Metabolic profiling of intra- and extracranial carotid artery atherosclerosis

Dina Vojinovic*, Sven J. van der Lee*, Cornelia M. van Duijn, Meike W. Vernooij, Maryam Kavousi, Najaf Amin, Aye Demirkan, M. Arfan Ikram, Aad van der Lugt, Daniel Bos

** These authors contributed equally to this work*

This chapter was published in Atherosclerosis. 2018 Mar 8;272:60-65.

The supplemental information for this paper is available online at https://doi.org/10.1016/j.atherosclerosis.2018.03.015

ABSTRACT

Background and aims

Increasing evidence shows that intracranial carotid artery atherosclerosis may develop under the influence of a differential metabolic risk factor profile than atherosclerosis in the extracranial part of the carotid artery. To further elucidate these differences, we investigated associations of a wide range of circulating metabolites with intracranial and extracranial carotid artery atherosclerosis.

Methods

From the population-based Rotterdam Study, blood samples from 1,111 participants were used to determine a wide range of metabolites by proton nuclear magnetic resonance (NMR). Moreover, these participants underwent non-contrast computed tomography of the neck and head to quantify the amount of extra- and intracranial carotid artery calcification (ECAC and ICAC), as a proxy of atherosclerosis. We assessed associations of the metabolites with ICAC and ECAC and compared the metabolic association patterns of the two.

Results

We found that one standard deviation (SD) increase in concentration of 3-hydroxybutyrate, a ketone body, was significantly associated with a 0.11 SD increase in ICAC volume (p-value = 1.8×10^{-4}). When we compared the metabolic association pattern of ICAC with that of ECAC, we observed differences in glycolysis-related metabolite measures, lipoprotein subfractions, and amino acids. Interestingly, glycoprotein acetyls were associated with calcification in both studied vessel beds. These associations were most prominent in men.

Conclusions

We found that a higher circulating level of 3-hydroxybutyrate was associated with an increase in ICAC. Furthermore, we found differences in metabolic association patterns of ICAC and ECAC, providing further evidence for location-specific differences in the etiology of atherosclerosis.

INTRODUCTION

Carotid artery atherosclerosis is established as the single most important cause of stroke worldwide.[1-4] Importantly, increasing evidence suggests that the specific location of carotid atherosclerosis, i.e. extracranial versus intracranial, harbors unique, differential information with regard to the risk of subsequent stroke.[3-4] In addition, it was also found that the contribution of traditional cardiovascular risk factors to intracranial carotid artery atherosclerosis is different from that to extracranial carotid artery atherosclerosis.[5-7] In particular, diabetes mellitus and insulin resistance, i.e. expressions of disrupted glucose and insulin metabolism, seem to play a more prominent role in the development of intracranial carotid artery atherosclerosis.[6,8,9] This apparent location-specific susceptibility to metabolic disturbances warrants further in-depth investigation of the metabolic underpinnings of carotid artery atherosclerosis. Interestingly, methods to perform such an in-depth investigation of large spectra of active metabolites in relation to disease have only recently become available.[10,11] With the use of nuclear magnetic resonance (NMR), metabolites can now be inexpensively and reproducibly quantified on a large-scale, which enables metabolomics studies in large population-based cohorts. Successful examples include metabolic profiling of type 2 diabetes,[12,13] and cardiovascular events.[14–17]

Applying a similar approach to carotid artery atherosclerosis may expose important metabolites contributing to the disease. To date, several inflammatory markers have been associated with different stages and manifestation of carotid artery atherosclerosis, such as interleukin-6 and tumor necrosis factor-α.[18,19] Ultimately, this knowledge may provide opportunities for the development of specific therapeutic and preventive strategies.

Hence, the aim of this study was to investigate associations of a broad range of metabolites with intracranial and extracranial carotid artery calcification (ICAC and ECAC), as a proxy of atherosclerosis, and to compare the metabolic association profile of ICAC with that of ECAC.

