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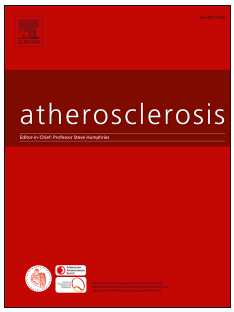


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# *Ldlr-/-* and *ApoE-/-* mice better mimic the human metabolite signature of increased carotid intima media thickness compared to other animal models of cardiovascular disease.

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**Abstract**

*Background and aims:* Preclinical experiments on animal models are essential to understand the mechanisms of cardiovascular disease (CVD). Metabolomics allows access to the metabolic perturbations associated with CVD in heart and vessels. Here we assessed which potential animal CVD model most closely mimics the serum metabolite signature of increased carotid intima-media thickness (cIMT) in humans, a clinical parameter widely accepted as a surrogate of CVD.

*Methods:* A targeted mass spectrometry assay was used to quantify and compare a series of blood metabolites between 1362 individuals (KORA F4 cohort) and 5 animal CVD models: *ApoE-/-*, *Ldlr-/-*, and klotho-hypomorphic mice (*kl/kl*) and SHRSP rats with or without salt feeding. The metabolite signatures were obtained using linear regressions adjusted for various co-variates.

*Results:* In human, increased cIMT [quartile Q4 *vs.* Q1] was associated with 26 metabolites (9 acylcarnitines, 2 lysophosphatidylcholines, 9 phosphatidylcholines and 6 sphingomyelins). Acylcarnitines correlated preferentially with serum glucose and creatinine. Phospholipids correlated preferentially with cholesterol (total and LDL). The human signature correlated positively and significantly with *Ldlr-/-* and *ApoE-/-* mice, while correlation with *kl/kl* mice and SHRP rats was either negative and non-significant. Human and *Ldlr-/-* mice shared 11 significant metabolites displaying the same direction of regulation: 5 phosphatidylcholines, 1 lysophosphatidylcholines, 5 sphingomyelins; *ApoE-/-* mice shared 10.

*Conclusions:* The human cIMT signature was partially mimicked by *Ldlr-/-* and *ApoE-/-* mice. These animal models might help better understand the biochemical and molecular

mechanisms involved in the vessel metabolic perturbations associated with, and contributing to metabolic disorders in CVD.

**Introduction**

Cardiovascular disease (CVD) is the leading cause of death worldwide (1). The major clinical cardiovascular events, myocardial infarction and stroke, are the symptomatic end-stage of a number of distinct pathophysiological processes affecting both the small and large vessels. Early markers are required to develop targeted strategies to prevent pathological complications of CVD. Complex structural and functional changes leading to endothelial dysfunction, medial vascular calcification, atherosclerosis and thrombosis are induced in response to different risk factors, including smoking, dyslipidaemia, diabetes, hypertension and chronic kidney disease (2-4) Assessment of carotid intima media thickness (cIMT) is accepted as a marker of early atherosclerosis and a good predictor of cardiovascular outcomes (5, 6).

Preclinical experiments on animal models are essential to understand the mechanisms of CVD progression and to develop preventive and therapeutic treatment strategies. However, the translational value of an animal model strongly depends on its ability to robustly reproduce important functional, structural, and molecular pathological features of human disease. Various animal models have been developed as means to display different well-known risk factors for accelerated human CVD (3). Among the most frequently used are the *Ldlr-/-* and *ApoE-/-* mice atherosclerotic models fed with high cholesterol diet to mimic human dyslipidaemia (7-9). Similar to patients with advanced chronic kidney disease, klotho hypomorphic mice (*kl/kl*) develop hyperphosphatemia and extensive medial vascular calcifications (10, 11). The stroke-prone spontaneously hypertensive rat (SHRSP), obtained by selective inbreeding of the Wistar–Kyoto (WKY) strain, is a well-characterized CVD model of high blood pressure, a characteristic which can be further increased by salt feeding (7, 9).

All these animal models exhibit artery thickening due to plaque formation (*ApoE-/-* and *Ldlr-/-* mice), medial vascular calcification (*kl/kl* mice) or hypertension (SHRSP and SHRSP-NaCl rats). Which of these animal models best mimics increased human cIMT remains an open question.

Typically, the suitability of animal models to mimic human disease is based on phenotypic similarity (e.g. dyslipidaemia, plaque size and number, blood pressure, etc.) and, at best, a few molecular features (eg increased circulating cytokines (12) or cell adhesion molecules (13)). However, this restricted number of observations only partially describes multifactorial diseases such as CVD.

Numerous metabolic cardiac and vessel perturbations are associated with and contribute to CVD. Metabolomics gives access to the metabolite and lipid compositions of biological fluids or biopsies reflecting the metabolic signature of individual patients and may serve as a valuable diagnostic and prognostic tool to manage CVD (14-16). Metabolomics also represents an opportunity to better understand the biological processes related to development and progression of atherosclerosis.

Here we assessed which animal model most closely mimics human CVD by using a metabolomics approach. For that we analysed the blood metabolome similarities that exist between human CVD and 5 recognized animal models of vascular lesions (*ApoE-/-*, *Ldlr-/-*, *kl/kl* mice and SHRSP rats with or without salt feeding).

