RHEUMATOLOGY

Rheumatology 2020;59:3369–3379
doi:10.1093/rheumatology/keaa125
Advance Access publication 20 April 2020

Original article

Circulating plasma metabolites and risk of rheumatoid arthritis in the Nurses' Health Study

Su H. Chu [1,3], Jing Cui[2,3], Jeffrey A. Sparks [2,3], Bing Lu[2,3], Sara K. Tedeschi[2,3], Cameron B. Speyer[2], LauraKay Moss[4], Marie L. Feser[4], Lindsay B. Kelmenson[4], Elizabeth A. Mewshaw[5], Jess D. Edison[5], Kevin D. Deane[4], Clary Clish[6], Jessica Lasky-Su[1,3], Elizabeth W. Karlson[2,3,*] and Karen H. Costenbader[2,3,*]

Abstract

Objectives. RA develops slowly over years. We tested for metabolic changes prior to RA onset using a large non-targeted metabolomics platform to identify novel pathways and advance understanding of RA development.

Methods. Two hundred and fifty-four incident RA cases with plasma samples drawn pre-RA onset in the Nurses' Health Study (NHS) cohorts were matched 1:2 to 501 controls on age, race, menopause/post-menopausal hormone use and blood collection features. Relative abundances of 360 unique, known metabolites were measured. Conditional logistic regression analyses assessed associations between metabolites and incidence of RA, adjusted for age, smoking and BMI, accounting for multiple comparisons. Subgroup analyses investigated seropositive (sero+) RA and RA within 5 years of sample collection. Significant metabolites were then tested in a female military pre-RA case–control study ($n = 290$).

Results. In the NHS, metabolites associated with RA and sero+RA in multivariable models included 4-acetamidobutanoate (odds ratio (OR) = 0.80/S.D., 95% CI: 0.66, 0.95), *N*-acetylputrescine (OR = 0.82, 95% CI: 0.69, 0.96), C5 carnitine (OR = 0.84, 95% CI: 0.71, 0.99) and C5:1 carnitine (OR = 0.81, 95% CI: 0.68, 0.95). These were involved primarily in polyamine and leucine, isoleucine and valine metabolism. Several metabolites associated with sero+RA within 5 years of diagnosis were replicated in the independent military cohort: C5 carnitine (OR = 0.55, 95% CI: 0.33, 0.92), C5:1 carnitine (OR = 0.62, 95% CI: 0.39, 0.99) and C3 carnitine (OR = 0.57, 95% CI: 0.36, 0.91).

Conclusion. Several metabolites were inversely associated with incidence of RA among women. Three short-chain acylcarnitines replicated in a smaller dataset and may reflect inflammation in the 5-year period prior to sero+RA diagnosis.

Key words: metabolomics, rheumatoid arthritis, pathogenesis, metabolites, carnitines

Rheumatology key messages

- Untargeted metabolic profiling of women pre-RA compared with controls identified several circulating metabolite abnormalities.
- Acylcarnitine depletion, related to cellular oxidation, inflammation and muscle-wasting, was observed among women pre-RA.
- Decreased cholesterol ester and polyamine concentrations pre-RA may reflect lipid catabolism and inflammation.

[1]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital, [2]Section of Clinical Sciences, Division of Rheumatology, Brigham and Women's Hospital, [3]Department of Medicine, Harvard Medical School, Boston, MA, [4]Department of Medicine, University of Colorado Denver, Denver, CO, [5]Walter Reed National Military Medical Center, Bethesda, MD and [6]Broad Institute of MIT and Harvard, Boston, MA, USA

Submitted 4 September 2019; accepted 6 February 2020

Correspondence to: Karen H. Costenbader, Section of Clinical Sciences, Division of Rheumatology, Brigham and Women's Hospital, Boston, MA 02115, USA. E-mail: kcostenbader@bwh.harvard.edu

*Elizabeth W. Karlson and Karen H. Costenbader contributed equally to this study.

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CLINICAL SCIENCE

Introduction

Metabolomics is the study of small molecules (metabolites) that are products or intermediates of metabolic activity. Metabolite relative abundances provide a snapshot of biological processes arising through genetic, environmental or pharmacological influences in a biosample [1]. RA is a chronic autoimmune disease that develops slowly, with elevated inflammatory markers and autoantibodies, including IL-6, RF and ACPA, detectable even 15 years before onset, and at higher concentrations within 5 years of onset [2]. A few small studies have examined metabolites in *prevalent* rheumatic diseases, including OA [3, 4], PsA [5] and RA [6, 7]. In a Swedish study, a 52-metabolite panel distinguished 25 RA from 20 PsA patients with high sensitivity and specificity [8]. Most studies have investigated RA years after onset, when multiple therapies have been received. Metabolomics of biosamples prior to RA may advance understanding of early events in pathogenesis not driven by longstanding disease or therapies.

We therefore conducted a metabolome-wide association study among women at risk of RA in the Nurses' Health Study (NHS) and aimed to replicate the strongest findings in pre-RA samples from women in the Department of Defense (DoD) cohort. Metabolic derangements pre-RA could provide critical information about specific and novel biological pathways targetable for prevention.