MATERIALS AND METHODS

Study population

Our study population consisted of participants from the Rotterdam Study, a prospective population-based cohort study among individuals aged 45 years and over, who are living in the well-defined Ommoord district in Rotterdam, the Netherlands.[20] The study started in 1990, with 7,983 participants (first Rotterdam Study cohort, RS-I), and was extended in 2000/2001 (RS-II, 3,011 participants) and 2006/2008 (RS-III, 3,932 participants).[20] All participants were invited for extensive re-examinations every 3–4 years. At each visit,

blood was drawn after overnight fasting. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study).[20] All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Population for analysis

Metabolites were available for two independent datasets of the Rotterdam Study. The first set encompassed all individuals from the RS-I cohort that participated in the fourth examination round at the study center (N = 2,975). Of these, 730 underwent a computed tomography (CT) scan to visualize calcification in the carotid arteries. The second dataset consisted of 768 participants from the RS-I, RS-II and RS-III cohorts of whom 381 also underwent a CT scan. This dataset was the subset of samples previously included in the Biobank-based Integrative Omics Studies Consortium (BIOS Consortium).[20,21] The CT scan was performed on average 4 months (interquartile range (IQR) 2–4 months) after metabolite measuring for the first Rotterdam Study dataset, and 6 years (IQR 5.9–6.2 years) before metabolite measuring for the second Rotterdam Study dataset.

Metabolite quantification

The metabolites were quantified from EDTA plasma samples using high-throughput proton Nuclear Magnetic Resonance (NMR) metabolomics (Nightingale Health, Helsinki, Finland). This method provides simultaneous quantification of metabolic measures, i.e. routine lipids, lipoprotein subclass profiling with lipid concentrations within 14 subclasses, fatty acid composition, and various low-molecular-weight metabolites including amino acids, ketone bodies and gluconeogenesis-related metabolites in molar concentration units. The lipoprotein subclasses include very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). In these subclasses, the concentration is measured as well as the subfraction of lipids, triglycerides, cholesterol esters, free cholesterol, and phospholipids. Details of the experimentation and applications of this NMR metabolomics platform have been described previously.[22,23] For this study we analyzed in total 166 non-derived metabolites that were measured across both cohorts.

Assessment of atherosclerosis

A 16-slice (n = 785) or 64-slice (n = 1,739) multidetector CT scanner (Somatom Sensation 16 or 64; Siemens, Forchheim, Germany) was used to perform non-enhanced scanning of intracranial and extracranial carotid arteries to visualize calcification as a proxy of atherosclerosis. Detailed information regarding the protocol and imaging settings is provided elsewhere.[4] ICAC was semi-automatically quantified from the horizontal seg-

ment of the petrous internal carotid artery to the top of the internal carotid artery.[8] Details of this quantification method were described previously.[4] Briefly, regions of interest were drawn in the course of the intracranial internal carotid arteries in consecutive CT sections. Next, calcification volumes were calculated by multiplying the number of pixels in excess of 130 Hounsfield units by the pixel size and the increment.[8] Calcification volumes in the extracranial internal carotid arteries were quantified using dedicated commercially available software (Syngo Calcium Scoring; Siemens).[4] All calcification volumes are expressed in cubic millimeters.

Other measurements

Information on cardiovascular risk factors was obtained by means of interview, physical examination or blood sampling. Hypertension was defined as a systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or use of medication for the treatment of hypertension.[24] Diabetes was defined as fasting plasma glucose levels above 7 mmol/L or use of medication indicated for the treatment of diabetes.[24] Hypercholesterolemia was defined as a total cholesterol ≥ 6.2 mmol/L or use of lipid-lowering medication.[24] BMI was calculated as weight in kilograms divided by square of height in meters. A history of cardiovascular disease was defined as previous myocardial infarction, percutaneous transluminal coronary angioplasty, coronary artery bypass graft or stroke.[8,24]

