continuous net reclassification improvement (NRI), as implemented in the nricens R package (https://cran.r-project.org/web/packages/nricens/index.html). Confidence intervals for NRI were computed by bootstrapping. We did not evaluate categorical NRI due to the lack of established categories for the 3-year risk of subsequent cardiovascular events. Model calibration was assessed by the Greenwod-Nam-D'Agostino goodness-of-fit test[24] across risk deciles. Deciles with <5 events were collapsed with the next decile to prevent bias.[24] An overview of the study design is shown in Figure 1. All statistical analyses were performed in R (version 3.3.2).

Results

A total of 1,511 patients with angiographically documented CAD were included in the analysis, 150 of whom suffered a subsequent cardiovascular event during 3 years of follow-up. The median follow-up was 2.1 years (interquartile range: 1.5-2.7 years) in UCORBIO and 11.0 years (5.7-12.2 years) in ANGES. Given the longer follow-up period in ANGES, follow-up data were truncated at 3 years. Both cohorts showed similar baseline characteristics in terms of age, sex, medical history, concentrations of routine lipids and medication (Table 1). A total of 72 metabolic measures were quantified in both cohorts using NMR spectrometry. Correlations between metabolic measures are displayed in Supplemental Figure 1.

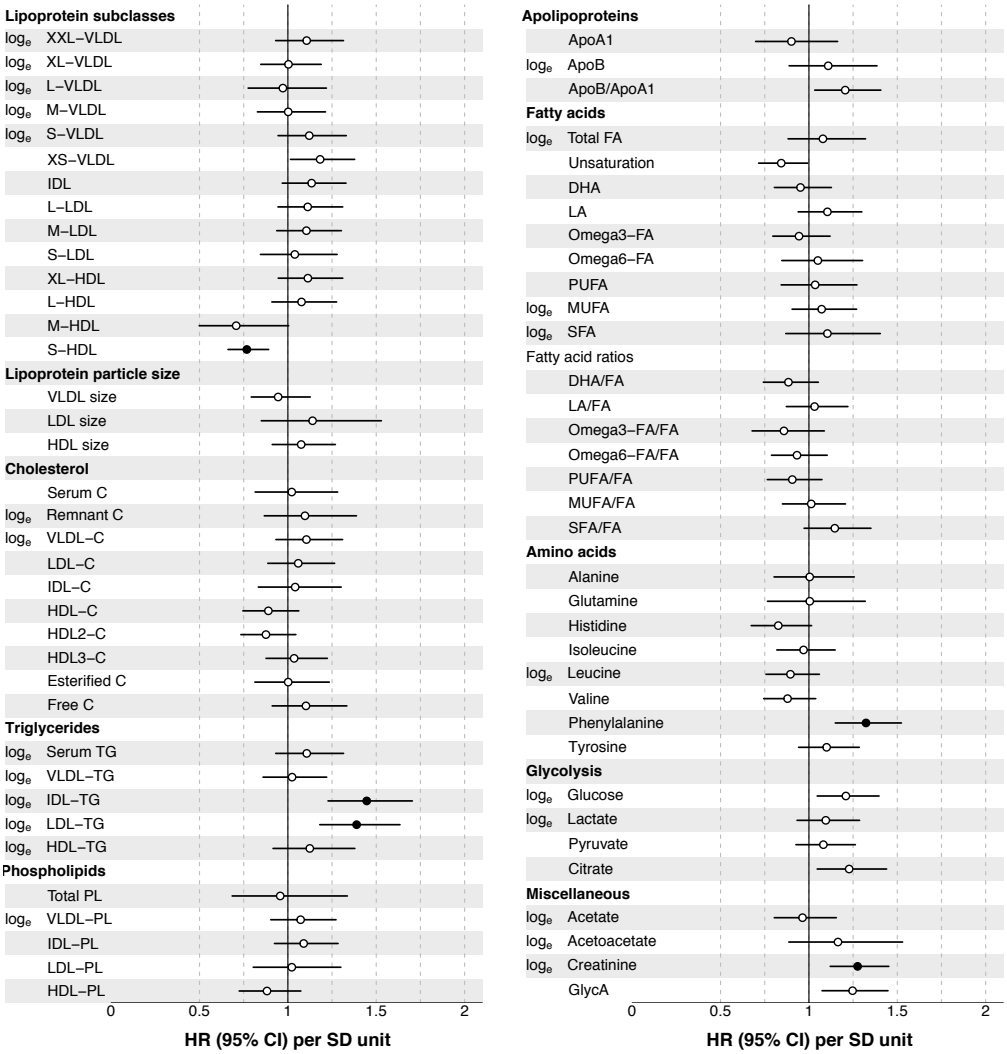
Associations with subsequent events

Associations with subsequent events were adjusted for age, hypertension, multivessel disease, myocardial infarction at inclusion, history of cerebrovascular accident, history of CABG, use of renin inhibitors and use of diuretics. Figure 2 displays overall associations of 72 metabolic measures with the 3-year risk of subsequent cardiovascular events. Effect sizes in SD units for UCORBIO and ANGES are given in Supplemental Table 2. We found significant associations for 5 metabolic measures: the concentration of small high-density lipoprotein particles (S-HDL-P; HR per SD: 0.77 [95% CI: 0.66, 0.89]), triglycerides in intermediate-density lipoprotein (IDL-TG; HR=1.45 [1.23, 1.70]) and low-density lipoprotein (LDL-TG; HR=1.39 [1.18, 1.63]), phenylalanine (HR=1.32 [1.15, 1.52]) and creatinine (HR=1.28 1.05, 1.45]). These metabolic measures showed comparable risk estimates in UCORBIO and ANGES (Figure 3), but smaller effect magnitudes for long-term outcome in ANGES with the same direction of effect (Supplemental Table 3). Interestingly, cholesterol measures were not associated with subsequent event risk (Figure 2).

Predictive performance

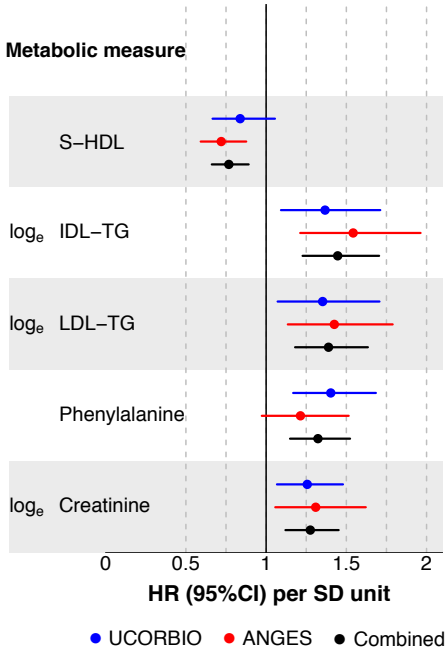
We first derived two reference risk scores in UCORBIO, comprising clinical and demographic variables. Model A was based on the variables that served as covariates in the exploratory analysis and was derived in UCORBIO using a boosting approach for model selection. Model B was based on traditional risk factors: age, sex, BMI, hypertension, diabetes mellitus, current smoking status, total cholesterol and HDL cholesterol. We subsequently added all 5 significant metabolic measures to Model A, and selected the biomarker combination with the best model fit, including S-HDL-P,

Figure 2: Illustration of the study design



HRs for 72 lipid and metabolite measures were analyzed separately in UCORBIO and ANGES and meta-analyzed using inverse variance weighting. Skewed metabolic measures were log$_e$ transformed. HRs are given per SD-unit adjusted for age, hypertension, multivessel disease, myocardial infarction at inclusion, history of cerebrovascular accident, history of CABG, use of renin inhibitors and use of diuretics. CABG: coronary artery bypass grafting; CI: confidence interval; HR: Hazard ratio; SD: standard deviation; Particle size: XXL: extremely large; XL: very large; L: large; M: medium; S: small; XS: very small; Lipids and metabolites: ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; C: cholesterol; DHA: decosahexaenoic acid; FA: fatty acids; GlycA: glycoprotein A; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LA: linoleic acid; LDL: low-density lipoprotein; PL: phospholipids; TG: triglycerides; VLDL: very low-density lipoprotein.

Figure 3: Metabolic measures in UCORBIO and ANGES



Metabolic measures significantly associated with subsequent cardiovascular events in UCORBIO and ANGES. HRs for five metabolic significantly associated with outcome. Skewed metabolic measures were $\log_e$ transformed. HRs are given per SD-unit adjusted for the variables detailed in Figure 2. CABG: coronary artery bypass grafting; CI: confidence interval; HR: hazard ratio; IDL-TG: triglycerides in intermediate-density lipoprotein; LDL-TG: triglycerides in low-density lipoprotein; S-HDL: small high-density lipoprotein particle concentration; SD: standard deviation.

Table 2: Predictive performance of biomarker scores in ANGES

Prediction model	Difference c-statistic (95% CI)	Reclassification	Continuous NRI (95% CI)
Model A	0.067 (0.028, 0.105) p=6x10^{-4} Biomarker score: 0.670 Reference score: 0.603	Events	27.6% (4.2%, 49.2%) p=0.02
		No events	33.2% (24.7%, 41.2%) p=3x10^{-15}
		Net	60.8% (34.7%, 83.7%) p=1x10^{-6}
Model B	0.103 (0.054, 0.153) p=4x10^{-5} Biomarker score: 0.651 Reference score: 0.548	Events	4.3% (-9.4%, 19.4%) p=0.56
		No events	35.4% (25.7%, 44.9%) p=5x10^{-13}
		Net	39.6% (23.3%, 57.9%) p=7x10^{-6}

Discrimination and reclassification for subsequent cardiovascular events in ANGES with and without metabolic biomarkers. Risk prediction (3-year absolute risk) in ANGES was based on two models derived in UCORBIO (Table 2). For both Model A and Model B, we compared risk estimates calculated from the reference score and the corresponding biomarker score to evaluate the added value of metabolic measures for risk assessment. Discrimination was assessed by comparing c-statistics. Reclassification was evaluated as continuous NRI. CI=confidence interval; NRI=net reclassification improvement.

LDL-TG and phenylalanine. We incorporated the selected metabolic measures into both models to obtain biomarker risk scores and compared their performance to the corresponding reference risk score. The coefficients used to calculate risk scores and absolute risk estimates for both models can be found in Supplemental Table 4. While calibration was good for the biomarker score of Model A, the reference score showed mediocre calibration (Supplemental Table 5). For Model B, both the reference risk score and the biomarker risk score showed sufficient calibration. Metabolic measures significantly improved the discriminative performance of 3-year risk estimates for both models, which was evaluated by comparing c-statistics (Table 2). The biomarker score of Model A showed the best discriminative performance with a c-statistic of 0.670 [95% CI: 0.611, 0.728]. Discrimination for traditional risk factors alone was not above chance, as indicated by the c-statistic for the reference score of Model B (0.548 [0.482, 0.614]). The biomarker risk scores of both models significantly enhanced continuous reclassification in patients with a subsequent event and event-free patients, with a net reclassification of 60.8% for Model A and 54.7% for Model B.

Discussion

Using NMR-based metabolic profiling in patients with established CAD from two European cohorts, we found five metabolic significantly associated with subsequent cardiovascular events. Phenylalanine, S-HDL-P and LDL-TG improved prediction of subsequent events relative to a clinical risk prediction model and traditional risk factors. These findings underscore the utility of high-throughput metabolic profiling for biomarker identification and extend data from previous NMR-based metabolic profiling studies on first CVD event risk[19] to secondary cardiovascular prevention. Thus, metabolic measures may improve risk stratification in cardiovascular patients and provide novel insights into the molecular mechanisms driving CAD progression.

We found the strongest associations with subsequent cardiovascular events for LDL-TG and IDL-TG. We observed very strong correlations between LDL-TG and IDL-TG (Supplemental Figure 1), reflecting their common metabolic pathway, in which lipolysis of IDL yields LDL. A study in CAD patients and matched controls demonstrated that LDL-TG was more strongly associated with subsequent cardiovascular events than LDL cholesterol.[25] In addition, higher LDL-TG concentrations coincided with increased concentrations of biological markers related to vascular damage. This association was independent of the inflammatory marker C-reactive protein, suggesting that elevated LDL-TG itself may stimulate inflammatory processes within the vasculature, which play a more prominent role in advanced CAD than in early phases of atherogenesis.[14] In line with this, patients with myocardial infarction show increased LDL-TG as compared to stable CAD patients, associated higher levels of oxidized LDL and inflammatory marker interleukin 6, whereas the TG content of VLDL does not differ between these patients.[26] Thus, LDL-TG and IDL-TG may be related to disease mechanisms particularly relevant to CAD progression and subsequent manifestations of CAD.

S-HDL was the only measure that was inversely associated with outcome. Numerous population-based cohort studies have consistently shown that HDL cholesterol is associated with reduced cardiovascular risk.[27] Interestingly, findings from experimental studies indicate that distinct HDL subpopulations differ in their anti-atherogenic properties. Particularly small, dense HDL particles are thought to exert potent cardioprotective effects due to their anti-oxidative and anti-inflammatory activity.[28] Prospective studies found inverse associations between small HDL particle concentration and cardiovascular risk, but also reported similar or even stronger effects for larger HDL particles,[29,30] suggesting that distinct HDL subclasses do not differ in their cardioprotective capacities. However, these findings were mainly derived from general populations without CAD at inclusion. Given the greater significance of vascular inflammation in advanced CAD, the anti-inflammatory properties of S-HDL may confer a protective effect in patients with established CAD, whereas larger HDL species may play a minor role in CAD progression. This is in line with results from a case-control study in cardiovascular patients, showing that small HDL particles were inversely associated with subsequent events, whereas larger HDL particles were not.[31]

In addition, the aromatic amino acid phenylalanine predicted subsequent cardiovascular events, paralleling findings from a large-scale NMR metabolic profiling study of incident cardiovascular risk in three population-based cohorts.[19] A case-control study showed that an amino acid score, which included phenylalanine, was associated with future cardiovascular disease and intima-media thickness (IMT),[32] a well-known marker of atherosclerosis. The latter finding was confirmed by a recent cross-sectional study, reporting a significant correlation between phenylalanine levels and IMT in randomly selected participants.[33] Furthermore, ischemic stroke patients show a higher serum phenylalanine to tyrosine ratio as compared to healthy controls.[34] Finally, phenylalanine, among other amino acids, has been linked to other cardiovascular risk factors, including diabetes mellitus[35] and obesity[36]. While several studies have related phenylalanine to cardiovascular risk factors and cardiovascular events, further research is required to establish the molecular processes linking this amino acid to the pathogenesis of CAD. Since higher phenylalanine levels not only predict cardiovascular events in patients with established CAD, but also in initially healthy individuals,[19] phenylalanine may be implicated in pathophysiological pathways common to primary and subsequent manifestations of CAD.

Finally, creatinine was associated with poor outcome, but was not selected for the biomarker risk scores, indicating that creatinine did not provide additional predictive value beyond other metabolites. Creatinine is a well-established marker of kidney function and also associated with cardiovascular risk[37-39] and short-term outcome[6] in patients with acute coronary syndrome. The association between chronic kidney disease and CAD is well documented. Pre-existing cardiovascular risk factors are known to contribute to renal dysfunction, suggesting common pathophysiological mechanisms. Conversely, chronic kidney disease has also been causally linked to the development of various cardiovascular risk factors, such as hypertension, dyslipidemia and chronic inflammation.[40] Thus, elevated creatinine levels may serve as a marker of increased cardiovascular risk resulting from renal dysfunction.

Interestingly, none of the routine or subclass cholesterol measures was associated with risk of subsequent events, which may be explained by the high degree of statin treatment in the study populations (Table 1). This finding was paralleled by the poor predictive performance of traditional risk factors in Model B. Consistent with our results, the prognostic value of traditional risk factors for secondary cardiovascular risk is low compared to circulating biomarkers.[13] Moreover, the presence of multiple traditional risk factors does not result in poorer outcome in patients with myocardial infarction.[14] These findings do not argue against a causal role of traditional risk factors in secondary cardiovascular risk. However, most secondary prevention therapies target these risk factors, thereby compromising their predictive value.[13] Thus, alternative risk indicators, such as biomarkers, are needed for risk stratification in secondary prevention. Current guidelines do not recommend the general use of biomarkers for clinical risk assessment, mainly because they do not or only marginally improve cardiovascular risk prediction beyond traditional risk factors in the general population.[7] By contrast, our findings demonstrate that a combination of three metabolic biomarkers substantially improves cardiovascular risk prediction in patients with established CAD. However, further studies are required to further assess the clinical utility of these biomarkers.

This study also has some limitations that deserve consideration. Although we combined data from two cohorts, limited statistical power may have prevented us from detecting biomarkers with smaller effect sizes. Furthermore, the predictive performance of the biomarker risk was moderate, mainly due to limited set of clinical and demographic variables that were available in both cohorts. Therefore, future metabolic profiling studies should also include other known markers of subsequent events. Moreover, metabolic profiles were only analyzed once after symptom onset. Metabolic biomarkers, however, are highly sensitive to physiological changes. Therefore, repeated blood sampling may be helpful to identify biomarkers across different stages of CAD progression and to define windows of vulnerability during which patients are at elevated risk for subsequent events.

Conclusions

Using high-throughput metabolic profiling, we identified LDL-TG, phenylalanine and S-HDL particles as biomarkers of subsequent events in patients with established CAD. These metabolic measures enhance risk prediction compared to demographic and clinical variables, underscoring the utility of metabolomics as a tool for biomarker discovery. Our findings provide novel insights into the pathophysiology of CAD progression and may help improve clinical risk assessment for secondary prevention. However, further research is needed to assess the clinical utility of these biomarkers.