**Materials and methods.**

***Cohort study design.*** The KORA study (Cooperative health research in the Region of Augsburg) consists of independent population-based samples from the general population

living in the region of Augsburg, Southern Germany. The study has been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent has been given by each participant. The informed consents given by KORA study participants does not cover data posting in public databases. However, data are available upon request from KORA gen (https://epi.helmholtz-muenchen.de/). Data requests can be submitted online and are subject to approval by the KORA Board. The study was reviewed and approved by the local ethics committee (Bayerische Landesärztekammer). The S4 survey (examination 1999-2001) consisted of standardized interviews, physical examinations and blood sampling. Only individuals between the ages of 25 and 74 were selected. For the current study, participants of the KORA F4 cohort (examination 2006-2008), a seven-year follow-up study of the KORA S4 cohort, were used (17).

Carotid intima media thickness (cIMT), used here as a surrogate for CVD, and the anthropometric and biological parameters of the participants were measured as previously described (17, 18) . An average of the left and right cIMT was used as the variable of interest in all human models. Valid cIMT data were obtained from 2714 individuals (cIMT group). A drug-free cIMT group of 1814 was isolated from the cIMT group after exclusion of the individuals treated with statins, fibrates, beta-blockers, ACE inhibitors, nitrates, lipid-lowering drugs or anti-hypertensives. The bottom (Q1) and the top (Q4) cIMT quartiles contained 687 and 675 individuals in cIMT group, and 611 and 273 individuals in the drug-free cIMT group. Sample characteristics of the individuals of the bottom (Q1) and the top (Q4) cIMT quartiles can be found in **Table 1**.

Lipid levels were determined in fasting fresh blood samples at most six hours after collection. In KORA F4 total cholesterol was measured using the cholesterol-esterase method (CHOL Flex, Dade-Behring, Germany). HDL and triglyceride levels were determined

using the TGL Flex and AHDL Flex methods (Dade-Behring, Germany), respectively, and LDL was measured by a direct method (ALDL, Dade-Behring, Germany). In KORA F4 the intra-assay coefficient of variation (CV) for repeated measurements was 1.85% (total cholesterol), 2.75% (triglycerides), 3.25% (HDL-C) and 2.7% (LDL-C).

***Animal models of CVD.*** *ApoE-/-* (n=23) and *Ldlr-/-* (n=22) and their wild-type controls (C57Bl6J; n=20) mice were purchased from Charles River at the age of 5 weeks. *ApoE-/-* and *Ldlr-/-* mice were fed a high cholesterol diet (1.2% of cholesterol, 6% fat and 2% sucrose (Ssniff, Soest, Germany) diet: TD.96335 mod. – 1.25 % cholesterol) from the age of 8 weeks to sacrifice (22 weeks). C57Bl6J mice were fed a regular chow all along the protocol. Blood samples were collected and stored at -80°C. The project was approved by the local (Inserm/UPS US006 CREFRE) and national ethics committees under the number 02604.02. *ApoE-/-* and *Ldlr-/-* mice exhibited dyslipidaemia (increased total cholesterol, LDL-cholesterol and triglycerides) when compared to controls, as previously described (7, 9)**.**

*Kl/kl* mice (n=10) and their corresponding wild-type mice (129Sv) (n=10) were described previously (19). Mice were fed a standard rodent chow and drinking water ad libitum. Mice were sacrificed at 7 weeks of age, and blood was collected and stored at -80°C. All animal experiments were conducted according to the recommendations of the Guide for Care and Use of Laboratory Animals of the National Institutes of Health as well as the German law for the welfare of animals, and reviewed and approved by the local government authority.

Inbred colonies of stroke-prone spontaneously hypertensive rats (SHRSP; n=28) and normo-tensive Wistar Kyoto rats (WKY; n=13) have been maintained at the University of Glasgow since 1991, as previously described (20, 21). Animals were housed under controlled conditions, fed standard rat chow (rat and mouse No. 1 maintenance diet, Special Diet

Services) and water ad libitum. At 18 weeks of age, SHRSP rats were administered 1% NaCl in their drinking water (SHRSP-NaCl; n=14) or provided with regular water (SHRSP; n=14) for three weeks. WYK rats (n=13) were maintained under regular water. Blood samples were collected at sacrifice (21 weeks) and stored at -80°C. All animal procedures were approved by the Home Office according to the Animals (Scientific Procedures) Act 1986, under Project Licence 7009021. SHRSP and SHRSP-NaCl rats showed increased systolic blood pressure and cardiac hypertrophy when compared to their control WKY rats as previously described (20, 21).

***Metabolite quantification in human and animal.*** A series of metabolites was quantified (µM) in serum samples from individuals of the KORA F4 cohort and in plasma from the animal models using a targeted quantitative and quality controlled metabolomics approach based on electrospray ionization tandem mass spectrometry with the Absolute*IDQ*™p150 (human) and Absolute*IDQ*™p180 (animals) kits (BIOCRATES Life Sciences AG, Innsbruck, Austria). Details of measurement, quality control and imputation of the KORA samples have been previously published (22). Sample preparation was performed according to the user manual. Samples were randomized, and multiple quality control samples were included in the measurement sequence. In animals, values lower than the limit of detection (LOD) were set to half the value of the LOD for that metabolite. More details about mass spectrometry measurements and data analysis have been previously described (23, 24) (25). The p180kit corresponds to the p150kit completed with biogenic amines and biliary acids. These 2 classes of metabolites were not considered to compare human and animals. A list of the metabolites and their abbreviations is given in **Supplementary Table 1**. The number of metabolites that passed quality control was 151 for human and 167 for the animal models;

149 metabolites were common between human and animals. Abbreviations of the metabolite classes are: ACs (acylcarnitines), AAs (amino acids), PCs (phosphatidylcholines), lysoPCs (lysophosphatidylcholines), SM (sphingomyelins). A further 16 metabolite sums and ratios were previously identified to be of interest and added to the dataset.