Statistical analysis

The distributions of metabolic measures were visually inspected for non-normality and were, if necessary, natural logarithmic transformed to obtain approximately normal distributions (**Supplementary Table 1**). The metabolites were scaled to standard deviation (SD) units to enable direct comparisons of effect estimates across the different measures. Because ICAC and ECAC volumes were non-normally distributed, we used natural logarithmic transformed values. To deal with calcium volumes of zero we added 1.0 mm^3 to the non-transformed values. Subsequently, we scaled these new values to SD units to unify reporting and interpretation of the results. To assess the relation of metabolites with ICAC and ECAC per dataset, we performed linear regression analysis while adjusting for age, gender, and lipid-lowering medication (Model 1). The associations were further adjusted for hypertension, diabetes, hypercholesterolemia, smoking, and BMI (Model 2). Finally, we additionally adjusted for history of cardiovascular disease (Model 3). The summary statistic results of the two datasets were meta-analyzed using inverse variance-weighted fixed-effect meta-analysis. Additionally, all analyses were performed in males and females separately.

As metabolic measures are highly correlated (median absolute correlation coefficient = 0.24, IQR = 0.11–0.50), we used the method of Li and Ji[25] to correct for multiple

testing. The method calculates the number of independent variables (and thus tests) in correlated measures. The 166 metabolites corresponded to 33 independent variables. Bonferroni correction was applied for the number of independent variables tested (*p*-value threshold for significance: $0.05/33 = 1.5 \times 10^{-3}$). All analyses were performed with R (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (http://www.R-project.org/)).

RESULTS

The characteristics of the study population are shown in **Table 1** and the summary statistics of metabolites are shown in **Supplementary Table 1**. Participants from the first dataset of Rotterdam Study (N = 730, 51.2% women, mean age 73.8 ± 5.5 years) were older than participants from the second dataset of Rotterdam Study (N = 381, 53%

Table 1. Descriptive characteristic of study population

	Rotterdam Study dataset 1[a]	**Rotterdam Study dataset 2**[a]
Number of Participants	730	381
Age at CT scan, years	73.8 ± 5.5	64.9 ± 3.2
Women	374 (51.2%)	202 (53.0%)
Diastolic blood pressure, mmHg	79.7 ± 11.4	81.3 ± 10.5
Systolic blood pressure, mmHg	151.2 ± 21.2	142.3 ± 18.0
Hypertension	406 (56.2%)	162 (43.7%)
Glucose, mmol/l	5.8 ± 1.4	5.6 ± 1.2
Participants at CT with diabetes	105 (14.4%)	29 (7.6%)
Total cholesterol, mmol/l	5.6 ± 1.0	5.8 ± 1.0
HDL-Cholesterol, mmol/l	1.4 ± 0.4	1.5 ± 0.4
Hypercholesterolemia	351 (48.5%)	186 (49.7%)
Smoking (never/past/current) (%)	206/403/91 (28.2/55.2/12.5)	110/200/59 (28.9/52.5/15.5)
BMI, kg/m^2	27.3 ± 4	27.8 ± 3.8
Participants at CT with cardiovascular disease	95 (13.1%)	27 (7.1%)
Participants at CT with coronary heart disease	72 (9.9%)	17 (4.5%)
Participants at CT with stroke	32 (4.4%)	11 (2.9%)
ICAC volume, median (IQR), cm^3	64.8 (13.0-205.6)	22.1 (3.8-75.4)
ECAC volume, median (IQR), cm^3	48 (3.1-176.7)	10.4 (0-60.4)

Abbreviations: BMI - body mass index; HDL - high-density lipoprotein; ICAC - intracranial carotid artery calcification; ECAC - extracranial carotid artery calcification; IQR - interquartile range.

[a] Values are means ± standard deviation for continuous variables and number (percentages) for dichotomous variables.

women, mean age 64.9 ± 3.2 years), resulting in differences in age-related clinical characteristics and average volume of calcifications (**Table 1**). However, the prevalence of ICAC was comparable being 83.0% and 80.6% in the first and second group respectively. The prevalence of ECAC was 79.9% in the first and 65.6% in the second dataset.

Table 2. Association of 3-hydroxybutyrate with ICAC volume.