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Supplement

Supplemental Table 1: Results for imputed and non-imputed metabolic measures

		UCORBIO (N=951)			ANGES (N=560)		
		miss. %	non, imputed HR (95% CI)	imputed HR (95% CI)	miss. %	non, imputed HR (95% CI)	imputed HR (95% CI)
Lipoprotein subclasses							
log_e	XXL-VLDL	0.3	1.13 (0.90, 1.42)	1.13 (0.90, 1.42)	1.4	0.97 (0.77, 1.21)	1.00 (0.80, 1.26)
log_e	XL-VLDL	0.1	1.10 (0.87, 1.40)	1.10 (0.87, 1.39)	1.4	0.96 (0.77, 1.21)	0.91 (0.74, 1.14)
log_e	L-VLDL	0.1	1.12 (0.89, 1.42)	1.12 (0.89, 1.42)	1.4	0.95 (0.75, 1.19)	0.91 (0.73, 1.14)
log_e	M-VLDL	0.1	1.11 (0.89, 1.39)	1.11 (0.89, 1.39)	1.4	0.97 (0.77, 1.23)	0.94 (0.75, 1.18)
log_e	S-VLDL	0.1	1.11 (0.89, 1.37)	1.10 (0.89, 1.37)	1.4	1.09 (0.86, 1.38)	1.14 (0.91, 1.44)
	XS-VLDL	0.2	1.10 (0.89, 1.34)	1.10 (0.90, 1.35)	1.4	1.19 (0.99, 1.43)	1.16 (0.95, 1.41)
	IDL	0.1	1.01 (0.81, 1.26)	1.01 (0.81, 1.26)	1.4	1.02 (0.81, 1.28)	1.05 (0.84, 1.31)
	L-LDL	0.1	0.98 (0.78, 1.23)	0.98 (0.79, 1.23)	1.4	0.99 (0.78, 1.25)	1.01 (0.81, 1.27)
	M-LDL	0.1	0.98 (0.79, 1.23)	0.99 (0.79, 1.23)	1.4	0.99 (0.78, 1.25)	1.00 (0.79, 1.26)
	S-LDL	0.1	0.96 (0.76, 1.20)	0.96 (0.76, 1.20)	1.4	0.96 (0.75, 1.21)	0.91 (0.72, 1.16)
	XL-HDL	0.2	1.00 (0.80, 1.24)	1.00 (0.80, 1.24)	1.4	1.10 (0.88, 1.38)	1.13 (0.91, 1.40)
	L-HDL	1.8	1.01 (0.80, 1.26)	1.03 (0.83, 1.29)	1.4	0.97 (0.77, 1.23)	1.02 (0.81, 1.28)
	M-HDL	0.1	0.76 (0.60, 0.96)	0.76 (0.60, 0.97)	1.4	0.64 (0.51, 0.79)	0.62 (0.50, 0.77)
	S-HDL	0.1	0.81 (0.66, 0.99)	0.81 (0.66, 0.99)	1.4	0.70 (0.58, 0.84)	0.72 (0.61, 0.87)
Lipoprotein particle size							
	VLDL size	0.1	1.08 (0.87, 1.34)	1.08 (0.87, 1.34)	1.4	0.87 (0.69, 1.11)	0.88 (0.69, 1.11)
	LDL size	0.1	1.13 (0.89, 1.44)	1.13 (0.89, 1.43)	1.4	1.28 (1.01, 1.62)	1.34 (1.07, 1.68)
	HDL size	0.1	0.99 (0.79, 1.24)	0.99 (0.79, 1.24)	1.4	1.03 (0.82, 1.29)	1.06 (0.85, 1.32)
Cholesterol							
	Serum C	0.1	0.94 (0.74, 1.18)	0.94 (0.74, 1.18)	1.4	0.88 (0.68, 1.13)	0.87 (0.68, 1.11)
log_e	Remnant C	0.1	1.04 (0.84, 1.30)	1.04 (0.84, 1.30)	1.4	0.97 (0.76, 1.22)	0.96 (0.76, 1.21)
log_e	VLDL-C	0.1	1.07 (0.86, 1.34)	1.07 (0.86, 1.34)	1.4	1.03 (0.82, 1.30)	1.02 (0.82, 1.29)
	LDL-C	0.1	0.94 (0.75, 1.19)	0.94 (0.75, 1.19)	1.4	0.94 (0.74, 1.20)	0.93 (0.73, 1.18)
	IDL-C	0.3	0.94 (0.75, 1.18)	0.95 (0.75, 1.19)	1.4	0.90 (0.71, 1.15)	0.89 (0.70, 1.14)
	HDL-C	0.1	0.85 (0.68, 1.07)	0.85 (0.68, 1.07)	1.4	0.80 (0.62, 1.03)	0.80 (0.62, 1.03)
	HDL2-C	0.1	0.86 (0.69, 1.09)	0.86 (0.69, 1.09)	1.4	0.79 (0.61, 1.01)	0.79 (0.62, 1.01)
	HDL3-C	0.1	0.86 (0.68, 1.09)	0.86 (0.68, 1.09)	1.4	1.02 (0.81, 1.28)	0.97 (0.77, 1.23)
	Esterified C	0.7	0.89 (0.70, 1.13)	0.91 (0.72, 1.15)	2.3	0.87 (0.68, 1.11)	0.86 (0.68, 1.10)
	Free C	0.7	1.03 (0.83, 1.27)	1.01 (0.81, 1.26)	2.3	0.95 (0.75, 1.21)	0.96 (0.76, 1.21)
Triglycerides							
log_e	Serum TG	0.1	1.16 (0.94, 1.44)	1.17 (0.94, 1.44)	1.4	1.07 (0.85, 1.34)	1.07 (0.85, 1.34)
log_e	VLDL-TG	0.1	1.13 (0.91, 1.40)	1.13 (0.91, 1.41)	1.4	1.00 (0.80, 1.26)	0.97 (0.78, 1.23)
log_e	IDL-TG	0.3	1.36 (1.10, 1.67)	1.35 (1.09, 1.66)	1.4	1.51 (1.22, 1.86)	1.58 (1.30, 1.93)
log_e	LDL-TG	0.1	1.28 (1.03, 1.58)	1.27 (1.03, 1.57)	1.4	1.41 (1.15, 1.73)	1.44 (1.19, 1.74)
log_e	HDL-TG	0.1	1.22 (0.99, 1.50)	1.22 (0.99, 1.50)	1.4	1.05 (0.83, 1.33)	0.98 (0.78, 1.24)
Phospholipids							
	Total PL	2.2	0.91 (0.72, 1.16)	0.97 (0.77, 1.21)	1.4	0.79 (0.61, 1.02)	0.79 (0.61, 1.02)
log_e	VLDL-PL	0.4	1.11 (0.90, 1.38)	1.11 (0.89, 1.37)	1.4	1.01 (0.80, 1.27)	1.00 (0.79, 1.25)
	IDL-PL	0.3	0.96 (0.77, 1.21)	0.97 (0.78, 1.21)	1.4	1.01 (0.80, 1.28)	1.00 (0.80, 1.26)
	LDL-PL	0.1	0.94 (0.75, 1.19)	0.94 (0.75, 1.19)	1.4	0.88 (0.68, 1.13)	0.89 (0.69, 1.14)
	HDL-PL	1.8	0.91 (0.72, 1.14)	0.90 (0.72, 1.13)	1.4	0.79 (0.62, 1.01)	0.80 (0.63, 1.02)

Supplemental Table 1 (continued)

		UCORBIO (N=951)			ANGES (N=560)		
		miss. %	non, imputed HR (95% CI)	imputed HR (95% CI)	miss. %	non, imputed HR (95% CI)	imputed HR (95% CI)
Apolipoproteins							
	ApoA1	0.1	0.89 (0.71, 1.13)	0.89 (0.71, 1.12)	1.4	0.72 (0.56, 0.94)	0.72 (0.56, 0.92)
log_e	ApoB	0.2	1.08 (0.87, 1.34)	1.08 (0.87, 1.34)	1.4	0.96 (0.76, 1.21)	0.97 (0.77, 1.23)
	ApoB/ApoA1	0.2	1.17 (0.96, 1.43)	1.17 (0.96, 1.43)	1.4	1.14 (0.92, 1.42)	1.20 (0.97, 1.47)
Fatty acids							
log_e	Total FA	0.5	1.07 (0.87, 1.33)	1.07 (0.86, 1.32)	2.5	0.92 (0.72, 1.17)	0.93 (0.74, 1.18)
	Unsaturation	1.1	0.80 (0.64, 0.99)	0.78 (0.63, 0.97)	2.5	0.83 (0.64, 1.06)	0.91 (0.72, 1.16)
	DHA	0.4	0.94 (0.75, 1.18)	0.94 (0.75, 1.18)	2.5	0.87 (0.67, 1.13)	0.88 (0.69, 1.13)
	LA	0.9	1.03 (0.83, 1.28)	1.02 (0.82, 1.27)	2.5	0.91 (0.71, 1.16)	0.96 (0.76, 1.22)
	Omega3-FA	0.6	0.94 (0.75, 1.19)	0.93 (0.74, 1.18)	2.5	0.83 (0.63, 1.08)	0.88 (0.68, 1.13)
	Omega6-FA	0.9	1.00 (0.80, 1.25)	1.00 (0.80, 1.25)	2.5	0.86 (0.67, 1.11)	0.87 (0.68, 1.12)
	PUFA	0.9	0.99 (0.79, 1.24)	0.99 (0.80, 1.24)	2.5	0.84 (0.65, 1.09)	0.86 (0.67, 1.11)
log_e	MUFA	0.6	1.05 (0.84, 1.31)	1.05 (0.84, 1.30)	2.5	1.00 (0.79, 1.27)	1.03 (0.82, 1.29)
log_e	SFA	1.1	1.14 (0.93, 1.41)	1.14 (0.92, 1.40)	2.5	0.93 (0.74, 1.18)	0.94 (0.74, 1.18)
Fatty acid ratios							
	DHA/FA	0.5	0.87 (0.69, 1.10)	0.86 (0.68, 1.09)	2.5	0.87 (0.68, 1.12)	0.92 (0.72, 1.17)
	LA/FA	0.9	0.97 (0.78, 1.21)	0.95 (0.76, 1.18)	2.5	0.95 (0.75, 1.20)	0.95 (0.76, 1.20)
	Omega3-FA/FA	0.6	0.83 (0.65, 1.07)	0.83 (0.64, 1.06)	2.5	0.84 (0.65, 1.08)	0.94 (0.74, 1.19)
	Omega6-FA/FA	0.9	0.90 (0.72, 1.12)	0.89 (0.71, 1.10)	2.5	0.86 (0.68, 1.08)	0.84 (0.67, 1.05)
	PUFA/FA	0.9	0.87 (0.70, 1.08)	0.86 (0.69, 1.06)	2.5	0.82 (0.65, 1.04)	0.83 (0.66, 1.05)
	MUFA/FA	0.6	0.99 (0.79, 1.23)	0.99 (0.80, 1.24)	2.5	1.17 (0.93, 1.48)	1.15 (0.91, 1.44)
	SFA/FA	1.1	1.36 (1.09, 1.71)	1.38 (1.11, 1.72)	2.5	1.05 (0.83, 1.33)	1.04 (0.83, 1.31)
Amino acids							
	Alanine	0.0	1.18 (0.96, 1.45)	1.18 (0.96, 1.45)	1.4	0.82 (0.64, 1.06)	0.82 (0.64, 1.04)
	Glutamine	0.5	1.10 (0.88, 1.38)	1.10 (0.88, 1.38)	1.6	0.84 (0.66, 1.07)	0.82 (0.65, 1.04)
	Histidine	0.2	0.82 (0.66, 1.03)	0.83 (0.66, 1.03)	1.4	0.65 (0.51, 0.83)	0.64 (0.50, 0.82)
	Isoleucine	0.5	1.01 (0.81, 1.25)	1.00 (0.80, 1.25)	1.8	1.00 (0.79, 1.27)	0.99 (0.78, 1.25)
log_e	Leucine	0.1	0.78 (0.63, 0.98)	0.79 (0.63, 0.98)	1.6	0.93 (0.73, 1.17)	0.96 (0.76, 1.22)
	Valine	0.4	0.82 (0.65, 1.04)	0.82 (0.65, 1.04)	1.8	0.86 (0.68, 1.10)	0.92 (0.73, 1.17)
	Phenylalanine	1.6	1.56 (1.30, 1.86)	1.58 (1.34, 1.87)	1.6	1.46 (1.20, 1.78)	1.44 (1.18, 1.75)
	Tyrosine	0.4	1.19 (0.96, 1.47)	1.17 (0.94, 1.44)	1.4	0.94 (0.74, 1.19)	0.96 (0.76, 1.22)
Glycolysis							
log_e	Glucose	0.2	1.28 (1.07, 1.54)	1.28 (1.07, 1.54)	1.4	1.16 (0.94, 1.42)	1.15 (0.94, 1.41)
log_e	Lactate	0.0	1.16 (0.95, 1.44)	1.16 (0.95, 1.44)	1.4	1.02 (0.81, 1.30)	1.05 (0.83, 1.33)
	Pyruvate	0.1	1.18 (0.97, 1.43)	1.18 (0.97, 1.43)	1.6	1.08 (0.86, 1.36)	1.09 (0.87, 1.36)
	Citrate	0.1	1.34 (1.13, 1.60)	1.34 (1.13, 1.60)	2.1	1.10 (0.88, 1.39)	1.11 (0.89, 1.39)
Miscellaneous							
log_e	Acetate	0.9	0.90 (0.70, 1.15)	0.91 (0.71, 1.15)	3.6	0.90 (0.70, 1.17)	0.90 (0.70, 1.16)
log_e	Acetoacetate	0.0	1.01 (0.81, 1.26)	1.01 (0.81, 1.26)	2.0	1.20 (0.95, 1.53)	1.16 (0.92, 1.47)
log_e	Creatinine	2.5	1.45 (1.26, 1.67)	1.44 (1.25, 1.65)	5.0	1.30 (1.06, 1.60)	1.26 (1.03, 1.53)
	GlycA	0.0	1.42 (1.20, 1.68)	1.42 (1.20, 1.68)	1.8	1.36 (1.11, 1.66)	1.31 (1.07, 1.61)

Unadjusted associations with subsequent cardiovascular events for imputed and non-imputed data. Skewed metabolic measures log_e transformed. HRs are given per SD-unit. CI: confidence interval; HR: hazard ratio; miss.: missing. Abbreviations for lipids and metabolites are as in Figure 2.

Supplemental Table 2: Results for UCORBIO and ANGES

		UCORBIO (N=951) HR (95% CI)	ANGES (N=560) HR (95% CI)	Combined (N=1,511) HR (95% CI)	p
Lipoprotein subclasses					
log_e	XXL, VLDL	1.15 (0.90, 1.48)	1.07 (0.84, 1.36)	1.11 (0.93, 1.31)	0.25
log_e	XL, VLDL	1.09 (0.85, 1.40)	0.94 (0.74, 1.18)	1.00 (0.85, 1.19)	0.97
log_e	L, VLDL	1.09 (0.84, 1.41)	0.87 (0.67, 1.12)	0.97 (0.78, 1.22)	0.80
log_e	M, VLDL	1.10 (0.87, 1.40)	0.90 (0.70, 1.17)	1.00 (0.83, 1.21)	0.98
log_e	S, VLDL	1.14 (0.90, 1.43)	1.1 0(0.85, 1.43)	1.12 (0.94, 1.33)	0.19
	XS, VLDL	1.24 (1.01, 1.52)	1.12 (0.89, 1.41)	1.18 (1.01, 1.38)	0.032
	IDL	1.21 (0.98, 1.49)	1.05 (0.83, 1.33)	1.13 (0.97, 1.33)	0.12
	L, LDL	1.18 (0.95, 1.47)	1.03 (0.81, 1.32)	1.11 (0.94, 1.31)	0.20
	M, LDL	1.18 (0.95, 1.47)	1.02 (0.79, 1.30)	1.1 (0.94, 1.30)	0.24
	S, LDL	1.15 (0.91, 1.44)	0.93 (0.72, 1.20)	1.04 (0.84, 1.28)	0.72
	XL, HDL	1.08 (0.86, 1.36)	1.14 (0.91, 1.44)	1.11 (0.95, 1.31)	0.20
	L, HDL	1.09 (0.87, 1.37)	1.06 (0.82, 1.37)	1.08 (0.91, 1.28)	0.39
	M, HDL	0.84 (0.66, 1.07)	0.59 (0.46, 0.76)	0.71 (0.50, 1.00)	0.053
	S, HDL	0.84 (0.67, 1.06)	0.72 (0.59, 0.88)	0.77 (0.66, 0.89)	$5.1x10^{-4}$
Lipoprotein particle size					
	VLDL size	1.01 (0.80, 1.28)	0.87 (0.67, 1.13)	0.94 (0.79, 1.13)	0.53
	LDL size	0.99 (0.79, 1.23)	1.33 (1.04, 1.70)	1.14 (0.85, 1.53)	0.38
	HDL size	1.07 (0.85, 1.34)	1.09 (0.85, 1.38)	1.08 (0.91, 1.27)	0.39
Cholesterol					
	Serum C	1.14 (0.91, 1.43)	0.90 (0.70, 1.17)	1.02 (0.81, 1.28)	0.85
log_e	Remnant C	1.23 (0.98, 1.55)	0.97 (0.75, 1.24)	1.10 (0.87, 1.39)	0.44
log_e	VLDL, C	1.18 (0.94, 1.49)	1.02 (0.79, 1.31)	1.11 (0.93, 1.31)	0.25
	LDL, C	1.15 (0.92, 1.44)	0.96 (0.75, 1.23)	1.06 (0.89, 1.26)	0.53
	IDL, C	1.16 (0.93, 1.45)	0.92 (0.72, 1.18)	1.04 (0.83, 1.30)	0.72
	HDL, C	0.94 (0.74, 1.19)	0.83 (0.64, 1.08)	0.89 (0.75, 1.06)	0.20
	HDL2, C	0.93 (0.73, 1.18)	0.81 (0.62, 1.06)	0.88 (0.73, 1.05)	0.14
	HDL3, C	1.02 (0.80, 1.29)	1.05 (0.83, 1.34)	1.04 (0.88, 1.22)	0.68
	Esterified C	1.11 (0.88, 1.39)	0.89 (0.69, 1.15)	1.00 (0.81, 1.23)	0.99
	Free C	1.21 (0.96, 1.52)	1.00 (0.78, 1.27)	1.10 (0.91, 1.33)	0.32
Triglycerides					
log_e	Serum TG	1.15 (0.92, 1.46)	1.05 (0.81, 1.36)	1.11 (0.93, 1.32)	0.25
log_e	VLDL, TG	1.11 (0.87, 1.40)	0.93 (0.72, 1.21)	1.02 (0.86, 1.22)	0.79
log_e	IDL, TG	1.37 (1.09, 1.71)	1.54 (1.21, 1.96)	1.45 (1.23, 1.70)	$1.1x10^{-5}$
log_e	LDL, TG	1.35 (1.07, 1.71)	1.42 (1.14, 1.79)	1.39 (1.18, 1.63)	$7.5x10^{-5}$
log_e	HDL, TG	1.24 (0.99, 1.55)	1.00 (0.78, 1.29)	1.12 (0.92, 1.38)	0.26
Phospholipids					
	Total PL	1.13 (0.90, 1.41)	0.80 (0.62, 1.04)	0.96 (0.68, 1.34)	0.79
log_e	VLD, PL	1.16 (0.92, 1.46)	0.97 (0.75, 1.26)	1.07 (0.90, 1.27)	0.43
	IDL, PL	1.17 (0.94, 1.46)	1.00 (0.78, 1.27)	1.09 (0.92, 1.28)	0.31
	LDL, PL	1.15 (0.91, 1.44)	0.90 (0.69, 1.16)	1.02 (0.80, 1.30)	0.86
	HDL, PL	0.97 (0.76, 1.22)	0.79 (0.61, 1.03)	0.88 (0.72, 1.07)	0.21