***Comparison of metabolite concentrations.*** Differences (% variation) in metabolite concentrations (μM) between groups were analysed using two-tailed unpaired Welch's t-tests (Prism 6, GraphPad), with statistical significance judged as a false discovery rate (FDR)-adjusted p value smaller than 5%.

***Regression analysis.*** Regression analyses were conducted by running linear regressions using R version 3.4.0 (https://www.R-project.org/) to take into account possible confounding factors in humans. The level of statistical significance was 5% (FDR-adjusted p value). The following comparisons were considered: Q4 vs Q1 in human cIMT and drug-free cIMT groups; *ApoE-/-* vs C57Bl6J mice; *Ldlr-/-* vs C57Bl6J mice; *kl/kl* vs 129Sv mice; SHRSP vs WKY rats; SHRSP-NaCl vs WKY rats.

For cIMT and drug-free cIMT, the linear regression model (m1) was: "scale(log(metabolite concentration)) = cIMT ($1^{st}$/$4^{th}$ quartiles, reference $1^{st}$) + age + sex + batch + BMI + smoking habit (current/ex/never) + alcohol consumption (g/day) + physical activity (active yes/no)" where batch is a variable indicating the batch in which the metabolites were analysed (2 batches total). The metabolite concentrations were natural log-transformed and scaled over all individuals. Due to missing data in the covariates, for the regression analysis 13 individuals were lost for the cIMT group (7 from Q1, 6 from Q4); and 8 from the drug-free cIMT group (6 from Q1, 2 from Q4).

For *ApoE-/-* and *Ldlr-/-* mice, the linear regression model was "scale(log(metabolite concentration)) = mouse model" where "mouse model" is a categorical variable indicating the model in question (*ApoE-/-* or *Ldlr-/-*) or the reference C57Bl6J mice. Scaling was done with reference to all knock-out mouse data together.

For rats, the linear regression model was "scale(log(metabolite concentration)) = rat model" where "rat model" is a categorical variable indicating the model in question (SHSRP or SHSRP-NaCl) or the reference WKY rats administered with regular water. Scaling was done with reference to all rat data together.

For *kl/kl* mice, the model was "scale(log(metabolite concentration)) = *kl/kl* mouse model + sex" where "*kl/kl* mouse model" is a categorical variable indicating either the hypomorphic *kl/kl* models or the reference WT model. Scaling was performed with reference to all *kl/kl* mouse data together.

The metabolite concentrations were natural log-transformed and scaled, with scaling performed over all animal models. Scaling was performed to enable better comparisons between the metabolites.

**Results.**

***Human clinical parameters.*** In the cIMT and drug-free cIMT groups, increased cIMT between the top and bottom quartiles (Q4 and Q1) was accompanied by significant increase in age, body mass index, and alcohol consumption, as well as by significant higher blood parameters (glucose, pressure, cholesterol, triglyceride, creatinine) although these parameters remained within a healthy range (**Table 1**).

***Human metabolite signature*.** Comparing serum metabolite concentrations (µM) between the top and bottom cIMT quartiles (Q4 and Q1) revealed 131 and 109 metabolites (including sums and ratios) that differed significantly (FDR-adjusted p<0.05) in the cIMT and drug-free cIMT groups, respectively (**Supplementary Table 2**)**.** The relative order of variation of the different classes of metabolites was ACs>H1>SMs>AAs>PCs>lysoPCs and ACs>SMs>H1>AAs>lysoPCs>PCs for cIMT and drug-free cIMT groups respectively (**Supplementary Table 3**).

Regression analysis of the cIMT group showed that 26 metabolites (9 ACs, 2 lysoPCs, 9 PCs and 6 SMs) associated significantly with cIMT (FDR adjusted p<0.05) after adjustment of various potential confounders (model m1) **(Table 2 and Supplementary Table 4)**. Pearson correlation showed that ACs preferentially correlated with plasma glucose and creatinine, while PCs and SMs preferentially correlated with total cholesterol and LDL-cholesterol (**Supplementary Fig. 1).**

When running the regression after having introduced serum LDL into model m1, 8 metabolites (3 PCs, 5 SMs) lost significance (**Supplementary Table 5**). Loss of significant metabolites (3 PCs, 2 SMs) was also observed by introducing serum total cholesterol separately into model m1 (**Supplementary Table 5**). Introducing other clinical parameters, including glycemia, did not reduce, or only marginally reduced, the number of significant metabolites (**Supplementary Table5**). When running model m1 in the drug-free cIMT group with the initial 26 significant metabolites, 6 of them lost significance (5 PCs, 1 lysoPC, FDR correction based on only the 26 significant metabolites) (**Supplementary Table 5**). These results demonstrate that LDL, total cholesterol and medication partially drive the association of some metabolites (some PCs and most SMs) with cIMT.