Models	Effect (±SE)[a]	P	N
Model 1 Age, sex and, lipid-lowering medication	0.107(±0.029)	1.76×10^{-4}	1095
Model 2 Model 1 + hypertension, diabetes, hypercholesterolemia, smoking, and BMI	0.092(±0.030)	2.10×10^{-3}	1059
Model 3 Model 2 + history of cardiovascular disease	0.092(±0.030)	2.02×10^{-3}	1054

[a] Effect estimates are SD change in ICAC per 1-SD 3-hydroxybutyrate concentration.

We found a significant association of 3-hydroxybutyrate, a glycolysis-related metabolite, with ICAC (one SD increase in the concentration of 3-hydroxybutyrate was related to a 0.11 SD increase in ICAC; *p*-value = 1.8×10^{-4}, **Table 2**). The effect estimates were consistent across the two datasets (first Rotterdam Study dataset: effect = 0.10, *p*-value = 6.5×10^{-3}; second Rotterdam study dataset; effect: 0.13, *p*-value = 9.06×10^{-3}). Further adjustments for traditional cardiovascular risk factors or history of cardiovascular disease did not influence the effect estimate (**Table 2**). We found no statistically significant association of any of the metabolites with ECAC (**Supplementary Table 2**).

When comparing the metabolic association pattern between ICAC and ECAC we found specific differences (**Fig. 1**, **Supplementary Table 2**). Among the glycolysis-related metabolic measures, 3-hydroxybutyrate which was significantly associated with ICAC, showed nominally significant association with ECAC (effect = 0.07, *p*-value = 0.02). Glucose was nominally significant associated with both ICAC (effect = 0.07, *p*-value = 0.01), and ECAC (effect = 0.06, *p*-value = 0.03), whereas citrate was nominally associated with ECAC (effect = 0.06, *p*-value = 0.03) (**Supplementary Table 2**). Interestingly, among lipoprotein subfractions, only triglycerides in medium-sized LDL were nominally associated with ICAC (effect = 0.06, *p*-value = 0.03, **Fig. 1A**, **Supplementary Table 2**), whereas total and free cholesterol and cholesterol esters in extra-large HDL showed nominally significant association with ECAC (**Fig. 1A**, **Supplementary Table 2**). Among the amino-acids, isoleucine was nominally associated with ICAC, and histidine was nominally associated with ECAC. Glycoprotein acetyls were associated with calcification volume in both studied vessel beds.