Supplemental Table 2 (continued)

		UCORBIO (N=951) HR (95% CI)	ANGES (N=560) HR (95% CI)	Combined (N=1,511) HR (95% CI)	p
Apolipoproteins					
	ApoA1	1.02 (0.81, 1.28)	0.79 (0.61, 1.02)	0.90 (0.70, 1.16)	0.42
log_e	ApoB	1.23 (0.98, 1.55)	0.98 (0.76, 1.27)	1.11 (0.89, 1.39)	0.36
	ApoB/ApoA1	1.23 (1.00, 1.52)	1.17 (0.93, 1.47)	1.21 (1.03, 1.41)	0.018
Fatty acids					
log_e	Total FA	1.19 (0.95, 1.49)	0.97 (0.76, 1.23)	1.08 (0.88, 1.32)	0.46
	Unsaturation	0.79 (0.63, 0.98)	0.93 (0.72, 1.20)	0.84 (0.72, 0.99)	0.042
	DHA	0.98 (0.78, 1.23)	0.92 (0.72, 1.18)	0.95 (0.80, 1.13)	0.57
	LA	1.18 (0.95, 1.47)	1.02 (0.80, 1.30)	1.10 (0.94, 1.30)	0.23
	Omega3, FA	0.97 (0.77, 1.22)	0.92 (0.71, 1.18)	0.94 (0.80, 1.12)	0.51
	Omega6, FA	1.16 (0.93, 1.45)	0.93 (0.73, 1.20)	1.05 (0.85, 1.30)	0.65
	PUFA	1.14 (0.91, 1.43)	0.92 (0.72, 1.19)	1.04 (0.84, 1.27)	0.74
log_e	MUFA	1.11 (0.88, 1.41)	1.02 (0.80, 1.31)	1.07 (0.90, 1.27)	0.42
log_e	SFA	1.24 (1.00, 1.55)	0.97 (0.76, 1.24)	1.10 (0.87, 1.40)	0.42
Fatty acid ratios					
	DHA/FA	0.82 (0.65, 1.05)	0.96 (0.74, 1.25)	0.88 (0.74, 1.05)	0.17
	LA/FA	1.05 (0.84, 1.32)	1.01 (0.79, 1.30)	1.03 (0.87, 1.22)	0.72
	Omega3, FA/FA	0.76 (0.58, 0.99)	0.96 (0.75, 1.25)	0.86 (0.68, 1.09)	0.21
	Omega6, FA/FA	0.98 (0.78, 1.23)	0.88 (0.69, 1.13)	0.93 (0.79, 1.10)	0.42
	PUFA/FA	0.93 (0.74, 1.16)	0.88 (0.68, 1.14)	0.91 (0.76, 1.07)	0.25
	MUFA/FA	0.96 (0.75, 1.22)	1.08 (0.84, 1.39)	1.01 (0.85, 1.21)	0.89
	SFA/FA	1.23 (0.99, 1.54)	1.05 (0.82, 1.34)	1.15 (0.97, 1.35)	0.10
Amino acids					
	Alanine	1.11 (0.91, 1.37)	0.88 (0.69, 1.13)	1.00 (0.80, 1.26)	0.97
	Glutamine	1.15 (0.92, 1.44)	0.87 (0.69, 1.11)	1.00 (0.77, 1.32)	0.97
	Histidine	0.92 (0.72, 1.17)	0.74 (0.58, 0.95)	0.83 (0.67, 1.02)	0.069
	Isoleucine	1.05 (0.83, 1.32)	0.89 (0.69, 1.14)	0.97 (0.82, 1.15)	0.72
log_e	Leucine	0.90 (0.71, 1.13)	0.89 (0.70, 1.14)	0.90 (0.76, 1.06)	0.2
	Valine	0.89 (0.71, 1.12)	0.87 (0.68, 1.11)	0.88 (0.74, 1.04)	0.13
	Phenylalanine	1.40 (1.17, 1.68)	1.21 (0.97, 1.51)	1.32 (1.15, 1.52)	$9.9x10^{-5}$
	Tyrosine	1.18 (0.96, 1.46)	1.01 (0.80, 1.27)	1.10 (0.94, 1.29)	0.23
Glycolysis					
log_e	Glucose	1.25 (1.03, 1.51)	1.16 (0.93, 1.45)	1.21 (1.05, 1.40)	0.0099
log_e	Lactate	1.14 (0.91, 1.43)	1.05 (0.83, 1.32)	1.10 (0.93, 1.29)	0.27
	Pyruvate	1.10 (0.90, 1.34)	1.06 (0.83, 1.35)	1.08 (0.93, 1.26)	0.32
	Citrate	1.28 (1.03, 1.59)	1.17 (0.92, 1.48)	1.23 (1.05, 1.44)	0.012
Miscellaneous					
log_e	Acetate	0.95 (0.75, 1.22)	0.98 (0.75, 1.28)	0.96 (0.80, 1.16)	0.69
log_e	Acetoacetate	1.02 (0.82, 1.26)	1.34 (1.06, 1.70)	1.16 (0.89, 1.53)	0.27
log_e	Creatinine	1.26 (1.07, 1.48)	1.31 (1.06, 1.62)	1.28 (1.12, 1.45)	$2.3x10^{-4}$
	GlycA	1.32 (1.10, 1.60)	1.13 (0.89, 1.44)	1.25 (1.07, 1.45)	0.0037

Associations with subsequent cardiovascular events for UCORBIO, ANGES adjusted for the same variables as in Figure 2. Results were combined using inverse variance weighting. Skewed metabolic measures were log_e transformed. HRs are given in SD units. CI: confidence interval; HR: hazard ratio. Abbreviations for lipids and metabolites are as in Figure 2.

Supplemental Table 3: Results for 3-year follow-up and long-term follow-up in ANGES

		3 years HR (95% CI)	max. follow, up HR (95% CI)
Lipoprotein subclasses			
log_e	XXL, VLDL	1.07 (0.84, 1.36)	1.09 (0.94, 1.26)
log_e	XL, VLDL	0.94 (0.74, 1.18)	1.03 (0.88, 1.21)
log_e	L, VLDL	0.87 (0.67, 1.12)	1.01 (0.86, 1.18)
log_e	M, VLDL	0.90 (0.70, 1.17)	1.01 (0.87, 1.17)
log_e	S, VLDL	1.10 (0.85, 1.43)	1.12 (0.96, 1.31)
	XS, VLDL	1.12 (0.89, 1.41)	1.14 (1.00, 1.30)
	IDL	1.05 (0.83, 1.33)	1.04 (0.91, 1.20)
	L, LDL	1.03 (0.81, 1.32)	1.02 (0.89, 1.19)
	M, LDL	1.02 (0.79, 1.30)	1.02 (0.88, 1.18)
	S, LDL	0.93 (0.72, 1.20)	0.98 (0.84, 1.14)
	XL, HDL	1.14 (0.91, 1.44)	1.09 (0.95, 1.26)
	L, HDL	1.06 (0.82, 1.37)	1.01 (0.87, 1.17)
	M, HDL	0.59 (0.46, 0.76)	0.83 (0.72, 0.96)
	S, HDL	0.72 (0.59, 0.88)	0.88 (0.77, 1.00)
Lipoprotein particle size			
	VLDL size	0.87 (0.67, 1.13)	0.98 (0.84, 1.14)
	LDL size	1.33 (1.04, 1.70)	1.17 (1.00, 1.37)
	HDL size	1.09 (0.85, 1.38)	1.04 (0.90, 1.21)
Cholesterol			
	Serum C	0.90 (0.70, 1.17)	0.98 (0.84, 1.13)
log_e	Remnant C	0.97 (0.75, 1.24)	1.06 (0.91, 1.22)
log_e	VLDL, C	1.02 (0.79, 1.31)	1.10 (0.95, 1.27)
	LDL, C	0.96 (0.75, 1.23)	0.98 (0.85, 1.14)
	IDL, C	0.92 (0.72, 1.18)	0.99 (0.85, 1.14)
	HDL, C	0.83 (0.64, 1.08)	0.93 (0.80, 1.08)
	HDL2, C	0.81 (0.62, 1.06)	0.92 (0.79, 1.07)
	HDL3, C	1.05 (0.83, 1.34)	1.00 (0.86, 1.16)
	Esterified C	0.89 (0.69, 1.15)	1.00 (0.86, 1.16)
	Free C	1.00 (0.78, 1.27)	0.99 (0.86, 1.14)
Triglycerides			
log_e	Serum TG	1.05 (0.81, 1.36)	1.09 (0.94, 1.27)
log_e	VLDL, TG	0.93 (0.72, 1.21)	1.04 (0.90, 1.22)
log_e	IDL, TG	1.54 (1.21, 1.96)	1.36 (1.17, 1.58)
log_e	LDL, TG	1.42 (1.14, 1.79)	1.27 (1.10, 1.47)
log_e	HDL, TG	1.00 (0.78, 1.29)	1.06 (0.91, 1.22)
Phospholipids			
	Total PL	0.80 (0.62, 1.04)	0.95 (0.82, 1.10)
log_e	VLDL, PL	0.97 (0.75, 1.26)	1.07 (0.92, 1.24)
	IDL, PL	1.00 (0.78, 1.27)	1.02 (0.89, 1.18)
	LDL, PL	0.90 (0.69, 1.16)	0.97 (0.83, 1.12)
	HDL, PL	0.79 (0.61, 1.03)	0.93 (0.80, 1.08)

Supplemental Table 3 (continued)

		3 years HR (95% CI)	max. follow, up HR (95% CI)
Apolipoproteins			
	ApoA1	0.79 (0.61, 1.02)	0.89 (0.77, 1.04)
log_e	ApoB	0.98 (0.76, 1.27)	1.03 (0.89, 1.20)
	ApoB/ApoA1	1.17 (0.93, 1.47)	1.12 (0.97, 1.30)
Fatty acids			
log_e	Total FA	0.97 (0.76, 1.23)	0.98 (0.84, 1.13)
	Unsaturation	0.93 (0.72, 1.20)	0.93 (0.80, 1.07)
	DHA	0.92 (0.72, 1.18)	0.95 (0.82, 1.10)
	LA	1.02 (0.80, 1.30)	1.00 (0.86, 1.15)
	Omega3, FA	0.92 (0.71, 1.18)	0.92 (0.79, 1.07)
	Omega6, FA	0.93 (0.73, 1.20)	0.95 (0.82, 1.10)
	PUFA	0.92 (0.72, 1.19)	0.93 (0.80, 1.08)
log_e	MUFA	1.02 (0.80, 1.31)	1.04 (0.90, 1.20)
log_e	SFA	0.97 (0.76, 1.24)	0.97 (0.84, 1.12)
Fatty acid ratios			
	DHA/FA	0.96 (0.74, 1.25)	0.96 (0.83, 1.12)
	LA/FA	1.01 (0.79, 1.30)	0.98 (0.84, 1.13)
	Omega3, FA/FA	0.96 (0.75, 1.25)	0.93 (0.80, 1.09)
	Omega6, FA/FA	0.88 (0.69, 1.13)	0.91 (0.79, 1.05)
	PUFA/FA	0.88 (0.68, 1.14)	0.90 (0.78, 1.04)
	MUFA/FA	1.08 (0.84, 1.39)	1.10 (0.95, 1.28)
	SFA/FA	1.05 (0.82, 1.34)	0.99 (0.86, 1.15)
Amino acids			
	Alanine	0.88 (0.69, 1.13)	1.00 (0.87, 1.16)
	Glutamine	0.87 (0.69, 1.11)	0.97 (0.84, 1.12)
	Histidine	0.74 (0.58, 0.95)	0.81 (0.69, 0.94)
	Isoleucine	0.89 (0.69, 1.14)	0.95 (0.82, 1.11)
log_e	Leucine	0.89 (0.70, 1.14)	0.96 (0.82, 1.11)
	Valine	0.87 (0.68, 1.11)	0.97 (0.83, 1.12)
	Phenylalanine	1.21 (0.97, 1.51)	1.25 (1.07, 1.46)
	Tyrosine	1.01 (0.80, 1.27)	1.09 (0.95, 1.26)
Glycolysis			
log_e	Glucose	1.16 (0.93, 1.45)	1.04 (0.90, 1.20)
log_e	Lactate	1.05 (0.83, 1.32)	1.13 (0.98, 1.31)
	Pyruvate	1.06 (0.83, 1.35)	1.07 (0.92, 1.23)
	Citrate	1.17 (0.92, 1.48)	1.03 (0.89, 1.20)
Miscellaneous			
log_e	Acetate	0.98 (0.75, 1.28)	0.93 (0.79, 1.09)
log_e	Acetoacetate	1.34 (1.06, 1.70)	1.14 (0.99, 1.32)
log_e	Creatinine	1.31 (1.06, 1.62)	1.09 (0.95, 1.25)
	GlycA	1.13 (0.89, 1.44)	1.08 (0.93, 1.25)

Results are adjusted demographic and clinical variables. Skewed metabolic measures were log_e transformed. HRs are given in SD units. CI: confidence interval; HR: hazard ratio. Abbreviations for lipids and metabolites are as in Figure 2.

Supplemental Table 4: Regression coefficients for subsequent cardiovascular events

	Reference score β (95% CI)	Biomarker score β (95% CI)
Model A		
Age, SD	0.41 (0.16, 0.66)	0.42 (0.16, 0.68)
Multivessel disease	0.33 (-0.15, 0.81)	0.27 (-0.22, 0.76)
Hypertension	0.45 (-0.12, 1.01)	0.43 (-0.13, 0.99)
Myocardial infarction (inclusion)	0.35 (-0.15, 0.85)	0.10 (-0.43, 0.62)
Cerebrovascular accident	0.35 (-0.23, 0.92)	0.30 (-0.28, 0.87)
CABG	0.78 (0.27, 1.30)	0.75 (0.23, 1.27)
Renin inhibitors	0.25 (-0.29, 0.79)	0.28 (-0.25, 0.82)
Diuretics	0.88 (0.38, 1.37)	0.80 (0.31, 1.29)
LDL-TG, SD	-	0.35 (0.12, 0.57)
Phenylalanine, SD	-	0.29 (0.10, 0.48)
S-HDL, SD	-	-0.20 (-0.41, 0.01)
Model B		
Age, SD	0.61 (0.34 -0.87)	0.53 (0.26, 0.80)
Male sex	-0.14 (-0.66, 0.39)	-0.05 (-0.59, 0.48)
Hypertension	0.77 (0.23, 1.30)	0.76 (0.22, 1.31)
BMI, SD	-0.05 (-0.31, 0.20)	-0.08 (-0.33, 0.17)
Diabetes mellitus	0.14 (-0.36, 0.65)	0.12 (-0.40, 0.63)
Current smoker	0.25 (-0.28, 0.79)	0.21 (-0.33, 0.76)
Total cholesterol, SD	0.21 (-0.06, 0.48)	-0.04 (-0.40, 0.32)
HDL-C, SD	-0.28 (-0.57, 0.01)	-0.04 (-0.35, 0.27)
LDL-TG, SD	-	0.37 (0.06, 0.69)
Phenylalanine, SD	-	0.31 (0.14, 0.49)
S-HDL, SD	-	-0.22 (-0.46, 0.03)

HRs and regression coefficients (β) for subsequent cardiovascular events were derived in UCORBIO. Risk prediction was based on two reference risk score, including selected clinical and demographic variables (Model A) or traditional cardiovascular risk factors (Model B). LDL-TG, S-HDL and phenylalanine were added to each model to obtain biomarker risk scores. Regression coefficients from UCORBIO were used to calculate absolute risk estimates in ANGES. Continuous variables were scaled to SD units. BMI: body mass index; CABG: coronary artery bypass grafting; CI: confidence interval; HDL-C: high-density lipoprotein cholesterol; HR: hazard ratio; LDL-TG: triglycerides in low-density lipoprotein; S-HDL: small high-density lipoprotein particle concentration; SD: standard deviation.