***Metabolite signatures of the animal models.*** In animals, plasma concentrations (μM) of 108, 125, 77, 68 and 29 metabolites (including sums and ratios) were significantly (FDR-adjusted p<0.05) different in *ApoE-/-*, *Ldlr-/-*, *kl/kl*, SHRSP and SHRSP-NaCl, respectively, compared to their respective controls **(Supplementary Table 2).** The relative order of level change of the different classes of metabolites was SM>PC>AC>lysoPC>AA>H1, SM>PC>lysoPC>AC>AA>H1, AA=PC>SM>AC>lysoPC>H1, AC>lysoPC>AA>H1>PC>SM and PC>=AC>lysoPC>=AA>SM>H1 in *ApoE-/-*, *Ldlr-/-*, *kl/kl*, SHRSP and SHRSP-NaCl, respectively (**Supplementary Table3**).

***Comparison between human and mouse models.*** To determine which animal model most closely mimics the human cIMT signature, the differences in metabolite concentrations in the animal models were analysed by linear regression (**Supplementary Table 4).** The Spearman correlations were then calculated between these linear regression coefficients and those obtained in humans (cIMT group) (**Fig. 1**). When taking into account all metabolites regardless of their significance, coefficients correlated positively and significantly between human and *Ldlr-*/- ($p$ <0.0001) (r=0.27, $p$ <0.002); positively with *ApoE-*/- mice (r=0.13, $p$=0.14); and negatively and non-significantly with *kl/kl* mice (r=-0.12, $p$=0.17), and with SHRSP (r=-0.01, $p$=0.93) and SHRSP-NaCl rats (r=-0.08, $p$=0.37) **(Fig. 1)**. When considering all metabolites with a nominal $p$ <0.05 in humans (45 metabolites), the results are similar (r=0.42, p=0.025; r=0.18, p=0.34; r=-0.14, p=0.47; r=-0.14, p=0.48; r=-0.14, p=0.47); and when considering only those 26 metabolites with FDR-adjusted $p$ <0.05 in human, none achieve significance but the effect sizes remain roughly unchanged.

For the 26 significant metabolites in humans, *Ldlr-/-* mice display 11 (5PCs, 1 lysoPC, 5SMs) significant metabolites showing the same regulation direction (i.e. 42% of the metabolite signature of humans) (**Table 2**) and *ApoE-/-* mice 10 (38%, 5PCs, 5SMs); *Kl/kl*, SHSRP and SHSRP-NaCl shared 5, 2 and 3 (19%, 8%, 12%) significant metabolites showing concordant direction with the cIMT group, respectively. If one discounts the statistical significance of the metabolites for the animal models (ie, one looks only at the directions of association of the 26 metabolites in the animal models), the rats and klotho mice show better concordance, but remain below the levels of the *Ldlr-/-* and *ApoE-/-* mice.

**Discussion**

In the present study, we revealed a series of blood metabolites [phospholipids (PCs, lysoPCs, SMs) and acylcarnitines (ACs)] associated with elevated human cIMT. We also found that, among several animal models of vascular lesions, *Ldlr-/-* and *ApoE-/-* mice are those that better mimic the human cIMT signature.

Phospholipids (glycerophospholipids and sphingophospholipids) are structural constituents of cell membranes, and major components of circulating lipoproteins. LDL particles are enriched with ceramide and SM, while HDL particles are enriched with phosphatidylcholine, phosphatidylethanolamine, and phosphatidylethanolamine-based plasmalogens (26). Changes in blood phospholipids reflect alteration of lipoprotein metabolism. PCs are glycerophospholipids with choline as a head group, and they are involved in phospholipid metabolism, cell signaling, membrane structure and cell energy and are mostly influenced by diet (27). SMs accumulate in atherosclerotic lesions and plasma in

dyslipidaemia and can be used to predict acute coronary syndrome (27-29). LysoPCs are mainly produced by lipoprotein-associated phospholipase A2 (30) and are major determinants of the pro-atherogenic activity of oxidized LDL (oxLDL) and increase proteoglycan synthesis, an important determinant of intimal thickening (31). We found that all the phospholipids present in the human cIMT signature are increased and correlated preferentially with total cholesterol and LDL cholesterol, two major risk factors for the development of atherosclerosis. In our participants, increased cIMT is accompanied by a reduction of HDL cholesterol and an increase in plasma LDL-cholesterol. Although these biological parameters remain within a healthy range, their significant change might reflect a pre-dyslipidaemia that might explain the increase in some of the blood phospholipids.

ACs are intermediates of fatty acid oxidation and their elevation in blood is indicative of impaired β-oxidation and has been associated with increased risk of obesity, insulin resistance, and type 2 diabetes, all recognized as risk factors of CVD (32, 33). We also observed that increased cIMT is accompanied by increased BMI, glycaemia, HOMA-IR and HbA1c. Although these parameters remain within the normal reference, their increase might nevertheless explain the elevation of blood ACs associated with increased cIMT.