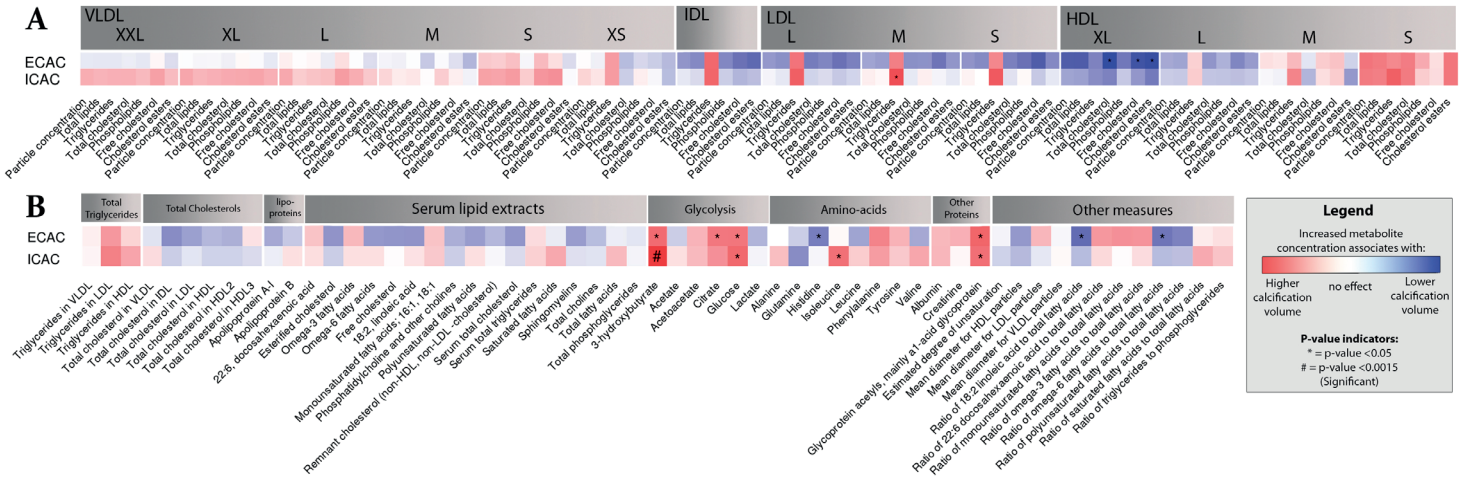

Figure 1. Metabolic association profiles of ICAC and ECAC. The colors represent standardized effect estimates of the metabolites with the calcification volume. The effect estimates of lipoprotein subfractions with calcification in all studied vessel beds are shown in Figure 1A grouped by classes of lipoproteins. The effect estimates of other metabolite measures are shown in Figure 1B grouped by type of metabolic measure. The effect estimates are adjusted for age, gender, and lipid-lowering medication.

When we stratified the analysis by sex the association of 3-hydroxybutyrate with ICAC was nominally significant in both men (effect = 0.13, *p*-value = 2.8×10^{-3}) and women (effect = 0.08, *p*-value = 0.04) (**Supplementary Figs. 1** and **2**, **Supplementary Tables 3** and **4**). Interestingly, the association of glycoprotein acetyls with ICAC and ECAC was mainly driven by men (**Supplementary Figs. 1** and **2**). However, after further adjustments for traditional cardiovascular risk factors or history of cardiovascular disease, glycoprotein acetyls in men were not associated with ICAC and ECAC (*p*-value > 0.05, **Supplementary Table 3**). Other metabolites that were significantly associated with ECAC in men were: the ratio of 18:2 linoleic acid to total fatty acids (effect = –0.17, *p*-value = 4.7×10^{-5}) and the ratio of omega-6 fatty acids to total fatty acids (effect = –0.15, *p*-value = 3.4×10^{-4}). These associations were not modified by the additional adjustments made in model 2 and 3 (**Supplementary Table 3**). No statistically significant associations were observed of metabolites with ECAC in women (**Supplementary Table 4**).

DISCUSSION

In this population-based study, we found that glycolysis-related metabolite measures were associated with a larger volume of ICAC. In particular, higher levels of 3-hydroxybutyrate substantially contributed to larger ICAC volumes. When comparing the metabolic association profile of ICAC with that of ECAC, we found specific differences in glycolysis-related metabolite measures, lipoprotein subfractions, and amino acids.

To our knowledge, this is the first study investigating associations of metabolomics with ICAC and ECAC. The most intriguing finding was the association of 3-hydroxybutyrate (also called beta-hydroxybutyric acid) with ICAC. The ketone 3-hydroxybutyrate is the most abundant of the three known ketone bodies (acetoacetate, 3-hydroxybutyrate, and acetone) that is produced by the liver during fasting and represents an alternative energy source for the brain.[26] In addition, fasting-induced 3-hydroxybutyrate has been found to enhance expression of the glucose transporter Glut1 in brain endothelial cells, which plays an important role in glucose transport across the blood-brain barrier.[27] In general, ketone bodies are considered to exert beneficial effects on brain functioning.[28] In this light, our finding that higher concentrations of 3-hydroxybutyrate relate to larger volumes of ICAC seems to contrast these beneficial effects, especially because ICAC is a risk factor for (subclinical) stroke, cognitive decline, and dementia.[29,30] Yet, a potential mechanism underlying this association may be found in the property of 3-hydroxybutyrate to form polymers known as Poly-(R)-3-hydroxybutyrates (PHB)s. These short-chain PHBs reside in the lipid core of lipoprotein(a) (Lp(a)), a lipoprotein with profound atherogenic effects and also causally related to coronary heart disease.[31-34]

