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Metabolic products of the intestinal microbiome and extremes of atherosclerosis

Chrysi Bogiatzi [a, b], Gregory Gloor [c], Emma Allen-Vercoe [d], Gregor Reid [e], Ruth G. Wong [c], Bradley L. Urquhart [f], Vincent Dinculescu [g], Kelsey N. Ruetz [f], Thomas J. Velenosi [f], Michael Pignanelli [h], J. David Spence [a, i, *]

[a] Stroke Prevention & Atherosclerosis Research Centre, Robarts Research Institute, Western University, London, Canada
[b] Dept. of Neurology, McMaster University, Hamilton, Canada
[c] Dept. Biochemistry, Schulich School of Medicine and Dentistry, Western University, London, Canada
[d] Dept. Molecular and Cell Biology, University of Guelph, Guelph, Canada
[e] Depts. of Urology and Microbiology, Schulich School of Medicine and Dentistry, Western University, London, Canada
[f] Dept. Physiology & Pharmacology, Schulich School of Medicine and Dentistry Western University, London, Canada
[g] Department of Radiology, University of Calgary, Calgary, Canada
[h] Schulich School of Medicine and Dentistry M.D. Candidate (CIHR Summer Research Training Program), Canada
[i] Divisions of Neurology and Clinical Pharmacology, Western University, London, Canada

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ABSTRACT

Background and aims: There is increasing awareness that the intestinal microbiome plays an important role in human health. We investigated its role in the burden of carotid atherosclerosis, measured by ultrasound as total plaque area.

Methods: Multiple regression with traditional risk factors was used to identify three phenotypes among 316/3056 patients attending vascular prevention clinics. Residual score (RES; i.e. the distance off the regression line, similar to standard deviation) was used to identify the 5% of patients with much less plaque than predicted by their risk factors (Protected, RES <-2), the 90% with about as much plaque as predicted (Explained, RES -2 to 2), and the 5% with much more plaque than predicted (Unexplained RES >2). Metabolic products of the intestinal microbiome that accumulate in renal failure – gut-derived uremic toxins (GDUT) – were assayed in plasma by ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry.

Results: Plasma levels of trimethylamine n-oxide (TMAO), *p*-cresyl sulfate, *p*-cresyl glucuronide, and phenylacetylglutamine were significantly lower among patients with the Protected phenotype, and higher in those with the Unexplained phenotype, despite no significant differences in renal function or in dietary intake of nutrient precursors of GDUT. In linear multiple regression with a broad panel of risk factors, TMAO ($p = 0.011$) and *p*-cresyl sulfate ($p = 0.011$) were significant independent predictors of carotid plaque burden.

Conclusions: The intestinal microbiome appears to play an important role in atherosclerosis. These findings raise the possibility of novel approaches to treatment of atherosclerosis such as fecal transplantation and probiotics.

1. Introduction

Khera and Kathiseran reviewed recently the concept that whereas LDL cholesterol may dominate atherosclerosis in a subset of the population, a quantitative blend of causal genetic and environmental factors underlies the majority of coronary artery disease cases [1]. Patients with chronic kidney disease are at very high risk of cardiovascular disease. Wheeler [2] estimated that the

Abbreviations: ADMA, asymmetric dimethylarginine; CKD, chronic kidney disease; DNA, deoxyribonucleic acid; FFQ, food frequency questionnaire; GDUT, gut-derived uremic toxins; RES, residual score in linear regression; RNA, ribonucleic acid; TMAO, trimethylamine N-oxide; TPA, total plaque area.

* Corresponding author. Stroke Prevention & Atherosclerosis Research Centre, Robarts Research Institute, Western University, 1400 Western Road, London, N6G 2V4, ON, Canada.
E-mail address: dspence@robarts.ca (J.D. Spence).
URL: http://www.imaging.robarts.ca/sparc

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cardiovascular risk of patients with end-stage renal failure was increased 17-fold. Gansevoort et al. [3] reported that life expectancy at age 55 was markedly reduced by renal failure; from 19.9 years with normal or only slightly impaired renal function to 5.6 years with severe renal impairment.

Uremic toxins that might increase cardiovascular risk in renal failure include high plasma levels of total homocysteine (tHcy), asymmetric dimethylarginine (ADMA), a nitric oxide antagonist, and thiocyanate [4]. A study by our group indicated that tHcy mediated only ~ 20% of the effect of uremic toxins on atherosclerosis [4]. It seems likely that other uremic toxins mediate a substantial proportion of the effect of renal failure on atherosclerosis. Many toxins that are eliminated by the kidney are produced by intestinal bacteria, and may be thought of as gut-derived uremic toxins (GDUT). The relatively recent recognition that the intestinal microbiome has profound effects on the host has revolutionized thinking about health and disease [5]. One area of particular interest is the interaction between diet, the intestinal microbiome, renal function and cardiovascular disease [6].