The 5 animal models of CVD that were compared to human were all expected to exhibit artery thickening due to plaque formation (*ApoE-/-* and *Ldlr-/-* mice), medial vascular calcification (*kl/kl*) or hypertension (SHRSP and SHRSP-NaCl rats). Nevertheless, *Ldlr-/-* and *ApoE-/-* mice are those that better mimicked the human cIMT signature. This is in agreement with the fact that atherosclerosis is the leading cause of cIMT elevation and arterial stiffness, due to lipid accumulation in the intima of vessel artery leading to calcification and plaque formation (34). We observed that *Ldlr-/-* correlated better than *ApoE-/-* with the human

cIMT signature. This is in agreement with the literature indicating that in human and *Ldlr-/-*, plasma cholesterol is mostly carried by LDL particles (35). Moreover, as in humans the development of atherosclerosis in the *Ldlr-/-* mouse is mainly due to dyslipidaemia, while atherosclerosis in *ApoE-/-* mice is also strongly associated with inflammation (8). Most of the metabolites that correlate between KORA study and Ldlr-/- mice are likely integral components of LDL particles (i.e. phospholipids and sphingolipids).

In contrast to *ApoE-/-* and *Ldlr-/-* mice, *kl/kl* mice and SHRSP rats did not mimic the human signature. *Kl/kl* mice exhibit medial vascular calcification, which develops due to hyperphosphatemia, comparable to the mineral bone disorder of chronic kidney disease patients (36, 37). Medial vascular calcification differs from atherosclerosis, although overlapping inflammatory pathways may exist (38). SHRSP is a model of arteriosclerosis secondary to high blood pressure (20, 21). Blood pressure was significantly elevated in the human cohort (4th quartile cIMT), but remained within a normal range. That might explain why we found a poor overlap with human cIMT. The current observations suggest that the metabolite signature strongly differs between atherosclerosis and medial vascular calcification due to hyperphosphatemia or chronic hypertension.

While we retrieved almost all the phospholipids (PCs, lysoPCs and SMs) of the human cIMT signature in *Ldlr-/-* and *ApoE-/-* signatures, we retrieved only 1 AC. As mentioned above, changes in blood phospholipids reflect alteration of lipoproteins metabolism, while increased levels of acylcarnitines are indicative of impaired β-oxidation and relate to increased risk of obesity, insulin resistance, and type 2 diabetes. *ApoE-/-* and *Ldlr-/-* are characterized by extensive atherosclerosis and dyslipidaemia (high plasma cholesterol and LDL cholesterol) but display no increase in body weight or glycaemia (7, 9). That might explain why blood ACs did not change in *ApoE-/-* and *Ldlr-/-*mice.

The fact that the hypercholesterolemia models Ldlr-/- and ApoE-/- mice better mimic the human metabolite profile was expected since LDL cholesterol is a major contributor to atherosclerosis development in humans. Nevertheless, we cannot exclude that knocking out LDLR or ApoE genes might have an influence on plasma metabolites independently of hypercholesterolemia.

One possible limitation of the present work is that we compared serum in human to plasma in animals. The coagulation cascade is indeed different between serum and plasma and could influence the concentrations of metabolites in these fluids. Nevertheless, we previously demonstrated that serum indeed contains higher concentrations of metabolites than plasma, but their topologies are very similar, with a high degree of correlation between the two fluids (39, 40). Therefore, while comparisons of the absolute concentrations of the metabolites can hardly be made, comparing the relative metabolite profiles between human serum and animal plasma is justifiable. Another limitation of the present work is that we screened a defined and limited number of metabolites due to the assay kits that were used. For example, some phosphatidylethanolamine species (not included in our assay kits) are increased in plasma from patients with non-calcified plaque with a possible association with de novo lipogenesis and type 2 diabetes (41 , 42 , 43) and are relatively abundant in the VLDL-fraction of lipoprotein (44). Therefore, it might be interesting to validate our findings by using other targeted or non-targeted metabolomics approaches to get access to a larger set of metabolites.

In conclusion, our work provides evidence that *Ldlr-/-* and *ApoE-/-* mice are interesting models to help better understanding the biochemical and molecular mechanisms involved in the vessel metabolic perturbations associated with and contributing to metabolic disorders

in CVD. These preclinical models should help in developing new preventive and therapeutic treatment strategies in human.

**Conflict of interest**

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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**Authors' contributions**

All authors participated in the conception and design, or analysis and interpretation of the data, contributed to drafting and revising the manuscript, and gave final approval of the version to be published.

**Legends of Figures**

**Figure 1.** Spearman correlations of the linear regression coefficients calculated for humans (Q4 *vs*. Q1 of cIMT) and those calculated for *ApoE-/-*, *Ldlr-/-*, *kl/kl*, SHRSP and SHRSP-NaCl *vs*. their respective controls. r (Spearman r); *p* (*p* value).