Another explanation for the relation of 3-hydroxybutyrate with ICAC may be impaired glucose tolerance. Impaired glucose tolerance is the (pre-) clinical state of diabetes mellitus type 2 (DM2) and is associated with an elevated risk of and a poor prognosis after cardiovascular events.[35,36] 3-hydroxybutyrate levels were found to be increased in individuals with impaired glucose tolerance and in patients with DM2, in whom it predicted worsening of hyperglycemia and incident DM2 in the next 5 years.[37] These data could hypothetically place 3-hydroxybutyrate in the pathway that leads from an impaired glucose tolerance to increased ICAC and eventually cardiovascular events. Another explanation may be that higher levels of 3-hydroxybutyrate compensate for defective transport of 3-hydroxybutyrate across the blood-brain barrier due to intracranial arteriosclerosis, i.e. reverse causation. Finally, a partial common genetic background might explain the relation between 3-hydroxybutyrate and ICAC.

We also compared the metabolic association patterns of ICAC with that of ECAC. The association between glycoprotein acetyls was observed with both ICAC and ECAC, and glycoprotein acetyls associated with calcifications in men. Attenuation of these associations in model 2, suggests that glycoprotein acetyls might in part reflect pathology related to cardiovascular risk. Levels of this protein are strongly associated with smoking and physical activity and glycoprotein acetyl concentration has been shown to be a strong predictor of 10-year mortality.[38,39] The protein is a marker of acute-phase reactions and may be implicated in this way in depression,[40] diabetes,[41] cardiovascular disease,[42] and cancer[43]. Furthermore, we observed specific differences that further underline the location-specific properties of atherosclerosis.[5,44,45] In addition to differences in glycolysis-related metabolic measures between ICAC and ECAC which are discussed above, another interesting difference we found was that higher concentrations of HDL subfractions were associated with lower volumes of ECAC, but not with ICAC.

The strength of our study includes the large sample with standardized assessments of metabolic measures and arterial calcification in intracranial and extracranial carotid artery, enabling comparisons of the metabolic association patterns of calcification in these two vessel beds. The metabolomics platform that we used contains a large proportion of lipoprotein or other lipid measures which provides an excellent opportunity to study atherosclerosis.[15,22,23,46] However, it should be acknowledged that many other metabolites can be measured using more detailed techniques,[47] which may also be of importance to atherosclerosis. There are also other limitations of our study that should be noted. First, even though calcification is a validated marker of atherosclerosis, non-calcified atherosclerotic disease was not taken into account. Especially, these non-calcified components of the atherosclerotic plaque may also be influenced by the studied metabolites.[48] Another limitation of the current study is that metabolites and CT scan

measures have not been taken at the same time. However, the results were concordant in the two datasets despite the time difference in the metabolites and CT scan measures. Finally, although we adjusted our analyses for various known potential confounders, residual confounding by unknown factors remains possible. We urge future replication efforts of our findings in independent datasets.

CONCLUSIONS

We found a prominent association between 3-hydroxybutyrate and the amount of ICAC. Investigation of the underlying biological mechanisms for the identified association should be the subject of future biological studies. When comparing the metabolic association profile of ICAC with that of ECAC, we found specific differences in glycolysis-related metabolite measures, lipoprotein subfractions, and amino acids, further corroborating the evidence for the existence of location-specific differences in the etiology of carotid artery atherosclerosis.