Supplemental Table 5: Model calibration in ANGES

Model A

Risk score	Decile	Total n	Events	Observed counts - adjusted for censoring	Expected count
Reference score (χ^2=16.9, df=7, p=0.02)	1 + 2	112	8	8.0	3.6
	3 + 4	112	8	8.1	6.7
	5	56	10	10.0	4.7
	6	56	8	8.1	5.6
	7	56	9	9.1	7.1
	8	56	9	9.0	9.3
	9	56	8	8.1	12.2
	10	56	12	12.2	20.4
Biomarker score (χ^2=10.5, df=7, p=0.16)	1 + 2	112	6	6.0	2.8
	3 + 4	112	8	8.1	5.9
	5	56	5	5.0	4.1
	6	56	9	9.1	5.4
	7	56	10	10.0	7.2
	8	56	6	6.1	9.5
	9	56	12	12.0	13.0
	10	56	16	16.5	22.5

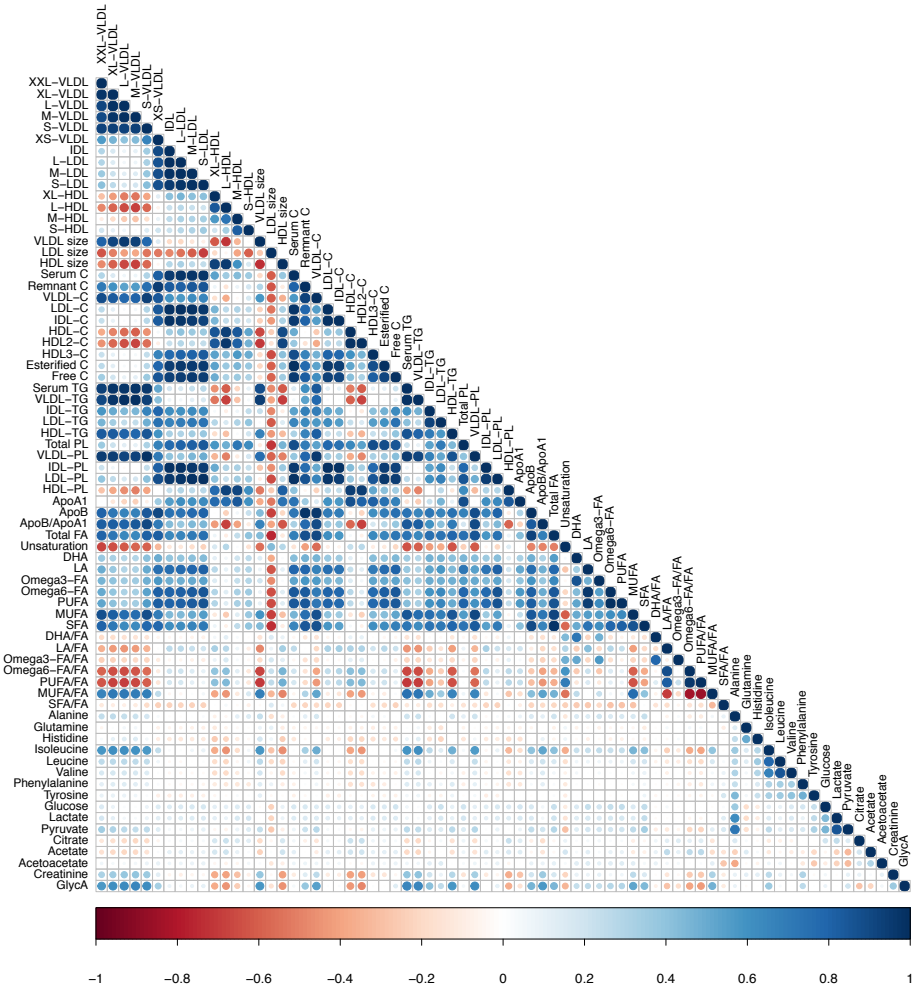
Model B

Risk score	Decile	Total n	Events	Observed counts - adjusted for censoring	Expected count
Reference score (χ^2=14.0, df=8, p=0.08)	1	56	6	6.0	2.1
	2	56	8	8.0	3.3
	3 + 4	112	8	8.0	9.2
	5	56	7	7.0	5.7
	6	56	10	10.1	6.6
	7	56	6	6.0	7.7
	8	56	8	8.0	9.3
	9	56	10	10.2	12.3
	10	56	9	9.1	15.2
Biomarker score (χ^2=12.5, df=6, p=0.05)	1 + 2	112	5	5.0	3.8
	3 + 4	112	14	14.0	7.6
	5 + 6	112	9	9.0	11.3
	7	56	9	9.1	7.8
	8	56	12	12.1	9.5
	9	56	6	6.1	12.2
	10	56	17	17.2	19.4

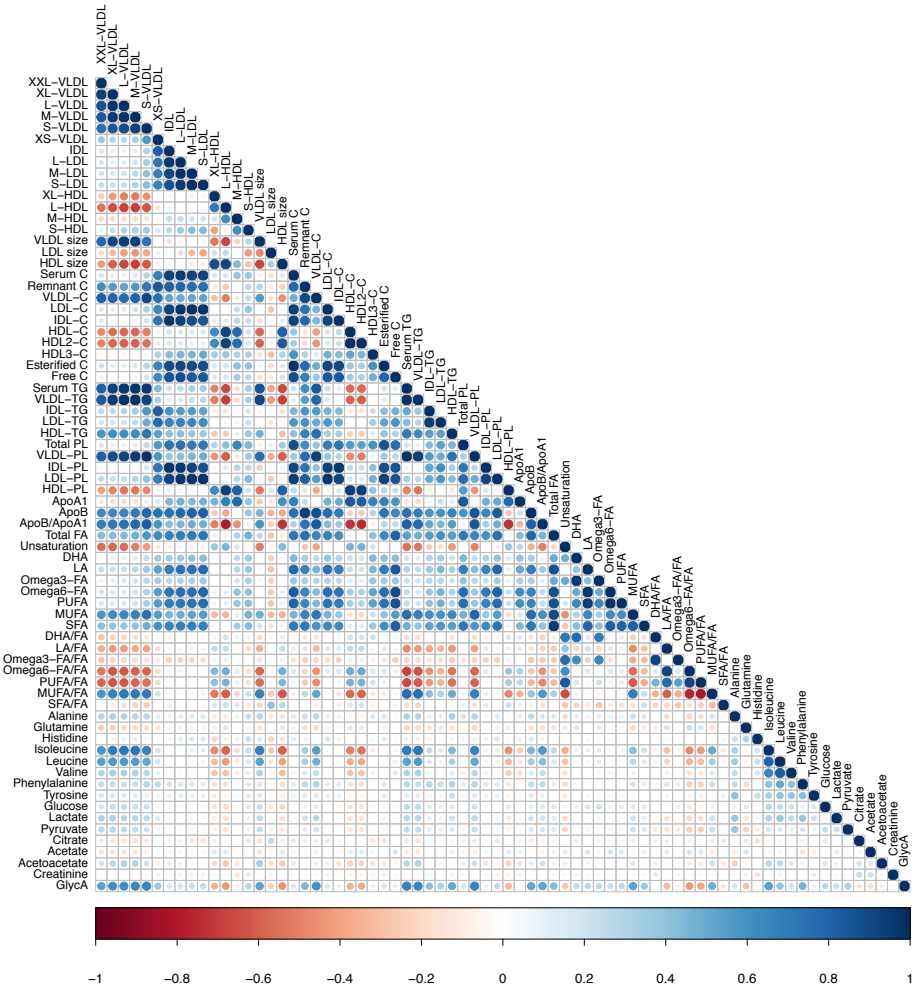
Calibration across risk deciles for the reference scores and biomarkers scores of Model A and Model B in ANGES. The reference risk scores were based on demographic and clinical variables, the biomarker risk scores additionally three biomarkers. Risk estimates in ANGES were computed based on regression coefficients derived in UCORBIO. Calibration was assessed by the Greenwod-Nam-D'Agostino goodness-of-fit test. Risk deciles with <5 events were collapsed with the next decile.

Supplemental Figure 1: Correlation heatmap of metabolic measures

UCORBIO



ANGES



Correlation heatmap of metabolic measures. Correlations were calculated using Spearman's rank correlation coefficient. Abbreviations for metabolic measures are as in Figure 2.

Chapter 6

Effect of metformin on metabolic profiles and relation with myocardial infarct size and left ventricular ejection fraction after myocardial infarction

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Abstract

Background: Left ventricular ejection fraction (LVEF) and infarct size (ISZ) are key predictors of long-term survival after myocardial infarction (MI). However, little is known about the biochemical pathways driving left ventricular dysfunction after MI. To identify novel biomarkers predicting post-MI LVEF and ISZ, we performed metabolic profiling in the GIPS-III randomized clinical trial. We also investigated the metabolic footprint of metformin, a drug associated with improved post-MI left ventricular function in experimental studies.

Results: Participants were ST-elevated MI (STEMI) patients who were randomly assigned to receive metformin or placebo for 4 months. Blood samples were obtained on admission, 24 h and 4 months post-MI. 233 lipoprotein and metabolite measures were quantified using nuclear magnetic resonance (NMR) spectrometry. LVEF and ISZ were assessed 4 months post-MI. 24 h post-MI measurements of HDL triglycerides (HDL-TG) predicted LVEF (β=1.90 [95% CI: 0.82, 2.98]; $p=6.4x10^{-4}$) and ISZ (β=-0.41; [-0.60, -0.21]; $p=3.2x10^{-5}$). Additionally, 24 h post-MI measurements of medium HDL-TG (β=-0.40 [-0.60, -0.20]; $p=6.4x10^{-5}$), small HDL-TG (β=-0.34 [-0.53, -0.14]; $p=7.3x10^{-4}$) and the triglyceride content of very large HDL (β=-0.38 [-0.58, -0.18]; $p=2.7x10^{-4}$) were associated with ISZ. After the 4-month treatment, the phospholipid content of very large HDL was lower in metformin vs. placebo treated patients (28.89% vs. 38.79%; $p=7.5x10^{-5}$); alanine levels were higher in the metformin group (0.46 mmol/l vs. 0.44 mmol/l; $p=2.4x10^{-4}$).

Conclusions: HDL triglyceride concentrations predict post-MI LVEF and ISZ. Metformin increases alanine levels and reduces the phospholipid content in very large HDL particles.

Clinical Trial Registration:
NCT01217307 (https://clinicaltrials.gov)

Introduction

Myocardial Infarction (MI) is one of the leading causes of global morbidity and mortality. While the survival after MI has improved due to ameliorated treatment strategies, including primary percutaneous interventions, the long-term outcome of MI in general remains poor with a 1-year risk for recurrent cardiovascular (CV) events of over 10%.[1] Left ventricular ejection fraction (LVEF) and infarct size (ISZ) are key predictors of long-term prognosis after MI.[2,3] However, treatment options for left ventricular dysfunction are limited and the biochemical mechanisms driving functional decline of the myocardium after MI are largely unknown.

Metformin, which is commonly used in the treatment of diabetes and more recently in insulin resistant conditions, has been found to preserve LVEF and to reduce ISZ in non-diabetic animal models of MI.[4] The GIPS-III clinical trial was designed to study the effects of metformin therapy on LVEF in non-diabetic ST segment Elevation MI (STEMI) patients undergoing PCI. However, in contrast to preclinical findings, metformin did not improve LVEF compared with placebo 4 months post-MI.[5]

This result may be explained by interindividual differences in metformin response, raising the possibility that metformin is effective in a subgroup of CV patients. Metabolic profiling has emerged as a powerful tool to explore drug effects and factors influencing drug response.[6-8] Metabolomics is a relatively novel field in 'omics' sciences, which uses high-throughput technologies, such as nuclear magnetic resonance (NMR) spectroscopy, to concurrently quantify a large number of small molecules in different tissues. While recent studies reported changes in lipid and amino acid concentrations after metformin treatment,[9-11] no study has yet used large-scale metabolic platforms to investigate the effects of metformin on a wide range of lipoprotein and metabolite measures at a time. Furthermore, metabolic profiling has been performed to improve diagnosis and prediction of CV events.[12,13] A recent study identified metabolic profiles which discriminate heart failure patients from healthy controls.[14] Metabolic profiling may thus help identify novel biomarkers of left ventricular function and ISZ to improve risk stratification in MI patients.

Metabolite concentrations can vary greatly over time and are highly sensitive to environmental influences. Lipid profiles have been shown to change shortly after MI and only gradually return to baseline after several weeks.[15] The predictive value of a biomarker may thus vary over time. We therefore studied metabolic markers of LVEF, ISZ and metformin response in the GIPS-III cohort at three different time points: baseline (on admission), 24 h post-MI and 4 months post-MI.

The objective of this ancillary study of the GIPS-III trial was to evaluate the effect of metformin on metabolic profiles in non-diabetic STEMI patients and to identify prognostic markers, which predict LVEF and ISZ 4 months post-MI. Furthermore we tested whether metformin improved LVEF and ISZ in subgroups of patients, as identified by metabolic profiling.

Methods

Study population
The GIPS-III study is a randomized trial that included 380 non-diabetic patients undergoing primary PCI for STEMI. Participants received a 4-month regimen with either metformin 500mg 2dd1 or matching placebo 2dd1. The design of the study has been previously described in more detail.[4,5] All patients provided written informed consent. The study complied with the Declaration of Helsinki and was approved by the ethics committee of the University Medical Center Groningen (the Netherlands) and national authorities (NCT01217307). The primary outcome measure was LVEF, the secondary outcome measure was ISZ. Both measures were assessed 4 months post-MI by MRI as described below.

Laboratory measurements
Non-fasting blood samples were obtained on admission (N=339), 24 h post-MI (N=329) and 4 months post-MI (N=316). Serum and EDTA anticoagulated plasma samples were stored at -80 °C until analyzed. Metabolic profiling was performed using a high-throughput ^{1}H NMR metabolomics platform.[16] We obtained a total of 233 concentrations and ratios of metabolic measures, including 168 lipoprotein subclass measures, 45 lipid related measures, 5 glycolysis related metabolites, 9 amino acids, 3 ketone bodies, 2 fluid balance related metabolites and 1 inflammatory marker. An overview of all NMR measures is given in Supplemental Table 1.

Cardiac magnetic resonance imaging
LVEF and ISZ were measured by cardiac magnetic resonance imaging (MRI) 4 months after MI as previously described in detail.[4,5] Independent cardiologists analyzed all MRI data and assessed LVEF and ISZ, blinded for treatment assignment.

Statistical analysis
Missing NMR measures were imputed using random forest imputation as implemented in the R package missForest.[17] Since most NMR measures showed skewed distributions, they were normalized using rank-based inverse normal transformation within each time point separately. Spearman's correlation coefficients were calculated from the metabolite concentrations for each time point (baseline, 24 h post-MI and 4 months post-MI) and plotted using the corrplot function of the corrplot package of R. The correlation plots are presented in Supplemental Figure 1. Since many metabolites were highly correlated, principal component analysis (PCA) was applied to estimate the number of independent tests for multiple testing correction, using the prcomp function in R. To additionally account for multiple testing at different time points, principal components were calculated across all three time points. The first 68 PCs explained over 95% of the variation in the metabolite data, yielding an adjusted significance level of $p<0.05/68=0.00074$.

Unpaired t-tests were performed to assess the effect of metformin treatment on lipoprotein and metabolite measures. To identify biomarkers predictive of LVEF and ISZ, we analyzed all NMR measures at each time point separately, using linear regression adjusted for known predictors of ventricular function and medication use:

age, sex, baseline N-terminal prohormone of brain natriuretic peptide (NTproBNP) levels, baseline creatine kinase (CK)-MB levels, myocardial blush grade, metformin treatment and statin treatment (4 months post-MI). To meet the assumption of normality of residuals, we tested different transformations. Since square-root transformation provided the best results, ISZ was square-root transformed. In addition we performed stratified analyses for LVEF and ISZ. According to current guidelines,[18] LVEF 52%-72% was categorized as normal ventricular function; LVEF 41-51% was defined as mildly abnormal and LVEF <41% as abnormal for men. Categories were LVEF 54%-74% for normal ventricular function, LVEF 41-53% as mildly abnormal and LVEF <41% for abnormal for women. ISZ was stratified by tertiles to obtain the same number of strata as with LVEF. Associations of NMR measures with LVEF categories and ISZ tertiles were assessed using multinomial logistic regression, which provides pairwise comparisons between each level of the outcome variable and a reference level. Finally we added the interaction term of metformin treatment and NMR measure to the linear regression models to identify subgroups of patients in whom metformin was effective. R (version 3.02 or higher, http://www.r-project.org/) was used for all statistical analyses.

Results

Patient characteristics and metabolic measures

A total of 380 patients received either metformin placebo treatment. Of these, 109 did not undergo MRI 4 months post-MI or did not provide utilizable scans due to insufficient quality. Details on metformin/placebo treatment, clinical parameters and conventional lipid and (apo)lipoprotein measures have been published elsewhere.[5,11] Briefly, metformin treatment resulted in a modest decrease in low-density lipoprotein cholesterol (LDL-C) without significant effects on total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, apolipoprotein B (apoB) and apolipoprotein A-I (apoA1) when the values after 4 months and after 24 h were compared (data not shown).[14] Metabolic profiles were quantified in a total of 376 patients. Baseline, 24 h post-MI and 4-month post-MI measurements were available from 339, 326 and 316 patients, respectively. Premature dropout was neither related to metformin treatment nor to mortality as none of the participants died before MRI.[5] A summary of all lipoprotein metabolite measures can be found in Supplemental Table 1. The correlation matrices revealed substantial correlation within lipoprotein subclasses, between amino acids and between fatty acids (Supplemental Figure 1).

Association of NMR measures with LVEF and ISZ

Results for all metabolic measures tested are shown in Supplemental Tables 2-7. None of the NMR measures was significantly associated with LVEF 4 months post-MI. No baseline NMR measure predicted LVEF. Patients with higher HDL triglyceride (TG) levels 24 h post-MI showed significantly better LVEF (β=1.90 [95% CI: 0.82, 2.97]; $p=6.4x10^{-4}$) after adjustment for metformin treatment, age, sex, baseline NTproBNP levels, baseline CK-MB levels, myocardial blush grade and statin use (Table 1). When LVEF was entered as categorical variable (normal, mildly abnormal, abnormal left

Table 1: Associations of selected NMR measures with LVEF and ISZ 24 h post–MI

	Unadjusted model		Ajusted model	
	β (95% CI)	p	β (95% CI)	p
LVEF (N=245)				
HDL-TG	1.84 (0.78, 2.89)	$7.4x10^{-4}$	1.90 (0.82, 2.97)	$6.4x10^{-4}$*
M-HDL-TG	1.70 (0.65, 2.75)	0.002	1.65 (0.55, 2.74)	0.003
XL-HDL-TG%	1.67 (0.56, 2.77)	0.003	1.82 (0.68, 2.96)	0.002
S-HDL-TG	1.51 (0.45, 2.57)	0.006	1.68 (0.58, 2.78)	0.003
Albumin	1.10 (0.04, 2.16)	0.044	1.25 (0.10, 2.40)	0.034
Phenylalanine	-0.90 (-2.01, 0.21)	0.113	-0.55 (-1.68, 0.58)	0.344
ISZ (N=231)				
HDL-TG	-0.42 (-0.60, -0.24)	$1.2x10^{-5}$*	-0.41 (-0.60, -0.22)	$3.2x10^{-5}$*
M-HDL-TG	-0.42 (-0.60, -0.23)	$1.4x10^{-5}$*	-0.40 (-0.60, -0.21)	$6.4x10^{-5}$*
XL-HDL-TG%	-0.37 (-0.56, -0.18)	$1.9x10^{-4}$*	-0.38 (-0.58, -0.18)	$2.7x10^{-4}$*
S-HDL-TG	-0.33 (-0.52, -0.14)	$6.8x10^{-4}$*	-0.34 (-0.54, -0.15)	$7.3x10^{-4}$*
Albumin	-0.33 (-0.52, -0.15)	$5.2x10^{-4}$*	-0.33 (-0.54, -0.13)	0.002
Phenylalanine	0.38 (0.18, 0.58)	$1.9x10^{-4}$*	0.34 (0.14, 0.55)	0.001

Results for metabolic measures that showed signifiacnt associations in the unadjusted model or the adjusted model, including age, sex, treatment, statin use, CKMB, NTproBNP and MBG as covariates. *Effects significant after correction for multiple testing ($p<7.4x10^{-4}$). CI: confidence interval; LVEF: left ventricular ejection fraction; ISZ: infarct size; HDL-TG: triglycerides in HDL particles; M-HDL-TG: triglycerides in medium HDL particles; XL-HDL-TG%: triglycerides to total lipids ratio in very large HDL particles; S-HDL-TG: triglycerides in small HDL particles.

ventricular function), 24 h post-MI measurements of HDL-TG (OR=0.36 [95% CI: 0.21, 0.61]; $p=1.8x10^{-4}$), medium (M-) HDL-TG (OR=0.37 [0.22, 0.63]; $p=2.3x10^{-4}$) and small (S-) HDL-TG (OR=0.35 [0.20, 0.61]; $p=2.1x10^{-4}$) significantly predicted normal vs. abnormal LVEF 4 months post-MI (Figure 1A, Table 2). Notably, all HDL-TG related measures showed a positive association with LVEF, suggesting a beneficial effect of increased triglyceride content in HDL. We found no association of 24 h post-MI measurements with mildly abnormal LVEF relative to normal LVEF. In addition, 24 h post-MI measurements of triglycerides (OR=0.39 [0.23, 0.66]; $p=5.2x10^{-4}$) and the cholesterol (OR=2.52 [1.48, 4.30]; $p=6.6x10^{-4}$) in very small (XS-) very low-density lipoprotein (VLDL) particles was associated with abnormal LVEF compared to normal left ventricular function. Finally, baseline measurements of the TG to total lipids ratio in large (L-) LDL (L-LDL-TG%) predicted abnormal LVEF (OR=0.37 [0.21, 0.65]; $p=6.2x10^{-4}$). Addition of a treatment x NMR measure interaction term did not reveal any patient subgroup in whom metformin improved LVEF (Table 3).