One key GDUT is trimethylamine n-oxide (TMAO), a compound that results from hepatic oxidation of trimethylamine [7]. Trimethylamine, which accounts for the fishy breath of uremic patients [8], is naturally abundant in fish [9] and is produced by intestinal bacteria from phosphatidylcholine [7] (largely from egg yolk), and carnitine [10] (largely from red meat). Hazen and colleagues reported that TMAO can cause atherosclerosis in rodent models [10,11], accumulates in renal failure and accelerates decline of renal function and cardiovascular events in patients with renal failure [12]. Levels of TMAO are normalized by renal transplantation [13]. Among patients referred for coronary angiography, levels of TMAO in the top quartile after a test dose of 2 hard-boiled eggs (each containing ~ 250 mg of phosphatidylcholine) were associated with a 2.5-fold increase in the 3-year risk of stroke, myocardial infarction or cardiovascular death [7]. Increased concentrations of TMAO correlate with both eGFR and coronary atherosclerosis in patients with chronic kidney disease (CKD) [13]. One mechanism that may be involved is that TMAO appears to increase thrombosis [14].

Other GDUTs including indoxyl sulfate, *p*-cresyl sulfate, *p*-cresyl glucuronide and phenylacetylglutamine, have also recently been implicated in mediating cardiovascular disease in CKD patients [15–18].

The aim of the present study was to analyze levels of GDUT in patients phenotyped by the difference between their measured carotid plaque burden and the plaque burden predicted by traditional coronary risk factors in linear regression. The quantitative trait is the residual score in linear multiple regression (similar to the distance off the regression line, in standard deviations). Such phenotyping was originally described as a way to reduce sample sizes for genetic studies of atherosclerosis [19,20], but in this study, it was employed to analyze effects of the intestinal microbiome. We hypothesized that patients with a high plaque burden not explained by traditional risk factors (Unexplained Atherosclerosis) would have higher levels of GDUT, and those with much less plaque than predicted by traditional risk factors (Protected) would have lower levels.

2. Materials and methods

2.1. Patient population

Study participants were patients of JDS, recruited from the Stroke Prevention Clinic and the Premature Atherosclerosis Clinic at University Hospital, London, Ontario, Canada. Measurement of carotid plaque burden (total plaque area, TPA) has been used in these prevention clinics since 1995, for risk stratification, genetic research and management of patients [14]. Residual scores were computed from a database of 3056 patients with measurement of their TPA and complete data for the risk factors used in the linear regression model. The original intent was to recruit patients at the extremes of atherosclerosis (unexplained and protected) using residual scores from the regression model with all 3056 patients. However, after they were recruited, an erroneous extremely high value of LDL-C for one of the subjects was found to have distorted the regression model. When this was corrected, the residual scores were recalculated, and we found we had recruited three groups: unexplained, explained and protected. In a way this accident worked out well, as we were able to compare all three groups.

Patients were grouped into three phenotypes: Protected (meaning the 5% extreme group that had much less carotid plaque than predicted by traditional risk factors, with very low residual scores below −2 and approaching −4); Explained (the 90% of patients whose plaque burden was largely explained by traditional risk factors, with residual scores between −2 and 2); and Unexplained (the 5% extreme with low levels of risk factors and a very high plaque burden, with residual scores > 2 and approaching 4). Residual scores were computed in a model in which the dependent variable in the model was total plaque area (normalized by a cube root transformation); backward (Wald) linear regression was performed (probability of F-to-remove ≥0.10) with age, sex, diabetes, smoking status, estimated glomerular filtration rate (eGFR CKD-Epi), systolic and diastolic blood pressures, total cholesterol, triglycerides, low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) as independent variables.

2.2. Ethics

Participants gave written informed consent to an ethics protocol approved by the Western University Health Sciences Research Ethics board (approval number 12107E).

2.3. Dietary assessment and nutrient analysis

Nutrient intake was assessed at the time of enrolment in the study by the Harvard Food Frequency Questionnaire (FFQ; also known as the Willett FFQ). We used the 4-page (Blue) grid, updated in 2011, a well-validated and widely accepted FFQ. Nutrient analysis was carried out at the Harvard School of Public Health, under the supervision of Prof. Walter Willett.

2.4. Calculation of TMAO precursors including L-carnitine

Total protein and amino acids consumed per day were determined by summation of their density in each item on the FFQ as described above. Total indigestible fiber determination was accomplished using biochemical and/or enzymatic methods approved by the Association of Analytic Communities (AOAC) [21]. Total choline-containing nutrients included the sum of water-soluble choline-containing compounds (free choline, glycerophosphocholine, phosphocholine) and lipid membrane associated choline (phosphatidylcholine, sphingomyelin) and total betaine (without supplementation).

The following items from the FFQ were used to estimate total free L-carnitine: “chicken sandwich”, “chicken without skin”, “chicken liver”, “liver”, “bacon”, “extra-lean hamburger”, “hamburger”, “pork”, “beef or lamb as a main dish”, “tuna”, “shrimp”, “dark fish”. Although not exhaustive for all sources of L-carnitine, these entries were selected because meat products are the most important sources of L-carnitine in human diets [22]. Concentrations of free L-carnitine in individual food items was estimated using values published by Demarquoy et al. [23], and