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**Table 1.** Characteristics of KORA F4 participants. cIMT (carotid intima media thickness). Q1 (first quartile cIMT). Q4 (last quartile cIMT). Categorical variables are presented as N (%), with p-value for the difference between Q1 and Q4 calculated using Fisher's exact test. Continuous variables are presented as mean (standard deviation), with p-value for the difference between Q1 and Q4 calculated using Welch's t-test.

| | **cIMT group** | | | **drug-free cIMT group** | | |
|---|---|---|---|---|---|---|
| | **Q1** | **Q4** | **P** | **Q1** | **Q4** | **P** |
| **Categorical characteristics** | | | | | | |
| Sex (men) | 271 (39.45%) | 393 (58.22%) | 5.00E-12 | 236 (38.63%) | 158 (57.88%) | 1.20E-07 |
| Current smoking | 155 (22.59%) | 76 (11.31%) | 3.20E-08 | 147 (24.1%) | 48 (17.71%) | 0.035 |
| Former smoking | 253 (36.88%) | 313 (46.58%) | 0.00034 | 216 (35.41%) | 112 (41.33%) | 0.097 |
| Physical activity (active) | 369 (53.71%) | 330 (49.11%) | 0.093 | 331 (54.17%) | 144 (53.14%) | 0.83 |
| Previous myocardial infarction | 5 (0.73%) | 39 (5.8%) | 3.90E-08 | 0 (0%) | 2 (0.74%) | 0.094 |
| Angina Pectoris | 25 (3.64%) | 53 (7.9%) | 0.00098 | 17 (2.78%) | 9 (3.32%) | 0.67 |
| Previous stroke | 4 (0.58%) | 26 (3.87%) | 2.40E-05 | 2 (0.33%) | 0 (0%) | 1 |
| Presence of cancer | 19 (2.77%) | 85 (12.65%) | 3.00E-12 | 15 (2.45%) | 23 (8.49%) | 0.00011 |
| Intake of anti-hypertensive medicine | 64 (9.32%) | 370 (54.9%) | 8.30E-78 | 0 (0%) | 0 (0%) | 1 |
| Intake of beta-blockers | 41 (5.97%) | 210 (31.16%) | 3.10E-35 | 0 (0%) | 0 (0%) | 1 |
| Intake of ACE-inhibitors | 23 (3.35%) | 162 (24.07%) | 2.10E-31 | 0 (0%) | 0 (0%) | 1 |
| Intake of statins | 21 (3.06%) | 147 (21.84%) | 3.30E-28 | 0 (0%) | 0 (0%) | 1 |
| Intake of fibrates | 1 (0.15%) | 5 (0.74%) | 0.12 | 0 (0%) | 0 (0%) | 1 |
| Intake of nitrates | 0 (0%) | 12 (1.78%) | 0.00021 | 0 (0%) | 0 (0%) | 1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Intake of lipid-lowering drugs | 23 (3.35%) | 153 (22.73%) | 1.10E-28 | 0 (0%) | 0 (0%) | 1 |
| Insulin therapy | 2 (0.29%) | 21 (3.12%) | 2.70E-05 | 0 (0%) | 2 (0.73%) | 0.095 |
| Intake of antidiabetic medication | 10 (1.46%) | 66 (9.81%) | 3.50E-12 | 3 (0.49%) | 7 (2.56%) | 0.012 |
| | | | | | | |
| **Continuous characteristics** | | | | | | |
| Age at examination | 44.01 (8.47) | 67.46 (8.48) | 2e-318 | 42.87 (7.43) | 64.09 (8.85) | 1.00E-128 |
| Body mass index (kg/m2) | 25.98 (4.59) | 29.14 (4.5) | 1.90E-35 | 25.59 (4.35) | 28.14 (3.91) | 5.70E-17 |
| Alcohol consumption (g/day) | 12.5 (17.55) | 15.22 (19.26) | 0.0067 | 12.63 (17.99) | 16.57 (20.9) | 0.0074 |
| Fasting glucose in serum (mg/dl) | 91.49 (11.41) | 104.97 (23.04) | 1.80E-38 | 90.27 (9.43) | 99.47 (20.13) | 5.70E-12 |
| HOMA-IR | 1.57 (4.92) | 3.05 (14.36) | 0.017 | 1.45 (5) | 3.46 (20.82) | 0.13 |
| HbA1c value (%) | 5.36 (0.39) | 5.81 (0.73) | 2.10E-41 | 5.32 (0.31) | 5.6 (0.58) | 2.40E-13 |
| Systolic blood pressure (mm Hg) | 113.97 (14.93) | 129.96 (19.38) | 1.20E-58 | 113.01 (14.46) | 130.18 (18.09) | 2.60E-36 |
| Diastolic blood pressure (mm Hg) | 73.24 (9.2) | 75.24 (10.21) | 0.00015 | 72.79 (9.12) | 77.44 (9.41) | 2.20E-11 |
| Pulse frequency (beats) | 73.35 (9.38) | 70.92 (10.76) | 9.20E-06 | 73.41 (9.17) | 72.16 (9.58) | 0.07 |
| Total cholesterol (mg/dl) | 203.71 (37.24) | 222.51 (40.66) | 1.80E-18 | 203.02 (37.37) | 234.19 (39.96) | 4.90E-25 |
| HDL cholesterol (mg/dl) | 58 (14.75) | 53.55 (13.48) | 7.40E-09 | 58.31 (14.47) | 55.07 (14.12) | 0.0019 |
| LDL cholesterol (mg/dl) | 125.28 (33.38) | 143.47 (35.97) | 2.10E-21 | 124.62 (33.28) | 154.05 (35.33) | 7.30E-28 |
| Triglycerides (mg/dl) | 107.3 (91.31) | 139.71 (87.92) | 3.70E-11 | 103.14 (91.47) | 135.7 (79.48) | 1.20E-07 |
| Serum creatinine (mg/dl) | 0.86 (0.17) | 0.96 (0.28) | 1.60E-14 | 0.85 (0.16) | 0.88 (0.16) | 0.003 |
| CKD-EPI | 103.03 (14.21) | 80.18 (15.95) | 2.50E-135 | 104.75 (12.6) | 87.33 (13.76) | 4.40E-55 |
| Serum albumin (g/l) | 45 (3.51) | 44.03 (3.11) | 9.60E-08 | 45.13 (3.5) | 44.29 (3.08) | 0.00041 |