REFERENCES

1. Gorelick, P.B., Wong, K.S., Bae, H.J. & Pandey, D.K. Large artery intracranial occlusive disease: a large worldwide burden but a relatively neglected frontier. *Stroke* **39**, 2396-9 (2008).
2. Arenillas, J.F. Intracranial atherosclerosis: current concepts. *Stroke* **42**, S20-3 (2011).
3. Qureshi, A.I. & Caplan, L.R. Intracranial atherosclerosis. *Lancet* **383**, 984-98 (2014).
4. Bos, D. *et al.* Intracranial carotid artery atherosclerosis and the risk of stroke in whites: the Rotterdam Study. *JAMA Neurol* **71**, 405-11 (2014).
5. Bos, D. *et al.* Genetic loci for coronary calcification and serum lipids relate to aortic and carotid calcification. *Circ Cardiovasc Genet* **6**, 47-53 (2013).
6. Lopez-Cancio, E. *et al.* Biological signatures of asymptomatic extra- and intracranial atherosclerosis: the Barcelona-AsIA (Asymptomatic Intracranial Atherosclerosis) study. *Stroke* **43**, 2712-9 (2012).
7. Allison, M.A., Criqui, M.H. & Wright, C.M. Patterns and risk factors for systemic calcified atherosclerosis. *Arterioscler Thromb Vasc Biol* **24**, 331-6 (2004).
8. Bos, D. *et al.* Intracranial carotid artery atherosclerosis: prevalence and risk factors in the general population. *Stroke* **43**, 1878-84 (2012).
9. Mazighi, M. *et al.* Autopsy prevalence of intracranial atherosclerosis in patients with fatal stroke. *Stroke* **39**, 1142-7 (2008).
10. Quehenberger, O. & Dennis, E.A. The human plasma lipidome. *N Engl J Med* **365**, 1812-23 (2011).
11. Inouye, M. *et al.* Metabonomic, transcriptomic, and genomic variation of a population cohort. *Mol Syst Biol* **6**, 441 (2010).
12. Wang, T.J. *et al.* Metabolite profiles and the risk of developing diabetes. *Nat Med* **17**, 448-53 (2011).
13. Mahendran, Y. *et al.* Glycerol and fatty acids in serum predict the development of hyperglycemia and type 2 diabetes in Finnish men. *Diabetes Care* **36**, 3732-8 (2013).
14. Shah, S.H., Kraus, W.E. & Newgard, C.B. Metabolomic profiling for the identification of novel biomarkers and mechanisms related to common cardiovascular diseases: form and function. *Circulation* **126**, 1110-20 (2012).
15. Wurtz, P. *et al.* High-throughput quantification of circulating metabolites improves prediction of subclinical atherosclerosis. *Eur Heart J* **33**, 2307-16 (2012).
16. Stegemann, C. *et al.* Lipidomics profiling and risk of cardiovascular disease in the prospective population-based Bruneck study. *Circulation* **129**, 1821-31 (2014).
17. Roberts, L.D. & Gerszten, R.E. Toward new biomarkers of cardiometabolic diseases. *Cell Metab* **18**, 43-50 (2013).
18. Ammirati, E and Fogacci, F, Clinical relevance of biomarkers for the identification of patients with carotid atherosclerotic plaque: Potential role and limitations of cysteine protease legumain. *Atherosclerosis* **257**:248-249 (2017).
19. Ammirati, E, Moroni, F, Norata, GD, *et al.* Markers of inflammation associated with plaque progression and instability in patients with carotid atherosclerosis, *Mediators Inflamm* **718329** (2015).
20. Hofman, A. *et al.* The Rotterdam Study: 2016 objectives and design update. *Eur J Epidemiol* **30**, 661-708 (2015).
21. Huan, T. *et al.* A meta-analysis of gene expression signatures of blood pressure and hypertension. *PLoS Genet* **11**, e1005035 (2015).
22. Soininen, P. *et al.* High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. *Analyst* **134**, 1781-5 (2009).