We did not find any association between NMR measures and ISZ at baseline and 4 months post-MI. In the adjusted model, HDL-TG (β=-0.41 [-0.60, -0.22]; $p=3.2x10^{-5}$), M-HDL-TG (β=-0.40 [-0.60, -0.21]; $p=6.4x10^{-5}$), very large (XL-) HDL-TG% (β=-0.38 [-0.58, -0.18]; $p=2.7x10^{-4}$) and S-HDL-TG (β=-0.34 [-0.54, -0.15]; $p=7.3x10^{-4}$) were

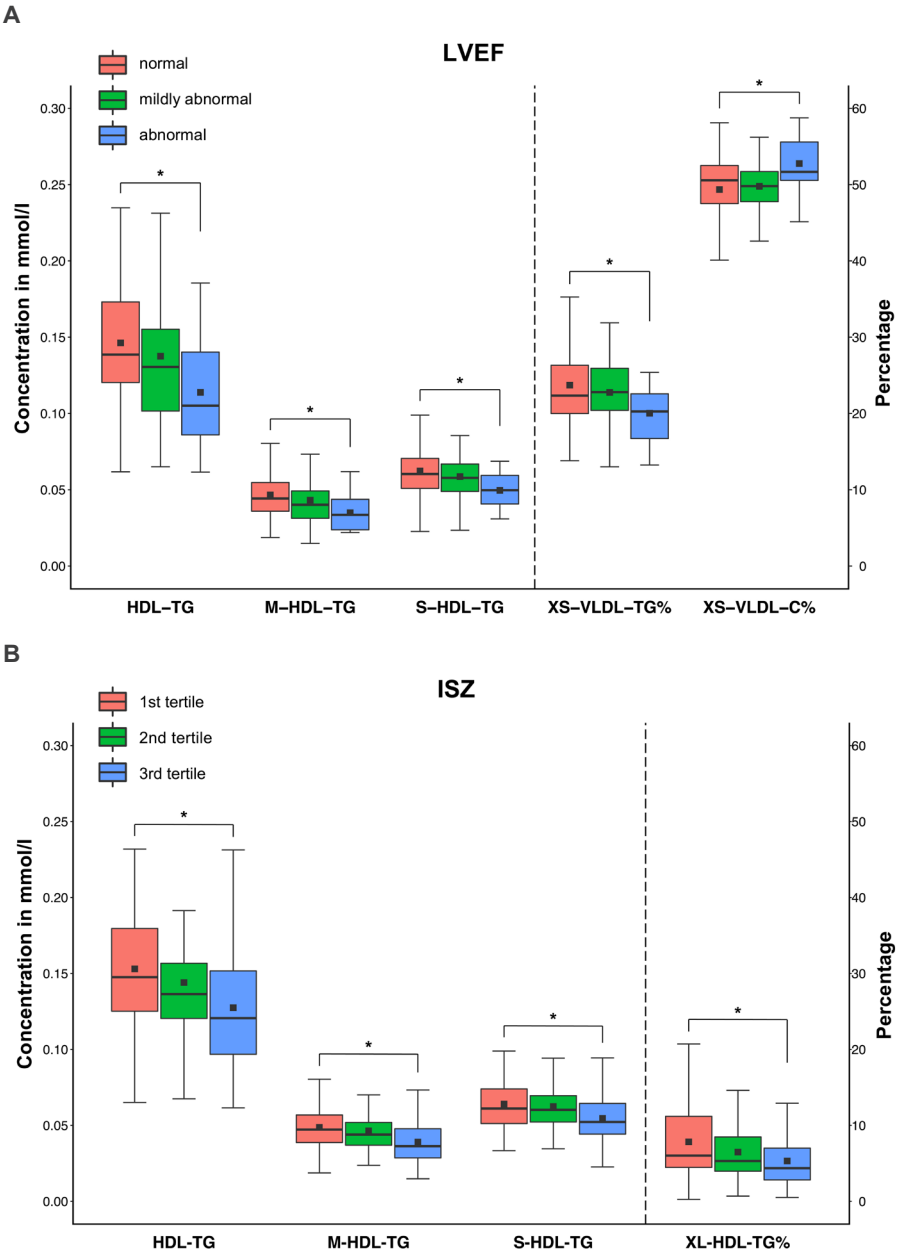
significantly associated with ISZ 24 h post-MI (Table 1). In addition, phenylalanine (β=0.38 [0.18, 0.58]; p=1.9x10^{-4}) and albumin (β=-0.33 [-0.52, -0.15]; p=5.2x10^{-4}) reached significance in the unadjusted model, but not in the adjusted model. Similarly, 24 h post-MI measurements of HDL-TG (OR=0.48 [0.33, 0.69]; p=9.2x10^{-5}), M-HDL-TG (OR=0.46 [0.31, 0.67]; p=6.2x10^{-5}), S-HDL-TG (OR=0.51 [0.35, 0.74]; p=3.9x10^{-4}) and XL-HDL-TG% (OR=0.49 [0.33, 0.72]; p=3.2x10^{-4}) predicted ISZ, when the first tertile was compared to the third tertile (Figure 1B, Table 2). Again, our findings suggest a beneficial effect of higher HDL-TG levels. We found no significant interactions between treatment and NMR measures (Table 3). As shown in Supplemental Table 8, HDL-TG, M-HDL-TG, S-HDL-TG and XL-HDL-TG% increased between baseline and 24 h post-MI and remained relatively stable between 24 h and 4 months post-MI, except for XL-HDL-TG%, which showed a moderate gain. Similar to HDL-TG, serum triglyceride levels increased between baseline and 24 h post-MI, but were decreased 4 months after MI.

Table 2: Associations of selected NMR measures with LVEF and ISZ categories

	OR (95% CI)	p	OR (95% CI)	p
LVEF	normal vs. mildly abnormal		normal vs. abnormal	
Baseline				
L-LDL-TG%	1.05 (0.76, 1.45)	0.774	0.37 (0.21, 0.65)	6.2x10^{-4}
24 h post-MI				
HDL-TG	0.70 (0.51, 0.96)	0.027	0.36 (0.21, 0.61)	1.8x10^{-4}
M-HDL-TG	0.72 (0.52, 1.00)	0.050	0.37 (0.22, 0.63)	2.3x10^{-4}
S-HDL-TG	0.74 (0.53, 1.02)	0.062	0.35 (0.20, 0.61)	2.1x10^{-4}
XS-VLDL-TG%	0.83 (0.61, 1.14)	0.247	0.39 (0.23, 0.66)	5.2x10^{-4}
XS-VLDL-C%	1.10 (0.82, 1.49)	0.523	2.52 (1.48, 4.30)	6.6x10^{-4}
ISZ	1st tertile vs. 2nd tertile		1st tertile vs. 3rd tertile	
24 h post-MI				
HDL-TG	0.78 (0.55, 1.11)	0.169	0.48 (0.33, 0.69)	9.2x10^{-5}
M-HDL-TG	0.88 (0.62, 1.25)	0.472	0.46 (0.31, 0.67)	6.2x10^{-5}
S-HDL-TG	0.92 (0.64, 1.30)	0.621	0.51 (0.35, 0.74)	3.9x10^{-4}
XL-HDL-TG%	0.75 (0.52, 1.07)	0.116	0.49 (0.33, 0.72)	3.2x10^{-4}

Associations of metabolic measures with LVEF categories (normal, mildly abnormal, abnormal) and ISZ categories (tertiles), and adjusted for age, sex, treatment, statin use, CKMB, NTproBNP and MBG. Results for pairwise comparisons are given Metabolic measures with at least one significant pairwise between-group comparison are shown. *Effects significant after correction for multiple testing (p<7.4x10^{-4}). CI: confidence interval; LVEF: left ventricular ejection fraction; ISZ: infarct size; L-LDL-TG%: triglyceride to total lipids ratio in large LDL particles; HDL-TG: triglycerides in HDL particles; M-HDL-TG: triglycerides in medium HDL particles; S-HDL-TG: triglycerides in small HDL particles; XL-HDL-TG%: triglycerides to total lipids ratio in very large HDL particles; XS-VLDL-TG%: triglycerides to total lipids ratio in very small VLDL particles; XS-VLDL-C%: cholesterol to total lipids ratio in very small VLDL particles.

Figure 1: Categorical analysis for LVEF and ISZ



Box plots comparing selected NMR measures (24 h post-MI) between distinct LVEF (**A**) and ISZ (**B**) categories. For all plots, the lower and the upper margins represent the first and third quartile, respectively. Vertical lines indicate median values; squares indicate mean values. The whiskers represent the lowest and the highest value within 1.5 interquartile ranges. Outliers are not shown. Differences between categories were assessed using multinomial logistic regression adjusted for treatment, age, sex, NTproBNP levels, CK-MB levels, myocardial blush grade, statin use. *Effects significant after correction for multiple testing ($p<7.4x10^{-4}$). Abbreviations are as in Table 2.

Table 3: Interaction between treatment and selected NMR measures

	Unadjusted model		Adjusted model	
	β (95% CI)	p	β (95% CI)	p
LVEF (N=245)				
Treatment X				
HDL-TG	1.15 (-0.96, 3.26)	0.285	0.94 (-1.18, 3.06)	0.387
M-HDL-TG	1.09 (-1.01, 3.19)	0.311	0.82 (-1.30, 2.94)	0.449
XL-HDL-TG%	0.71 (-1.50, 2.92)	0.529	0.60 (-1.61, 2.82)	0.593
S-HDL-TG	0.73 (-1.42, 2.87)	0.506	0.68 (-1.46, 2.82)	0.534
Albumin	0.36 (-1.77, 2.49)	0.740	0.38 (-1.74, 2.51)	0.725
Phenylalanine	-1.02 (-3.25, 1.22)	0.373	-1.24 (-3.51, 1.03)	0.286
ISZ (N=231)				
Treatment X				
HDL-TG	-0.07 (-0.44, -0.30)	0.701	-0.04 (-0.41, 0.34)	0.856
M-HDL-TG	-0.19 (-0.56, 0.18)	0.316	-0.14 (-0.52, 0.24)	0.465
XL-HDL-TG%	0.04 (-0.35, 0.42)	0.849	0.04 (-0.35, 0.44)	0.827
S-HDL-TG	-0.04 (-0.42, 0.35)	0.857	-0.03 (-0.41, 0.36)	0.891
Albumin	-0.06 (-0.43, 0.32)	0.762	-0.05 (-0.43, 0.33)	0.804
Phenylalanine	0.01 (-0.39, 0.40)	0.977	0.04 (-0.36, 0.45)	0.834

Association of treatment x NMR measure interaction (24 h post-MI) with LVEF and ISZ. Results are shown for the unadjusted model and adjusted model including age, sex, statin use, CKMB, NTproBNP and MBG as covariates. CI: confidence interval; LVEF: left ventricular ejection fraction; ISZ: infarct size; HDL-TG: triglycerides in HDL particles; M-HDL-TG: triglycerides in medium HDL particles; XL-HDL-TG%: triglycerides to total lipids ratio in very large HDL particles; S-HDL-TG: triglycerides in small HDL particles.

Effects of metformin of metabolic profiles

Results for all lipoprotein and metabolite measures are shown in Supplemental Table 9. To assess baseline differences in metabolic profiles, we compared NMR measures between the treatment group and controls at baseline. We did not find any difference between the two groups at baseline. Table 4 summarizes metabolic measurements for 24 h post-MI and 4 months post-MI. 24 h post-MI, after the administration of the first doses of the treatment. Both alanine (median: 0.49 mmol/l vs. 0.46 mmol/l; $p=9.0x10^{-4}$) and pyruvate (median: 0.16 mmol/l vs. 0.14 mmol/l; p=0.001) displayed trends towards increased concentrations in the metformin group compared to the placebo group. After the 4-month treatment period, alanine levels were significantly elevated in metformin-treated patients (median: 0.46 mmol/l vs. 0.44 mmol/l; $p=2.4x10^{-4}$). In addition, the phospholipids to total lipids ratio in XL-HDL particles (XL-HDL-PL%) was significantly reduced in the metformin group compared to the placebo group (median: 28.89% vs. 38.79%; $p=7.5x10^{-5}$).

Table 4: Treatment effects of metformin

	Placebo Median (IQR)	Metformin Median (IQR)	p
24 h post-MI	N=170	N=159	
Alanine in mmol/l	0.46 (0.09)	0.49 (0.09)	$9.0x10^{-4}$
Pyruvate in mmol/l	0.14 (0.05)	0.16 (0.07)	0.001
XL-HDL-PL%	36.11 (17.52)	33.98 (14.65)	0.908
4 months post-MI	N=159	N=157	
Alanine in mmol/l	0.44 (0.08)	0.46 (0.09)	$2.4x10^{-4}$*
Pyruvate in mmol/l	0.10 (0.04)	0.11 (0.04)	0.006
XL-HDL-PL%	38.79 (19.50)	28.89 (23.90)	$7.5x10^{-5}$*

Effects of treatment on selected NMR measures 24 h post-MI and 4 months post-MI. *Significant effects ($p<7.4x10^{-4}$). IQR: inter-quartile range; XL-HDL-PL%: phospholipids to total lipids ratio in very large HDL particles.

Discussion

We used NMR spectrometry-based lipoprotein and metabolite measures to evaluate the effects of metformin on metabolic profiles of non-diabetic MI patients and to study prognostic metabolic measures predicting LVEF and ISZ 4 months post-MI. Moreover we investigated whether metabolic profiling could be used to identify subgroups of patients in whom metformin was effective. After the 4-month treatment period, we found higher alanine levels and lower XL-HDL-PL% in metformin-treated patients as compared to controls. Remarkably, higher triglyceride levels in HDL and several HDL subclassess measured 24 h post-MI were associated with a favorable outcome, as inferred from higher LVEF and smaller ISZ 4 months post-MI. Moreover, categorical analysis of LVEF revealed that besides HDL-TG, the composition of XS-VLDL (24 h post-MI) and L-LDL (baseline) was associated with abnormal left ventricular function 4 months post-MI. We could not identify metabolic profiles associated with treatment benefits from metformin.

Similar to our results, the CAMERA study, a clinical trial investigating the effects of metformin on different amino acids, found substantially increased alanine levels 18 months after treatment onset.[13] Alanine plays a crucial role in the alanine-glucose cycle, in which alanine released by muscle tissue is transported to the liver before it is converted into pyruvate for gluconeogenesis. Findings from animal studies suggest that metformin reduces gluconeogenesis by inhibiting hepatic alanine uptake and by hampering fat-induced changes in the glycolysis metabolic pathway.[19,20] As a result of reduced uptake into the liver, blood alanine levels may rise in metformin-treated patients. Interestingly, we observed a trend towards increased alanine levels in the metformin group 24 h post-MI, suggesting rapid effects of metformin on gluconeogenesis.

Numerous randomized controlled trials have studied the effects of metformin treatment on lipid levels in patients with type 2 diabetes. A recent study in diabetic patients found that metformin lowered total cholesterol and LDL-C.[21] Another study in patients at risk for diabetes reported changes in lipoprotein subclasses after one year of metformin treatment, with reduced particle concentrations of small LDL and elevated concentrations of large LDL, small HDL and large HDL.[9] In our recent report, we observed modest decreases in LDL cholesterol, no change in apolipoprotein B, and as a result a small decrease in LDL particle size.[11] In the present study, which used a different NMR-based method, only the phospholipid content of large HDL particles was decreased in response to metformin.

We also tested whether lipoprotein characteristics and NMR measures at baseline, 24 h post-MI and 4 months post-MI were associated with 4 months post-MI LVEF and ISZ. We found that increased HDL-TG levels measured 24 h post-MI were associated with a greater LVEF. In addition, decreased HDL-TG, M-HDL-TG, XL-HDL-TG% and S-HDL-TG measured 24 h post-MI predicted higher ISZ. Categorical analysis of LVEF and ISZ provided similar results with more favorable outcomes for patients with higher HDL-TG levels. No NMR measure showed a significant interaction with metformin treatment, suggesting that there was no metabolic subgroup of patients in whom metformin was effective.

Our findings suggest beneficial effects of higher triglyceride levels in HDL and in HDL subclasses measured 24 h post-MI on ISZ and LVEF. Clinical studies have identified low admission triglyceride levels as a risk factor for recurrent CV events and mortality in STEMI patients.[22,23] Likewise, low triglyceride levels are associated with a poor prognosis in stroke patients.[24] This contrasts with findings from large-scale case-control and prospective cohort studies indicating that hypertriglycemia is a strong predictor of CV events, even independent of cholesterol levels.[25,26] These epidemiological findings, however, apply to individuals who were not studied during the course of an acute coronary event. Similarly paradoxical findings have been obtained for plasma cholesterol levels. While hypercholesterolemia is an established CV risk factor in the general population, admission LDL-C levels <70 mg/dl are associated with higher mortality and incidence of heart failure in statin-naïve STEMI patients.[27] The pathogenic mechanisms underlying recurrent CV events shortly after an acute event are still poorly understood. It is possible that in the acute setting HDL-TG plays a distinct role on CV outcome .