| | | | | | | |
|---|---|---|---|---|---|---|
| IMT left of A. carotis communis | 0.69 (0.05) | 1.05 (0.09) | 0 | 0.69 (0.05) | 1.04 (0.08) | 3.80E-203 |
| IMT right of A. carotis communis | 0.7 (0.05) | 1.04 (0.09) | 0 | 0.7 (0.05) | 1.03 (0.08) | 5.30E-200 |
| IMT mid A. carotis communis | 0.69 (0.04) | 1.05 (0.08) | 0 | 0.69 (0.04) | 1.03 (0.07) | 1.70E-216 |

**Table 2.** Comparison of coefficients for the regressions between Q4 vs Q1 of cIMT (cIMT group) and the regressions for each animal model vs their respective controls. Showing only those metabolites statistically significant for the cIMT comparison (FDR-adjusted P<0.05). Regression coefficients (Coef) and P values (Pval) are from the linear regressions of the cIMT analysis and for each animal model (full results Table S4). Empty cells correspond to metabolites that are present in human but not in animals. Metabolite abbreviations are given in Table S1.

| | cIMT Q4 vs Q1 | | *ApoE-/-* | | *Ldlr-/-* | | *kl/kl* | | SHSRP | | SHSRP-NaCl | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Coef. | Pval | Coef. | Pval | Coef. | Pval | Coef. | Pval | Coef. | Pval | Coef. | Pval |
| C12-DC | 0.25 | 1.2E-03 | | | | | | | | | | |
| C16:2-OH | 0.34 | 1.2E-03 | | | | | | | | | | |
| C3-DC (C4-OH) | 0.39 | 2.9E-04 | | | | | 1.37 | 9.6E-04 | | | | |
| C5-DC (C6-OH) | 0.41 | 5.2E-05 | | | | | | | | | | |
| C5-M-DC | 0.35 | 1.2E-03 | | | | | | | | | | |
| C3-DC-M (C5-OH) | 0.43 | 6.2E-05 | | | | | | | | | | |
| C5-1 | 0.34 | 1.4E-03 | | | | | | | | | | |
| C5-1-DC | 0.51 | 1.9E-06 | | | | | | | | | | |
| C6:1 | 0.6 | 1.1E-08 | | | | | | | | | | |
| PC aa C38:3 | 0.34 | 1.8E-03 | 0.02 | 8.2E-01 | 1.76 | 4.3E-18 | -0.15 | 2.8E-01 | 0.04 | 7.3E-01 | 0.26 | 2.0E-01 |
| PC aa C38:4 | 0.25 | 4.3E-02 | 0.85 | 5.2E-05 | 2.86 | 4.8E-17 | 0.01 | 9.7E-01 | -0.13 | 4.3E-01 | 0.36 | 1.7E-01 |
| PC aa C40:4 | 0.39 | 6.3E-04 | 0.84 | 1.5E-07 | 2.79 | 1.8E-20 | 0.16 | 4.6E-01 | 0.04 | 8.0E-01 | 0.67 | 4.1E-02 |
| PC aa C40:5 | 0.26 | 4.0E-02 | 0.95 | 7.0E-11 | 3.02 | 3.4E-25 | 0.37 | 3.9E-02 | 0.11 | 2.9E-01 | 0.67 | 1.4E-02 |
| PC aa C42:2 | -0.25 | 3.9E-02 | 0.59 | 4.0E-06 | 2.28 | 9.8E-20 | 1.25 | 1.9E-04 | -0.02 | 9.7E-01 | 0.53 | 7.9E-02 |
| PC ae C30:2 | -0.23 | 3.9E-02 | | | | | | | | | | |
| PC ae C32:2 | -0.25 | 1.8E-02 | 0.85 | 2.2E-13 | 1.02 | 1.3E-15 | 0.22 | 8.0E-02 | -0.38 | 3.6E-06 | -0.15 | 2.3E-01 |
| PC ae C38:4 | 0.26 | 1.8E-02 | 2.21 | 3.1E-19 | 2.9 | 1.4E-23 | 0.94 | 9.3E-04 | -0.64 | 7.0E-03 | -0.06 | 8.8E-01 |
| PC ae C42:3 | -0.27 | 1.1E-02 | -1.09 | 1.4E-09 | 0.88 | 2.3E-05 | 0.44 | 5.3E-02 | 0.27 | 8.2E-02 | 0.61 | 2.8E-02 |
| lysoPC a C14:0 | 0.27 | 2.0E-02 | | | | | | | | | | |
| lysoPC a C26:1 | 0.27 | 7.7E-03 | 0.18 | 5.4E-01 | 1.24 | 4.9E-04 | 0.07 | 8.9E-01 | -0.43 | 7.9E-02 | -0.54 | 1.1E-01 |
| SM (OH) C22:1 | 0.26 | 1.5E-02 | 1.52 | 6.9E-13 | 1.93 | 3.1E-13 | 0.1 | 5.7E-01 | -0.63 | 2.0E-05 | -0.36 | 4.1E-02 |
| SM (OH) C24:1 | 0.26 | 1.8E-02 | 1.24 | 2.1E-17 | 1.5 | 1.3E-22 | 0.31 | 3.4E-02 | 0.27 | 4.2E-04 | 0.3 | 4.2E-04 |
| SM C18:0 | 0.3 | 6.5E-03 | 2.43 | 1.2E-21 | 2.59 | 3.7E-23 | 0.86 | 5.7E-03 | -0.42 | 5.2E-02 | -0.09 | 6.9E-01 |
| SM C24:0 | 0.3 | 5.5E-03 | 1.5 | 4.0E-14 | 2.32 | 6.9E-18 | -0.12 | 5.7E-01 | -0.48 | 9.7E-05 | -0.29 | 6.9E-02 |
| SM C24:1 | 0.23 | 3.1E-02 | 2.36 | 4.7E-27 | 2.51 | 2.3E-27 | -0.44 | 1.0E-02 | -0.67 | 9.9E-06 | -0.5 | 3.5E-03 |
| SM C26:0 | 0.41 | 2.9E-04 | | | | | | | | | | |