23. Soininen, P., Kangas, A.J., Wurtz, P., Suna, T. & Ala-Korpela, M. Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. *Circ Cardiovasc Genet* **8**, 192-206 (2015).
24. Odink, A.E. *et al.* Risk factors for coronary, aortic arch and carotid calcification; The Rotterdam Study. *J Hum Hypertens* **24**, 86-92 (2010).
25. Li, J. & Ji, L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity* **95**, 221-227 (2005).
26. Owen, O.E. *et al.* Brain metabolism during fasting. *J Clin Invest* **46**, 1589-95 (1967).
27. Tanegashima, K. *et al.* Epigenetic regulation of the glucose transporter gene Slc2a1 by -hydroxybutyrate underlies preferential glucose supply to the brain of fasted mice. *Genes to Cells* **22**, 71-83 (2017).
28. Rahman, M. *et al.* The beta-hydroxybutyrate receptor HCA2 activates a neuroprotective subset of macrophages. *Nat Commun* **5**, 3944 (2014).
29. Bos, D. *et al.* Intracranial Carotid Artery Atherosclerosis and the Risk of Stroke in Whites The Rotterdam Study. *Jama Neurology* **71**, 405-411 (2014).
30. Bos, D. *et al.* Atherosclerotic calcification is related to a higher risk of dementia and cognitive decline. *Alzheimers & Dementia* **11**, 639-647 (2015).
31. Kettunen, J. *et al.* Genome-wide study for circulating metabolites identifies 62 loci and reveals novel systemic effects of LPA. *Nat Commun* **7**, 11122 (2016).
32. Tregouet, D.A. *et al.* Genome-wide haplotype association study identifies the SLC22A3-LPAL2-LPA gene cluster as a risk locus for coronary artery disease. *Nature Genetics* **41**, 283-285 (2009).
33. Schunkert, H. *et al.* Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. *Nature Genetics* **43**, 333-U153 (2011).
34. Reusch, R.N. Poly-(R)-3-hydroxybutyrates (PHB) are atherogenic components of lipoprotein Lp(a). *Medical Hypotheses* **85**, 1041-1043 (2015).
35. Barr, E.L.M. *et al.* Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance - The Australian diabetes, obesity, and lifestyle study (AusDiab). *Circulation* **116**, 151-157 (2007).
36. Kurihara, O. *et al.* Impact of Prediabetic Status on Coronary Atherosclerosis A multivessel angioscopic study. *Diabetes Care* **36**, 729-733 (2013).
37. Mahendran, Y. *et al.* Association of Ketone Body Levels With Hyperglycemia and Type 2 Diabetes in 9,398 Finnish Men. *Diabetes* **62**, 3618-3626 (2013).
38. Fischer, K. *et al.* Biomarker profiling by nuclear magnetic resonance spectroscopy for the prediction of all-cause mortality: an observational study of 17,345 persons. *PLoS Med* **11**, e1001606 (2014).
39. Singh-Manoux, A. *et al.* Association between inflammatory biomarkers and all-cause, cardiovascular and cancer-related mortality. *CMAJ* (2016).
40. Harley, J. *et al.* Orosomucoid influences the response to antidepressants in major depressive disorder. *J Psychopharmacol* **24**, 531-5 (2010).
41. El-Beblawy, N.M.S. *et al.* Serum and Urinary Orosomucoid in Young Patients With Type 1 Diabetes: A Link Between Inflammation, Microvascular Complications, and Subclinical Atherosclerosis. *Clinical and Applied Thrombosis-Hemostasis* **22**, 718-726 (2016).
42. Carriere, I. *et al.* Biomarkers of inflammation and malnutrition associated with early death in healthy elderly people. *Journal of the American Geriatrics Society* **56**, 840-846 (2008).

43. Bruno, R. *et al.* alpha-1-acid glycoprotein as an independent predictor for treatment effects and a prognostic factor of survival in patients with non-small cell lung cancer treated with docetaxel. *Clinical Cancer Research* **9**, 1077-1082 (2003).
44. Bos, D. *et al.* Calcification in Major Vessel Beds Relates to Vascular Brain Disease. *Arteriosclerosis Thrombosis and Vascular Biology* **31**, 2331-2337 (2011).
45. Bos, D. *et al.* Comparison of Atherosclerotic Calcification in Major Vessel Beds on the Risk of All-Cause and Cause-Specific Mortality: The Rotterdam Study. *Circ Cardiovasc Imaging* **8** (2015).
46. Wurtz, P. *et al.* Metabolite profiling and cardiovascular event risk: a prospective study of 3 population-based cohorts. *Circulation* **131**, 774-85 (2015).
47. Wishart, D.S. *et al.* HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res* **41**, D801-7 (2013).
48. Stary, H.C. *et al.* A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb Vasc Biol* **15**, 1512-31 (1995).