VLDL is the most important triglyceride carrier in plasma. The triglyceride content of VLDL showed substantial correlation with HDL-TG 24 h post-MI (Supplemental Figure 1B). However, only the triglyceride content of very small VLDL particles was associated with LVEF categories. In addition, the TG content of large LDL particles at baseline predicted abnormal LVEF 4 months post-MI. Inhibition of fatty acid uptake by relocation of FAT/CD36 may reduce intracellular fatty acid concentrations,[28] resulting in increased extracellular fatty acid levels and diminished lipolysis of lipid-bound triglycerides. This may initially lead to triglyceride enrichment of VLDL and LDL particles, which subsequently transfer excess triglycerides to HDL particles in exchange for cholesteryl esters by the action of cholesteryl ester transfer protein

(CETP), thereby increasing the triglyceride content in HDL.[29] In line with this, blood samples of MI patients collected immediately after diagnosis show strong triglyceride enrichment of HDL2 particles.[30] Higher plasma HDL-TG levels could thus be consequent to inhibition of fatty acid uptake, and coincide with diminished fatty acid oxidation and prevention of further myocardial damage.[31] Larger triglyceride-rich particles are converted to small VLDL subclasses as a result of lipase-mediated delipidation,[32] suggesting that triglyceride enrichment may be secondary to initial triglyceride uptake of large VLDL. Larger VLDL particles may be delipidated rapidly, which may explain why the association of triglycerides with LVEF was limited to very small VLDL 24 h post-MI. Similarly, a major proportion of LDL-TG is derived from large VLDL,[32] which may partly result from CETP-mediated delipidation of large VLDL. Taken together, early metabolic changes after MI could reflect adaptive mechanisms that promote functional recovery.

We observed associations of LVEF categories with 24 h post-MI measurements of XS-VLDL-TG% and XS-VLDL-C%. However, these NMR measures did not significantly predict LVEF when LVEF was analyzed as a continuous variable. The regression model with continuous outcome assumes linearity between NMR measures and LVEF, whereas categorical analysis of LVEF in combination with multinomial logistic regression renders the model sensitive to non-linear associations. As shown in Figure 1A, HDL-TG, M-HDL-TG and S-HDL-TG follow a linear trend across the three LVEF categories, whereas XS-VLDL-TG% and XS-VLDL-C% display non-linear trends.

Limitations

The GIPS-III trial was originally designed to assess differences in LVEF between metformin treated patients and controls. We conducted 68 independent tests, raising the possibility that our study was not powered to detect smaller changes. However, we were able to detect a significant effect for alanine levels, which were only slightly increased in the metformin group (median difference: 0.03 mmol/l), demonstrating sufficient power to perform a metabolic profiling analysis. In addition, all patients received intravenous heparin before PCI when baseline blood samples were drawn. Heparin stimulates lipolysis and hence acutely reduces plasma triglyceride levels,[33] which is in line with the marked increase in triglyceride levels between baseline and the other time points (Supplemental Table 8). STEMI patients routinely receive heparin before PCI, rendering the results for baseline measurements relevant to clinical settings. These findings measurements should nevertheless be interpreted with caution. Moreover, we performed metabolic profiling in non-fasting blood samples, warranting further research to substantiate our findings under fasting conditions. However, the NMR platform used in our study mainly quantifies lipid measures, which change only slightly after food consumption and show similar associations with cardiovascular risk in fasting and non-fasting individuals.[34]

Conclusions

In summary, our study suggests that metformin treatment started directly after presentation with STEMI produces changes in alanine and XL-HDL-PL% as assessed after 4 months. Higher triglyceride levels in HDL and in HDL subclasses measured 24 h post-MI were predictive of better LVEF and smaller ISZ 4 months post-MI. HDL-TG may thus serve as an early biomarker of left ventricular dysfunction in STEMI patients. However, further studies are required to substantiate the clinical significance of HDL-TG in CV risk prediction and to investigate the biological mechanism underlying associations of metabolic biomarkers with recurrent CV events. Our findings emphasize the utility of high-throughput metabolic profiling as a tool to study drug effects and to identify prognostic biomarkers of LVEF and ISZ.

Supplemental materials are available online.

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Chapter 7

Statin effects on metabolic profiles: data from the PREVEND IT trial

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Abstract

Background: Statins lower cholesterol by inhibiting HMG-CoA reductase, the rate limiting enzyme of the metabolic pathway that produces cholesterol and other isoprenoids. Surprisingly little is known about their effects on metabolite and lipoprotein subclass profiles. We therefore investigated the molecular changes associated with pravastatin treatment compared to placebo administration, using a nuclear magnetic resonance (NMR)-based metabolomics platform.

Results: We performed metabolic profiling of 231 lipoprotein and metabolite measures in the PREVEND IT study, a placebo-controlled randomized clinical trial designed to test the effects of pravastatin (40 mg once daily) on cardiovascular risk. Metabolic profiles were assessed at baseline and after 3 months of treatment. Pravastatin lowered low-density lipoprotein cholesterol (LDL-C; change in SD units: -1.01 [95% CI: -1.14, -0.88]), remnant cholesterol (change in SD units: -1.03 [-1.17, -0.89]) and apolipoprotein B (apoB; change in SD units: -0.98 [-1.11, -0.86]) with similar effect magnitudes. In addition, pravastatin globally lowered levels of lipoprotein subclasses, with the exception of high-density lipoprotein (HDL) subclasses, which displayed a more heterogeneous response pattern. The lipid lowering effect of pravastatin was accompanied by selective changes in lipid composition, particularly in the cholesterol content of very low-density lipoprotein (VLDL) particles. In addition, pravastatin reduced levels of several fatty acids, but had limited effects on fatty acid ratios.

Conclusions: These randomized clinical trial data demonstrate the widespread effects of pravastatin treatment on lipoprotein subclass profiles and fatty acids.

Clinical Trial Registration:
NCT03073018 (https://clinicaltrials.gov)

Introduction

Statins hamper cholesterol production in the liver through inhibition of HMG-CoA reductase, which, in turn, stimulates hepatic synthesis of low-density lipoprotein (LDL) receptors as a compensatory mechanism. These receptors bind to apoB-rich lipoproteins and facilitate their absorption by hepatocytes, leading to a further reduction in plasma cholesterol levels.[1] The cardiovascular risk reduction achieved through statins is believed to primarily result from their LDL cholesterol (LDL-C) lowering properties.[2] Lowering of LDL-C has therefore been identified as the primary treatment target of statin therapy.[3] However, statins act early in the mevalonate pathway and have the potential to extensively modify the metabolic profile in addition to their effect on cholesterol metabolism. This has led to the hypothesis that statins may provide cardioprotective benefits beyond LDL-C reduction. While there is mounting evidence underpinning the therapeutic capacities of such pleiotropic statin effects,[4-6] little is known about the underlying molecular pathways.

Nuclear magnetic resonance (NMR)-based metabolic profiling has evolved into a versatile high-throughput tool for biomarker discovery that allows simultaneous quantifications of numerous molecules, ranging from amino acids to a variety of lipoprotein subclass measures. Metabolic profiling has been widely used both in epidemiology and in drug research.[7-9] Better characterization of the metabolic footprint of statins may provide novel insights into their mechanisms of action and help guide drug discovery. A recent study of four observational population-based cohorts investigated the longitudinal effects of statins on metabolic profiles by comparing users to non-users, followed by confirmatory Mendelian randomization analysis.[9] Besides cholesterol lowering, statins influenced fatty acid levels, whereas amino acids and other metabolites were not substantially altered. While this study revealed extensive changes in routine lipid measures, little is known about the effect of statins on lipoprotein subclass profiles, even though mounting evidence suggests distinct roles for lipoprotein subclasses in the pathophysiology of cardiovascular disease.[10-12] In addition, no study has yet comprehensively investigated the metabolic effects of statin therapy in a placebo-controlled randomized setting. Here we present the first data on pravastatin treatment derived from the Prevention of Renal and Vascular End-stage Disease Intervention Trial (PREVEND IT) study, a randomized placebo-controlled clinical trial. In addition to previously quantified parameters, including lipids, fatty acids, amino acids and glycolysis metabolites, we report results for over 160 measures of lipoprotein subclasses. An overview of lipoprotein subclasses is given in Table 1.

Materials and methods

Participants

Details on the PREVEND IT study have been published elsewhere.[13] Briefly, PREVEND IT is a double-blind, placebo-controlled clinical trial, in which participants were randomized to receive 20 mg fosinopril or matching placebo and 40 mg pravastatin or matching placebo. PREVEND IT participants were recruited from

Table 1: Average particle size of lipoprotein subclasses

Lipoprotein	Subclass	Average particle diameter (in nm)*
VLDL	XXL	>75
	XL	64.0
	L	53.6
	M	44.5
	S	36.8
	XS	31.3
IDL		28.6
LDL	L	25.5
	M	23.0
	S	18.7
HDL	XL	14.3
	L	12.1
	M	10.9
	S	8.7

Average particle size of lipoprotein sublcasses expressed as diameter in nm. The values above are adapted from [8]. *Cut points for size ranges can be approximated by the midpoint between the average diameters of two consecutive lipoprotein subclasses, e.g. the lower bound of XS-VLDL is approximately 30 nm. HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; XXL: extremely large; XL: very large; L: large; M: medium; S: small; XS: very small.

the PREVEND program, which investigated the influence of microalbuminuria on cardiovascular and renal risk. The main inclusion criteria for PREVEND IT were a urine albumin concentration of >10 mg/l in one morning spot sample and at least once a concentration of 15 to 300 mg/24 h in two successive 24-hour urine samples, a blood pressure of <160/100 mm Hg, no hypertensive treatment and a total serum cholesterol concentration <8.0 mmol/l (or <5.0 mmol/l in case of prior myocardial infarction) and no lipid-lowering treatment. 864 subjects were randomized to receive study medication (see above) after giving informed consent. Blood samples for metabolic profiling were limited by sample availability and could be obtained in 394 participants at baseline and after 3 months of treatment. The study was approved by the Institutional Review Board and was conducted in according to the guidelines of the Declaration of Helsinki.

Laboratory measurements

Fasting blood samples were drawn before treatment onset (baseline) and at the 3-month medical review (N=394). Metabolic profiling was performed in EDTA anticoagulated plasma samples using high-throughput ^{1}H NMR metabolomics (Brainshake Ltd, Helsinki, Finland).[8] This method provides accurate quantification of 231 lipoprotein and metabolite measures, including routine lipids, lipoprotein profiles with 14 lipoprotein subclasses, glycolysis related metabolites, amino acids, ketone bodies,

fluid balance related metabolites and one inflammatory marker (Supplemental Table 1). Recent studies have demonstrated that NMR measurements quantified with this platform are in good agreement with routine clinical chemistry assays.[8] Representative coefficients of variation for this platform have been reported elsewhere.[14]

Statistical analysis
Correlations between different lipoprotein and metabolite measures were calculated using Spearman's correlation coefficients. The effect of statin treatment on each NMR measure was assessed by linear regression on the change during the treatment period, similarly as previously described.[9] The effect estimate (regression coefficient) of this regression model can be interpreted as the longitudinal change of a NMR measure attributable to pravastatin treatment. To facilitate comparison between different lipoprotein and metabolite measures, differences between pre- and post-treatment values were scaled to baseline SD units. Consequently, statin effects on NMR measures are expressed in baseline SD units. We additionally performed a sensitivity analysis adjusted for sex as the pravastatin group showed a higher percentage of male patients. Since many NMR measures were highly correlated (see Supplemental Table 1), we accounted for multiple testing by correcting the nominal level of significance for the number of independent tests, which was estimated by the method of Li and Ji,[15] using the matrix spectral decomposition (matSpD) tool (http://gump.qimr.edu.au/general/daleN/matSpD/). The number of independent tests was estimated to be 85, yielding a corrected significance threshold of 0.05/85=0.00059.

Results

Baseline characteristics and NMR measures
Baseline characteristics of all patients included in this study are listed in Table 2. Of 394 participants, 195 received pravastatin and 199 placebo during the 3-month treatment period. A summary of all 231 lipoprotein and metabolite measures can be found in Supplemental Table 1. NMR and available clinical chemistry measures showed strong correlations for baseline and post-treatment measurements (Supplemental Table 2), indicating consistency between different analytical methods. Heat maps of correlations between NMR measures are displayed in Supplemental Figure 1, revealing substantial correlation within lipoprotein subclasses, between amino acids and between fatty acids.

Statin effects
We compared longitudinal changes of NMR measures between the pravastatin group and controls, using linear regression. To facilitate comparison between different measures, differences between pre- and post-treatment values were scaled to baseline SD units. After the 3-month treatment period, a total of 150 NMR measures were significantly altered ($p<0.00059$) between the pravastatin group and the control group. Absolute concentration changes are given for all lipoprotein and metabolite measures in Supplemental Table 3. Additional sensitivity analysis adjusted for sex provided similar findings, suggesting that our results were not confounded by the imbalance in

Table 2: Average particle size of lipoprotein subclasses

Variable	Placebo (n=199)	Pravastatin (n=195)
Age (years)	50.6±11.1	51.5±11.5
Male	121 (60.8)	141 (72.3)
BMI (kg/m2)	26.5±4.5	26.3±4.1
Current smoker	79 (39.7)	82 (42.1)
SBP (mm Hg)	130.6±17.3	131.6±18.3
DBP (mm Hg)	75.8±9.9	76.6±9.4
Cholesterol (mmol/l)	5.9±1.0	5.9±1.1
HDL (mmol/l)	1.0±0.3	1.0±0.3
LDL (mmol/l)	4.1±0.9	4.2±1.0
Triglycerides (mmol/l)	1.3 (0.9, 1.9)	1.4 (0.9, 1.9)
Glucose (mmol/l)	4.9 (4.5, 5.3)	4.9 (4.5, 5.3)
Creatinine (µmol/l)	84.0±15.1	86.4±13.2
Medication use		
Beta-blockers	4 (2.0)	0 (0.0)
Nitrate	2 (1.0)	0 (0.0)
Diuretics	4 (2.0)	0 (0.0)
Calcium channel blockers	0 (0.0)	1 (0.5)
Digoxin	1 (0.5)	2 (1.0)

Baseline characteristics. Discrete variables are expressed as absolute count (%) and continuous variables as mean±SD or median (interquartile range). Lipids, glucose and creatinine as measured by clinical chemistry. SD: standard deviation; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; SD: standard deviation.

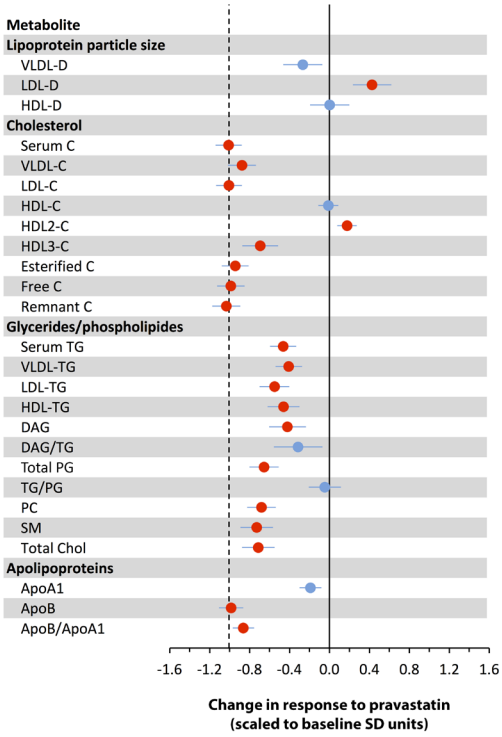
sex ratio between the pravastatin group and the control group (Supplemental Table 4). As compared with placebo, pravastatin reduced levels of conventional lipid measures (Figure 1), including total serum cholesterol (change associated with pravastatin in SD units: -1.01 [95% CI: -1.14, -0.88]; $p=7.3x10^{-41}$), LDL-C (change in SD units: -1.01 [-1.13, -0.88]; $p=6.7x10^{-42}$) and total serum triglycerides (change in SD units: -0.46 [-0.60, -0.33]; $p=1.8x10^{-11}$), whereas HDL-C levels were not affected by statin treatment (change in SD units: -0.01 [-0.11, 0.09]; p=0.829). However, pravastatin significantly increased cholesterol in large lipid-rich HDL2 particles (change in SD units: 0.18 [0.08, 0.27]; p=0.00048) and decreased cholesterol in small less dense HDL3 particles (change in SD units: -0.69 [-0.87, -0.51]; $p=3.1x10^{-13}$).

Moreover, pravastatin treatment markedly lowered remnant cholesterol levels (change in SD units: -1.03 [-1.17, -0.89]; $p=2.0x10^{-38}$), which reflects the total cholesterol content in very large-density lipoprotein (VLDL; change in SD units: -0.88 [-1.02, -0.74]; $p=2.1x10^{-29}$) and intermediate-density lipoprotein (IDL; change in SD units: 1.03 [-1.16, -0.89]; $p=1.3x10^{-39}$). The effect of pravastatin on apolipoprotein B (apoB; change in SD units: -0.98 [-1.11, -0.86]; $p=1.1x10^{-44}$) was comparable to the change in LDL-C. Pravastatin globally lowered levels of VLDL, LDL and IDL subclasses (Figure

2), whereas changes in HDL subclasses were less consistent, with significant increases across large HDL subclasses measures and a reduction in small and very large HDL-C.