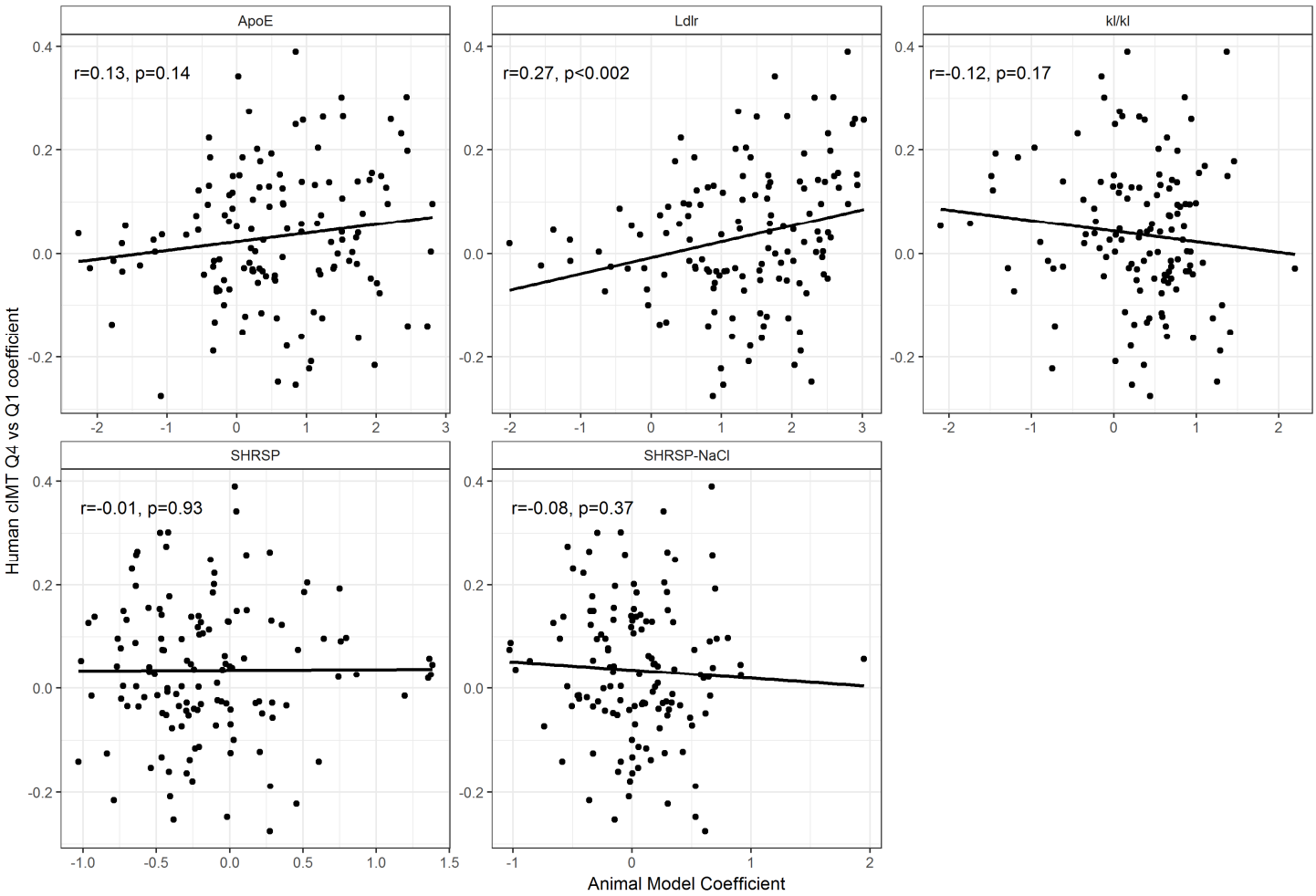


**Figure 1**

**Highlights**

- Metabolomics allows access to the metabolic perturbations associated with cardiovascular disease (CVD)
- Increased carotid intima-media thickness (cIMT) is a surrogate of CVD
- Human blood metabolite signature of increased cIMT is made of acylcarnitines and phospholipids
- *LDLR-/-* and *ApoE-/-* mice mimicked the phospholipid part of cIMT signature