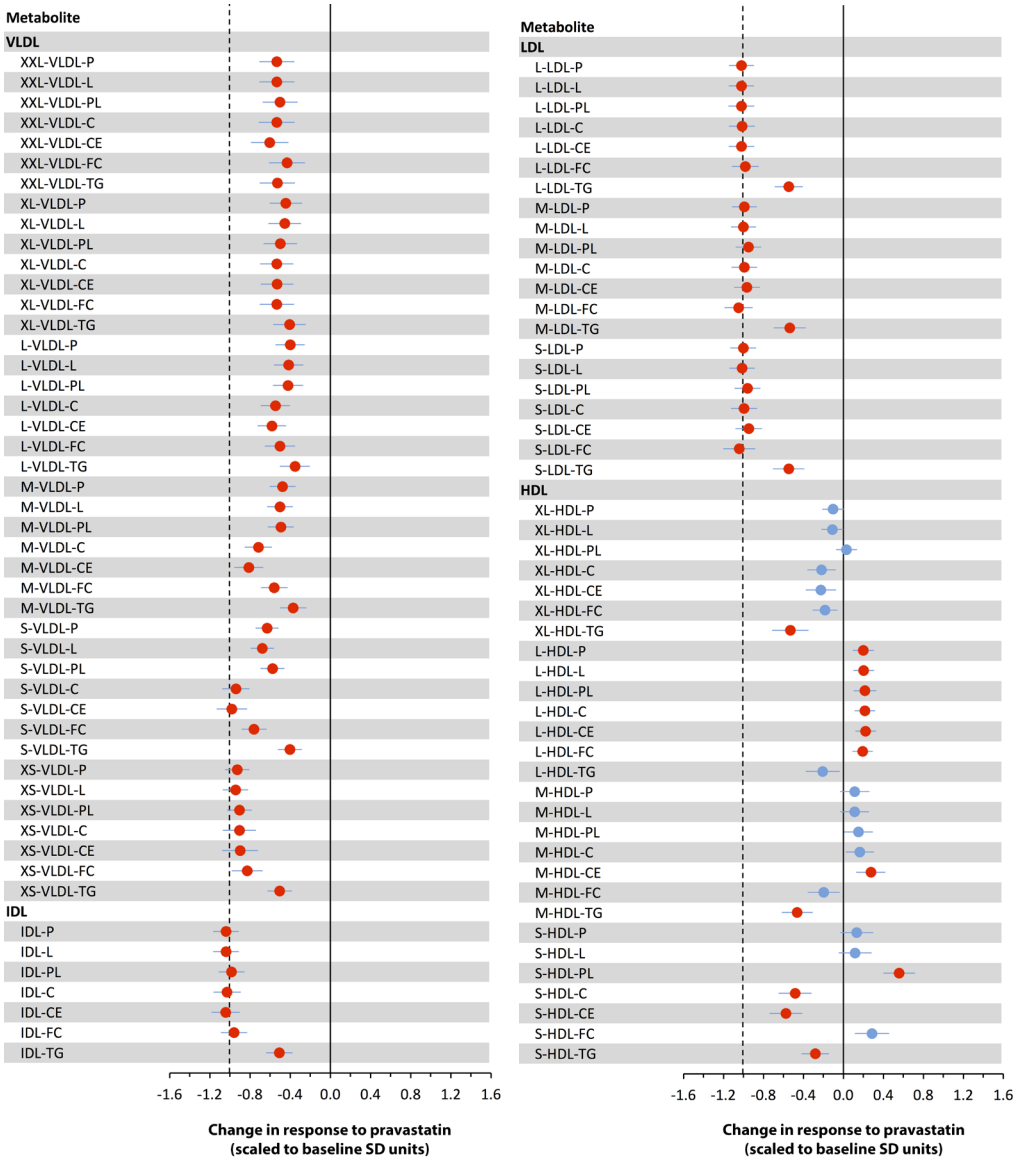
Particle concentrations of all VLDL, IDL and LDL subclasses decreased in response to statin treatment. IDL was the subclass with the greatest change in particle concentration (change in SD units: -1.04 [-1.17, -0.91]; $p=7.6x10^{-45}$). In addition, we analyzed the lipid composition of different lipoprotein subclasses, expressed as the ratio of individual lipid concentrations to the total lipid concentration (Figure 3). Pravastatin treatment markedly lowered the cholesterol and cholesteryl ester to total lipids ratio in IDL and across all LDL subclasses, concomitant with an elevated relative content of free cholesterol and phospholipids in LDL. Furthermore, pravastatin selectively reduced cholesterol ratios in small and medium VLDL particles.

Figure 1: Lipids and lipid-related NMR measures



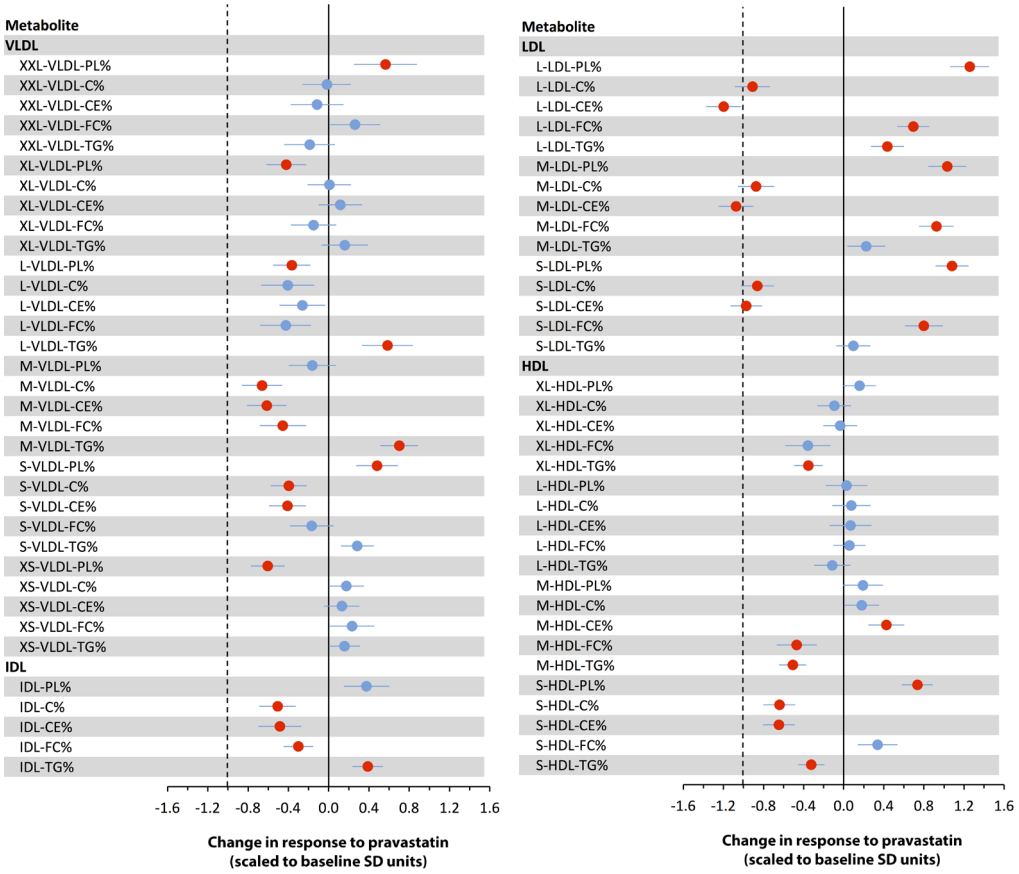
Concentration changes in lipids and lipid-related measures associated with pravastatin treatment (n=195) compared with placebo treatment (n=199). Effect estimates indicate changes over the treatment period (3 months) associated with pravastatin treatment in baseline SD-units. Error bars represent 95% confidence intervals. The dotted line shows the effect estimate for LDL-C. Red marks indicate significant changes ($p<0.00059$). HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; D: diameter; C: cholesterol; TG: triglycerides; DAG: diacylglycerol; PG: phosphoglycerides; PC: phosphatidylcholine; SM: sphingomyelins; Total Chol: total cholines; apoA1: apolipoprotein A1; apoB: apolipoprotein B.

Figure 2: Lipid concentrations in different liporotein suclasses



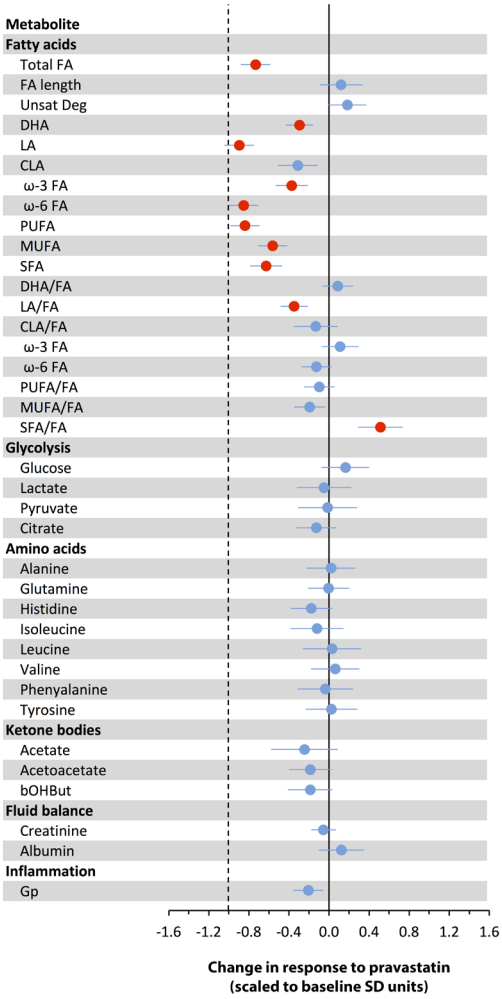
Changes in lipid concentrations across lipoprotein subclasses associated with pravastatin treatment (n=195) compared with placebo treatment (n=199). Effect estimates indicate changes over the treatment period (3 months) associated with pravastatin treatment in SD-units. Error bars represent 95% confidence intervals. The dotted line shows the effect estimate for LDL-C. Red marks indicate significant changes ($p<0.00059$). XXL: extremely large; XL: very large; L: large; M: medium; S: small; XS: very small; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; P: particle concentration; L: total lipids; PL: phospholipids; C: cholesterol; CE: cholesteryl esters; FC: free cholesterol; TG: triglycerides

Figure 3: Lipid composition of lipoprotein subclasses



Changes in lipid composition of lipoprotein subclasses associated with pravastatin treatment (n=195) compared with placebo treatment (n=199). Effect estimates indicate changes over the treatment period (3 months) associated with pravastatin treatment in baseline SD-units. Error bars represent 95% confidence intervals. The dotted line shows the effect estimate for LDL-C. Red marks indicate significant changes (p<0.00059). %: lipid concentration relative to total lipd concentration; XXL: extremely large; XL: very large; L: large; M: medium; S: small; XS: very small; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; P: particle concentration; L: total lipids; PL: phospholipids; C: cholesterol; CE: cholesteryl esters; FC: free cholesterol; TG: triglycerides.

Figure 4: Fatty acids, amino acids and other metabolites



Concentration changes in fatty acids, amino acids and other metabolites associated with pravastatin treatment (n=195) compared with placebo treatment (n=199). Effect estimates indicate changes over the treatment period (3 months) associated with pravastatin treatment in baseline SD-units. Error bars represent 95% confidence intervals. The dotted line shows the effect estimate for LDL-C. Red marks indicate significant changes ($p<0.00059$). FA: fatty acids; Unsat Deg: degree of unsaturation; DHA: docosahexaenoic acid; LA: linoleic acid; CLA: conjugated linoleic acid; ω-3 FA: omega-3 fatty acids; ω-6 FA: omega-6 fatty acids; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids; bOHbut: 3-hydroxybutyrate; Gp: glycoprotein acetyls.

In parallel with cholesterol and triglycerides, pravastatin lowered fatty acid concentrations (Figure 4), particularly ω-6 fatty acids (change in SD units: -0.85 [-1.00, -0.71]; $p=3.5\times10^{-26}$), total polyunsaturated fatty acids (PUFA, change in SD units: -0.84 [-0.98, -0.69]; $p=3.4\times10^{-26}$). By contrast, pravastatin treatment only altered the saturated fatty acid to total fatty acid ratio (SFA/FA; change in SD units: 0.51 [0.29, 0.74]; $p=9.4\times10^{-6}$) and the linoleic acid to total fatty acid ratio (LA/FA; change in SD units: -0.35 [-0.48, -0.21]; $p=7.2\times10^{-7}$), but produced no changes in other fatty acid ratios. Glycolysis-related metabolites, amino acids and other metabolites remained unchanged.

We next evaluated the effect of pravastatin on correlations between different NMR measures. Results are illustrated in a correlation difference map that provides a post-

treatment comparison between the pravastatin group and controls (Supplemental Figure 2). Pravastatin induced negative associations between the relative cholesterol content of medium HDL and cholesterol levels in small VLDL, IDL and LDL. We observed similar, but weaker effects for absolute cholesterol concentrations in medium HDL. Conversely, pravastatin strengthened or induced positive correlations between the phospholipid to total lipids ratio in medium HDL and lipid concentrations in other lipoproteins. Furthermore, correlations between absolute lipid concentrations and the relative lipid content were altered across VLDL subclasses. Finally, lactate and pyruvate showed weaker associations with lipid concentrations in VLDL following pravastatin treatment.

Discussion

This is the first placebo-controlled NMR-based study to assess metabolic changes associated with statin treatment, using data from the PREVEND IT trial. Our study adds to previous findings from observational NMR studies and additionally explored statin-induced changes in over 160 novel measures of lipid concentrations and lipid composition for 14 lipoprotein subclasses. Besides the well-known effects on LDL-C, statins altered a wide range of lipids and concentrations of fatty acids. These findings are supported by observational studies comparing statin users to non-users,[9,16] and fit with previous clinical trial data on fatty acids.[17] By contrast, pravastatin treatment only altered LA/FA and SFA/FA, but had no effect on other fatty acid ratios. In addition, pravastatin globally lowered levels of lipoprotein subclasses, except for HDL concentrations, which displayed a more intricate response pattern. Detailed lipid profiling revealed that the substantial lowering of VLDL-C, IDL-C and LDL-C was paralleled by more selective changes in lipid composition of different lipoprotein particles. Finally amino acids and other metabolites were not affected by statin treatment.

Statins not only act on LDL, but also on other apoB-rich lipoproteins. In our study, pravastatin reduced apoB and LDL-C with similar effect magnitudes. ApoB has been proposed as a more robust cardiovascular risk marker than LDL-C, supporting the use of apoB as an alternative treatment target for statin therapy.[18,19] Consistent with previous findings,[9,20] statin treatment substantially lowered cholesterol in apoB-containing, triglyceride-rich remnant particles, including IDL and VLDL. Since VLDL is the main carrier of triglycerides, remnant cholesterol is strongly associated with triglyceride levels. Although triglycerides are well-established markers of cardiovascular risk, their relationship with atherogenesis is not straightforward.[21] By contrast, remnant cholesterol is likely to play a causal role in cardiovascular disease risk.[22,23] In line with this, remnant cholesterol is associated with both ischemic heart disease and low-grade inflammation.[24] Compared with VLDL-C and IDL-C, HDL-C showed a more complex response to statin treatment, with cholesterol depletion of small HDL3 particles and slight cholesterol enrichment of larger HDL2 particles. Prospective cohort studies have consistently reported inverse associations between HDL-C levels and risk of cardiovascular disease,[25] whereas findings from recent

Mendelian randomization studies[26,27] and the failure of HDL-raising drugs to improve cardiovascular outcomes[28] may argue against a causal role for HDL-C in cardiovascular disease per se. Findings from experimental studies suggest that HDL3 and HDL2 differ in their cardioprotective capacities.[29] However, the relationship between different HDL subclasses and cardiovascular risk remains a matter of debate as results from observational studies are inconclusive.[30]

It has been suggested that small dense LDL particles are more atherogenic than larger LDL species as they are readily taken up by the arterial wall, are cleared from circulation at reduced rates due to their low affinity for LDL receptors and are more susceptible to oxidation, promoting the formation of atherosclerotic plaques.[10,12] This is supported by large cohort studies, demonstrating that concentrations of small rather than large LDL particles are associated with future cardiovascular risk after adjustment for non-lipid risk factors.[31-33] However, effect estimates for small LDL particles are not superior to total LDL concentrations and do not improve risk prediction beyond routine lipid measures.[31] Moreover, a systematic review of NMR-based studies found no association of LDL subclasses with cardiovascular disease after adjustment for other lipid measurements.[34] Experimental findings suggest that, similar to LDL particles, the atherogenic capacity of VLDL may depend on particle size as large VLDL subpopulations are unable to enter the arterial wall and are thus less likely to contribute to the formation of atherosclerotic plaques.[35] However, there is little evidence from clinical studies that smaller and larger VLDL particles differ in their atherogenic potential. While different lipoprotein subclasses may play distinct roles in the pathophysiology of cardiovascular disease, pravastatin treatment lowered lipoprotein particle concentrations and lipid concentrations across VLDL, IDL and LDL subclasses, which may be an indirect consequence of enhanced clearance and/or reduced synthesis of these lipoproteins.

While the cholesterol to total lipids ratio was decreased in IDL and all LDL subpopulations, pravastatin selectively reduced the cholesterol content of small and medium VLDL, raising the possibility that statins specifically target potentially atherogenic VLDL subpopulations.[35] At the same time, pravastatin lowered the triglyceride to total lipids ratio across all HDL subpopulations, but increased the triglyceride content of several VLDL and LDL subclasses as well as IDL. These changes in lipid composition may be attributable to statin effects on the reverse cholesterol transport pathway, in which cholesteryl ester transfer protein (CETP) transfers cholesteryl esters from HDL to triglyceride-rich, apoB-containing lipoproteins (LDL, IDL and VLDL) in exchange for triglycerides.[36] The statin-induced decrease in lipoprotein concentrations is associated with reduced CETP activity, resulting in cholesterol enrichment of HDL and cholesterol depletion of apoB containing lipoproteins.[37-39] Consistent with reduced CETP activity, pravastatin induced negative correlations between the cholesterol content of medium HDL and cholesterol levels in non-HDL particles. Correlation coefficients for other HDL subpopulations, however, were only moderately altered after pravastatin treatment.

The relative reduction in LDL cholesterol was associated with no or only minor changes in the triglyceride content of LDL particles. By contrast, there was triglyceride

enrichment of IDL as well as medium and large VLDL particles. Statin-induced lowering CETP activity may also hamper TG transfer from VLDL and IDL to LDL,[40] which would explain the increased relative triglyceride content of IDL and VLDL. Besides lowering the cholesterol content of IDL and LDL, pravastatin treatment led to a relative increase in phospholipids and free cholesterol, which may result from reduced enzymatic cholesterol esterification due to blocked cholesterol synthesis.[41] Taken together, detailed analysis of lipoprotein subclasses revealed selective changes in lipid composition, whereas lipid concentrations were reduced across all VLDL, IDL and LDL subclasses, following pravastatin treatment.

Several studies have shown that besides lipid lowering, statins alter fatty acid levels.[9,16,17] Since the vast majority of circulating fatty acids are bound in triglycerides, cholesteryl esters and phospholipids,[17] the reduction in fatty acid levels associated may result from the statin-induced decrease in lipoproteins providing the main source of circulating lipids. Alternatively, statins may interfere with fatty acid metabolism through different molecular pathways. Simvastatin treatment increases metabolic indices indicating elevated activity of elongases and desaturases,[17] two enzymes that catalyze the formation of highly unsaturated long-chain fatty acids. Moreover, statin treatment may stimulate hepatic uptake and beta-oxidation of fatty acids by enhancing expression of peroxisome proliferator-activated receptors (PPARs).[42] We observed elevated SFA/FA and reduced LA/FA, but no effects on other fatty acid ratios, which more appropriately reflect fatty acid metabolism than fatty acid concentrations given the lipoprotein-lowering effect of statins. By contrast, a recent observational study reported stronger effects on docosahexaenoic acid (DHA)/FA, whereas SFA/FA was unchanged after statin treatment.[9] In this study, however, information on statin type and dosage was not available. Consistent with our findings, data from a clinical trial suggest that simvastatin does not enhance DHA/FA.[17] Interestingly, studies comparing different statins reported that pravastatin, in contrast to other statins, did not influence selected fatty acid ratios, indicating that changes in fatty acid metabolisms depend on the statin type.[43,44] While the decrease in LA/FA is supported by other studies,[9,17] the underlying metabolic processes remain unclear. Statins increase lecithin:cholesterol acyltransferase (LCAT) activity, which synthesizes cholesteryl esters from cholesterol and fatty acids.[45] Since LA is the preferential substrate of LCAT, elevated LCAT activity would be consistent with higher LA/FA. Collectively, changes in absolute fatty acid levels are mainly driven by statin-induced lipid lowering, whereas statin effects on fatty acid metabolism remain uncertain and may differ between statins.

Pyruvate and lactate showed weaker correlations with VLDL-related measures after pravastatin treatment, whereas absolute concentrations of these two metabolites remained unchanged. In addition to producing lactate, pyruvate is involved in glucose and fatty acid metabolism by forming acetyl-coenzyme A, which is involved in fatty acid synthesis.[46] Fatty acids, in turn, are joined with glycerol to form triglycerides, the main component of VLDL. Pyruvate and lactate as a metabolic product of pyruvate are thus associated with enhanced hepatic VLDL synthesis and consequently should show a positive correlation with serum VLDL levels. This is in line with the correlation patterns of pyruvate and lactate in the placebo group (Supplemental Figure 2B).

Statins, however, facilitate hepatic uptake of non-HDL particles, including VLDL, by increasing LDL receptor activity.[1] The resulting decrease in VLDL levels coupled with unchanged pyruvate and lactate levels is consistent with weaker correlations in the pravastatin group (Supplemental Figure 2A).

Our study was powered to detect a large number of significant changes in lipoprotein and metabolite measures after pravastatin treatment, underscoring the strengths of a placebo controlled randomized setting with pre/post treatment comparisons, which limits potential sources of confounding to a minimum. We report associations for 231 NMR measures, including over 160 novel measures of lipid concentrations and lipid composition for different lipoprotein subclasses. No other study has assessed the effect of statins on lipoprotein subclasses in such detail. However, further research is warranted to confirm our findings on lipoprotein subclasses as we did not replicate our results in an independent study. In comparison with a recent observational study that used the same NMR metabolomics platform,[9] we observed more moderate effects of statin treatment on several lipid measures, including LDL-C, apoB and apoA1. Würtz et al. compared statin users, who commenced statin treatment, to non-users. While information on statin type and dosage was not available for this study, all statin users had an indication for statin therapy, such as hypercholesterolemia, suggesting that many of them underwent aggressive treatment. In our study, however, participants were randomly assigned to a moderate dose of a relatively week statin,[47] which may account for the lower effect estimates.

In conclusion, metabolic profiling in a randomized clinical trial revealed causal associations of statin treatment with globally reduced lipid levels across lipoprotein subclasses, accompanied by more selective changes in the lipid composition of lipoproteins. Additionally, pravastatin treatment lowered fatty acid concentrations, but had limited effects on fatty acid ratios. In line with previous findings,[9] statin treatment did not alter concentrations of non-lipid measures, such as amino acids and glycolysis-related metabolites, suggesting that these metabolites do not reflect pleiotropic statin effects. Our findings demonstrate that high-throughput metabolic profiling is emerging as a powerful tool to dissect a drug's metabolic footprint, providing important information that may be used to improve current treatments.

Supplemental materials will be available online soon.

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Chapter 8

Summary and discussion

This chapter summarizes the findings detailed in the previous chapters and discusses the potential of biomarkers in clinical risk stratification and drug discovery, followed by an outlook on emerging developments in omics research.

Summary

Chapters 2, **3** and **4** present findings from biomarker studies in patients with established atherosclerotic disease. In **Chapter 2**, we identified common molecular pathways for childhood obesity and atherosclerotic disease complexity in adults. Childhood obesity may promote the development of cardiovascular disease by predisposing individuals to cardiovascular risk factors in later life.[1] Moreover, both obesity and atherosclerosis are characterized by inflammatory processes associated with increased monocytosis and activation of inflammatory monocytes.[2,3] Thus, monocytes may provide a pathophysiological link between both diseases. We compared monocyte gene expression in obese children to lean controls and subsequently studied the association of differentially expressed genes with complexity of coronary atherosclerosis in adults (SYNTAX score). We observed downregulation of IMPDH2 and TMEM134 expression both in obese children and obese adults. Downregulation of TMEM134 was also related to a higher SYNTAX score. Obese children display alterations in monocyte gene expression associated with atherosclerotic burden in adulthood.

In **Chapter 3**, we investigated whether loss of chromosome Y was related to atherosclerotic plaque characteristics and clinical outcome in patients with carotid atherosclerosis. Recent evidence indicates that loss of chromosome Y is associated with smoking[4] and predicts cancer and all-cause mortality[5]. Genetic variation in chromosome Y is associated with altered gene expression in immune cells[6] and may contribute to the risk of coronary artery disease via immune-regulatory pathways,[7] suggesting that loss of chromosome Y may be related to severity of atherosclerosis and clinical outcome after carotid endarterectomy. Loss of chromosome Y was associated with larger atheroma size and independently predicted major cardiovascular events, but was not related to plaque phenotype and inflammation. Loss of chromosome Y may contribute to adverse cardiovascular events through mechanisms other than inflammation.

While traditional cardiovascular risk factors, such as smoking, hypertension, diabetes, obesity and hyperlipidemia, are important predictors of cardiovascular events in the general population,[8-10] their ability to predict secondary manifestations of atherosclerotic disease is limited.[11-13] Several biomarkers have been associated with prognosis of vascular disease, including routinely measured hematological parameters.[14] However, it is unknown whether hematological parameters improve prediction of recurrent vascular events beyond established assessment tools for secondary prevention, such as the SMART score. As detailed in **Chapter 4**, we found four parameters that predicted recurrent events independently of SMART score variables. Of these, lymphocyte % improved both reclassification and discrimination compared to the SMART risk score. The remaining chapters report findings from nuclear magnetic resonance (NMR)-

based metabolomics studies. To identify further biomarkers for risk stratification in secondary prevention, we performed metabolic profiling in two European cohorts that enrolled patients with angiographically documented coronary artery disease (**Chapter 5**). Triglyceride in IDL and LDL, phenylalanine, creatinine and the concentration of small HDL particles were associated with subsequent cardiovascular events. The combination of LDL triglyceride, phenylalanine and small HDL particles improved prediction of clinical outcome compared to a clinical risk prediction model and conventional cardiovascular risk factors.

Left ventricular ejection fraction (LVEF) and infarct size are predictors of survival after myocardial infarction (MI). Metformin, which is commonly used in the treatment of diabetes, has emerged as a candidate drug to preserve LVEF and reduce infarct size following MI.[15] However, recent clinical trial data indicate that metformin does not improve LVEF in MI patients compared to placebo treatment.[16] **Chapter 6** presents a metabolic profiling study in the GIPS-III clinical that was designed to investigate the effect of metformin therapy on LVEF in non-diabetic ST-elevated MI patients. We measured metabolic profiles at different time points to study the systemic effects of metformin treatment and to establish prognostic biomarkers for the prediction of LVEF and infarct size 4 months post-MI. HDL triglyceride measured 24 h post-MI predicted LVEF and infarct size 4 months post-MI. Additionally triglyceride measures in different HDL subclasses were associated with infarct size. After the 4-month treatment period, the metformin group showed increased alanine levels and a reduced phospholipid content of very large HDL particles. Taken together, this study identified potential earlier biomarkers of ventricular dysfunction in MI patients and provided a detailed characterization of the metabolic effects of metformin.

Statins are thought to primarily reduce cardiovascular risk through their LDL cholesterol lowering action. However, statins act early in the mevalonate pathway, raising the possibility that statins exhibit cholesterol-independent effects. Furthermore, there is growing evidence that different LDL and HDL lipoprotein subclasses may vary in their contribution to cardiovascular risk.[17-19] However, little is known about statin effects on lipoprotein subclass profiles. In addition to previously quantified lipoprotein and metabolite measures,[20] we studied the longitudinal effect of pravastatin treatment on over 160 novel measures of lipid concentrations and lipid composition for 14 lipoprotein subclasses in the PREVEND IT clinical trial (**Chapter 7**). Besides cholesterol measures, pravastatin extensively altered lipids measures and absolute concentrations of fatty acids, whereas most fatty acid ratios were not affected by statin treatment. Moreover, pravastatin globally lowered concentrations of lipoprotein subclasses, with the exception of HDL. The lipid lowering effect of pravastatin was accompanied by more selective alteration in the lipid composition of different lipoprotein subclasses. Amino acids and glycolysis-related metabolites remained unchanged. Thus, pravastatin exhibits widespread effects on lipid concentrations and fatty acids, along with more selective changes in lipoprotein composition.

Discussion

Reliable identification of individuals at risk and detailed understanding of molecular disease mechanisms are crucial for prevention, prognosis and treatment of cardiovascular disease (CVD). In this thesis, we studied cardiovascular biomarkers in different clinical settings and evaluated their ability to improve risk stratification in secondary prevention. Moreover, we used high-throughput metabolic profiling to study the molecular signature of different drugs.

Biomarkers in risk assessment
Whether or not the biomarkers described in this thesis will be useful in clinical practice has to be determined by further research. In recent year, numerous potential cardiovascular biomarkers have emerged, but only few of them have been sufficiently validated to support their clinical application. Several criteria have been proposed to evaluate the clinical utility of a candidate biomarker: (1) Methods for biomarker analysis must provide accurate and reliable measurements and high-throughput at reasonable cost. (2) Novel biomarkers must be consistently associated with disease and disease outcome across studies and add information to established clinical assessment tools. (3) The routine use of a biomarker should improve clinical management of patients.[21]

High-throughput omics technologies allow inexpensive measurement of candidate biomarkers in large quantities, using standardized assays to ensure reproducibility. In **Chapter 5**, **6** and **7**, we used a NMR-based metabolomics platform that has been widely applied in epidemiological studies and drug research with a total of over 400,000 profiled samples. This platform has been validated against routine clinical chemistry assays[22] and enables reliable quantification of a wide range of lipoprotein and metabolite measures (coefficients of variation published in [23]). In **Chapter 4**, we tested the ability of hematological parameters to predict recurrent vascular events. These parameters are routinely measured by most hematology analyzers approved for clinical use and do not rely on expensive equipment for analysis.

The reproducibility of measurements also depends on the variability and temporal resolution of a biomarker. Even though the genome undergoes structural changes over time (see **Chapter 3**), it is considered relatively stable, reflecting the heritable components of disease susceptibility. By contrast, many circulating biomarkers, such as lipoproteins and metabolites, are highly sensitive to environmental influences and pathophysiological changes. On the one hand, this creates a particular need for standardization as biomarker concentrations may be influenced by sampling conditions, including nutritional state of patients, drug use, storage conditions and storage duration. On the other hand, such biomarkers offer the possibility of studying drug effects and disease progression over time. In cardiovascular prevention, the detailed analysis of temporal fluctuation patterns may help define windows of vulnerability in which individuals are at elevated short-term risk for cardiovascular events and require immediate treatment.[24] While the studies described in **Chapters 4** and **5** used single measurements, in **Chapter 6**, we analyzed metabolic profiles at multiple time points to study the effects of metformin and to identify biomarkers

of left ventricular dysfunction after MI. Interestingly, only 24 h measurements were significantly associated with outcome, underscoring the added value of repeated sampling. More studies, such as BIOMArCS[24], are required to follow biomarker patterns over the course of CVD progression, using multiple sampling time points. Finally, comparison of biomarker dynamics across different age and disease groups may improve our understanding of how pathophysiological changes in childhood contribute to cardiovascular risk in later life (**Chapter 1**).

Our findings need to be confirmed by larger studies to evaluate the clinical potential of the novel biomarkers described in this thesis. Biomarker development will greatly benefit from biobanks, such as UK Biobank[25], that allow fast replication in very large samples and enhance reproducibility through data sharing and open access. The diagnostic and prognostic utility of a biomarker also depends on the added value it provides compared to established clinical tools. In **Chapter 4**, we assessed the incremental value of hematological parameters compared to the SMART risk score. However, clinical studies often do not collect all the information required for the use of existing tools. In **Chapter 5**, we therefore compared a combination of biomarkers to traditional cardiovascular risk factors and other disease-related variables rather than commonly used risk prediction models. To enhance biomarker development, clinical studies should put more emphasis on the collection of information used by established tools for diagnosis and prognosis. Moreover, existing tools should be complemented with validated candidate biomarkers to improve clinical care.

From biological pathways to drug discovery
Besides their potential in clinical risk assessment, omics biomarkers yield insights into disease mechanisms, which may help guide preclinical drug discovery. In this thesis, we identified different biomarkers of cardiovascular disease progression that could serve as therapeutic targets in the future. However, our observational findings cannot establish a causal link between biomarkers and cardiovascular outcome, raising the possibility of reverse causation. Additionally, biases and confounding may have affected our results. The causal role of biomarkers in cardiovascular disease progression can be examined by conducting a Mendelian randomization analysis, which uses genetic variants as proxies for modifiable exposures (e.g. biomarkers) that are less susceptible to confounding.[26] Future research could use this technique to assess the potential of our findings for drug discovery.

Omics profiling has not only emerged as an effective tool to study disease mechanisms, but can also provide a detailed characterization of molecular drug effects (**Chapter 6** and **7**). This information can be used in combination with data from epidemiological omics studies to optimize and complement established treatments. In **Chapter 5**, we identified several lipoprotein and metabolite measures associated with subsequent cardiovascular events in CAD patients. However, established cardiovascular drugs, such as statins, do not target all of these biomarkers (**Chapter 7**). Thus, secondary prevention therapies could be specifically developed to act on pathophysiological pathways that are not affected by current cardiovascular treatments.

Future directions

The growing availability of large-scale clinical databases offers the opportunity to develop personalized algorithms for clinical decision making and to identify disease subgroups for drug development based on clinical and biomarker profiles, sparking international initiatives to combine data resources. For example, the BigData@Heart consortium joins medical and omics data on over 25 million healthy individuals and cardiovascular patients across Europe.[27] In cardiovascular medicine, current therapeutic approaches mainly focus on treating symptoms rather underlying molecular mechanisms and, as a result, apply the same set of treatments to etiologically heterogeneous subgroups of patients ("one size fits all" medicine). Consequently, patients often receive inefficacious therapies, leading to poor outcomes and unnecessary health costs. Therefore, BigData@Heart aims to refine phenotype and outcome definitions in acute coronary syndrome, atrial fibrillation and heart failure, using omics biomarkers and big data analytics (e.g. machine learning), to identify patient subgroups with similar molecular characteristics. Such molecular sub-phenotypes can be used to improve diagnosis and risk stratification, identify novel drug targets and to inform treatment selection. Collectively, the combination of omics profiling and Big Data analytics holds the promise of stimulating drug discovery and transforming cardiovascular medicine towards personalized care.

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Appendix

Samenvatting

Hart- en vaatziekten gaan gepaard met hoge kosten voor de gezondheidszorg en maatschappelijke productiviteit. Dit onderstreept de noodzaak van biomarkers die ons helpen onderliggende pathofysiologische mechanismen van hart- en vaatziekten beter te begrijpen en die personen met een hoog cardiovasculair risico kunnen identificeren om ze vroegtijdig te behandelen. Deze patiënten kunnen daardoor mogelijk eerder en beter behandeld worden. Daarnaast kunnen biomarkers worden gebruikt om effecten van geneesmiddelen te bestuderen en bestaande cardiovasculaire therapieën te verbeteren. In **Hoofdstuk 2** van dit proefschrift kijken we naar de associatie van veranderde genexpressie in spiercellen van kinderen met obesitas met vaatziekten bij volwassen cardiovasculaire patiënten. **Hoofdstuk 3** onderzoekt de associatie van verlies van het Y-chromosoom met atherosclerotische plaquekenmerken en klinische uitkomsten bij patiënten met atherosclerose. **In Hoofdstuk 4** testen we of hematologische parameters (gemeten tijdens routinemetingen) kunnen voorspellen welke patiënten een hoger risico hebben op een recidiverende vasculaire aandoeningen. In de overige hoofdstukken bestuderen we metabolietprofielen bij cardiovasculaire patiënten. **Hoofdstuk 5** presenteert bevindingen van een prospectieve studie in patiënten die lijden aan coronaire hartziekten. Hierin hebben we biomarkers geïdentificeerd die beter voorspellen welke patiënten later opnieuw een cardiovasculaire aandoening krijgen. In **Hoofdstuk 6** gebruiken we klinische gegevens om het effect van metformine op metabolietprofielen te onderzoeken. Verder kijken we in dit hoofdstuk naar de associatie van biomarkers met infarctgrootte en verminderde pompfunctie van de linkerhartkamer na een myocardinfarct. In **Hoofdstuk 7** onderzoeken we het effect van statinegebruik op metabolietprofielen, met behulp van gegevens uit een gerandomiseerde klinische studie.

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List of publications

(Shared) first authorships

Eppinga RN, Kofink D, Dullaart RP, Dalmeijer GW, Lipsic E, van Veldhuisen DJ, et al. Effect of metformin on metabolites and relation with myocardial infarct size and left ventricular ejection fraction after myocardial infarction. Circ Cardiovasc Genet. 2017;10:e001564.

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Curriculum vitae

Daniel Kofink was born in Ludwigsburg (Germany). He obtained a Bachelor of Science degree in Psychology from Heidelberg University (Germany). After his undergraduate studies, he went on to study neuroscience at Utrecht University. During his Master's studies, he completed internships at the UMC Utrecht and the University of Cambridge (United Kingdom). After his return to the Netherlands, he completed his thesis on the role of epigenetic modifications in the development of psychiatric disorders. Moreover, he began to work as a research assistant at the Department of Psychiatry (UMC Utrecht). During this time, he became particularly interested in genetics and Big Data analysis. In 2014, he started a PhD in Prof. Folkert Asselbergs' lab at the UMC Utrecht, where he worked on omics biomarkers of cardiovascular disease until 2017. After his graduation, he will continue to collaborate with Prof. Asselbergs within the BigData@Heart Innovative Medicine Initiative, a large-scale international consortium which aims to improve clinical outcomes in patients with atrial fibrillation, acute coronary syndrome and heart failure, using omics biomarkers and big data analytics.