Methods

Primary study subjects

Participants were from the NHS and NHS II (NHSII), large prospective cohorts of US female nurses. The NHS began in 1976, recruiting 121 700 female nurses 30–55 years of age, and NHSII began in 1989, recruiting 116 608 female nurses 25–42 years of age. In both cohorts, medical history, diet and lifestyle factors were assessed every 2 years via self-reported questionnaires. Plasma samples were collected 1989–90 in the NHS and 1996–99 in the NHSII.

Incident RA diagnoses after blood collection were confirmed among nurses who self-reported RA by obtaining and reviewing medical records, as described [9]. Criteria for confirmation of RA included (i) RA classification by 1987 ACR [10] or 2010 ACR/EULAR criteria [11], and (ii) confirmation of diagnosis by medical record review by two rheumatologists. Each incident RA case with available plasma >3 months prior to RA onset (pre-RA) was matched to two controls based on age, race, menopausal status, post-menopausal hormone use and fasting status, time of day, and date of blood draw. An overview of the study design is available in Figure 1, and a review of the nested case–control selection process can be seen in Supplementary Fig. S1, available at *Rheumatology* online.

This protocol was approved by the Brigham and Women's Hospital and the Harvard T.H. Chan School of Public Health Institutional Review Boards. Participant consent for blood sample use was obtained and participants were given the opportunity to opt-out of all 'omics-based research'.

Collection of clinical covariates and metabolomics profiling

Clinical covariates

Covariates assessed on questionnaires immediately preceding sample collection included cigarette smoking (cumulative pack years) and BMI. Seropositive (sero+) RA status was determined based on the presence of RF or ACPA by chart review, or by anti-CCP antibodies in available blood samples by second generation Diastat ELISA (Axis-Shield Diagnostics, Dundee, UK) or the Bioplex multiplatform assay platform (Bio-Rad Laboratories, Hercules, CA, USA), as described [12, 13].

Tissue sample collection and metabolomics profiling

We included 767 plasma samples (NHS, 511; NHSII, 256) with plasma available >3 months prior to RA onset for metabolomic profiling. Blood samples were collected in heparin tubes, shipped overnight with an icepack to a central laboratory, processed into plasma aliquots and stored in a liquid nitrogen freezer. Most samples ($>95\%$) were received <24 h after blood draw. Non-targeted, global metabolite profiles were generated at the Broad Institute (Cambridge, MA, USA). Three complimentary liquid chromatography–tandem mass spectrometry (LC-MS/MS) metabolomics methods were applied: hydrophilic interaction liquid chromatography coupled with positive ion mode MS detection to measure amines and polar metabolites (HILIC-positive); C8 chromatography coupled with positive ion mode MS detection to measure polar and non-polar lipids (C8-positive); and C18 chromatography coupled with negative ion mode MS detection to measure free fatty acids, bile acids and metabolites of intermediate polarity (C18-negative) [14, 15]. Briefly, LC-MS/MS systems comprised of Nexera X2 U-HPLC systems (Shimadzu Corp.; Marlborough, MA, USA) coupled to Q Exactive or Exactive Plus orbitrap mass spectrometers (Thermo Fisher Scientific; Waltham, MA, USA), obtained high resolution mass profiling data. Raw LC-MS/MS data were processed using TraceFinder software (version 3.3, Thermo Fisher Scientific) and Progenesis QI (Nonlinear Dynamics; Newcastle upon Tyne, UK) for feature detection and signal integration. Metabolites were identified by matching to authentic reference standards and samples.

For quality control (QC), 85 pooled plasma samples were interspersed at intervals of approximately 20 participant samples to assess temporal drift in instrument response and batch effects on the metabolomics platforms. Metabolites with mean coefficient of variation $>25\%$, an intra-class correlation of $<40\%$, poor reproducibility after delays in processing or low within-person stability were excluded [14]. In metabolites with missingness in $\leq 25\%$ of the subjects, missing values were imputed with half of the minimum value observed per

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metabolite to avoid excluding metabolites with true missingness patterns (e.g. xenobiotics). Subjects with ≥10% missingness in metabolites were excluded; 755 subject plasma samples passed QC processing for a total of 254 pre-RA cases and 501 matched controls. Metabolite levels were log-transformed and Pareto-scaled within cohort and then pooled. A total of 360 known metabolites, that is, those with assigned identification numbers in the Human Metabolome Database, were included in analyses.

External replication study sample

The DoD Serum Repository was started in 1985 to monitor the health of US military personnel, collecting samples at enlistment, deployment and every other year of service. Possible RA cases were identified based on ≥2 International Classification of Disease codes for RA in the medical record, and ≥1 rheumatologist encounter. A total of 346 RA cases meeting 1987 ACR criteria by chart review, or receipt of an RA diagnosis from a rheumatologist were identified. Serum samples were obtained from military personnel in a variety of settings, from medical facilities and clinics to warehouses. Blood collection protocols, serum processing and storage varied; all specimens were sent to the nearest medical facility for processing to serum, transported to the DoD Serum Repository facilities and shipped to a central lab for storage at −30°C [16]. Controls were matched based on RA diagnosis index date, sex, race, enlistment region and sample storage duration. Subject data were abstracted, including demographics, smoking status, height and weight, in standardized form. Female subjects were included in this analysis, for a final replication sample size of 290.

Metabolomics profiling of DoD samples was conducted at the Broad Institute using the same C18-negative and HILIC-positive methods applied to NHS samples. A total of 59 QC samples were interspersed throughout the DoD samples, each with three replicates. QC filtering procedures above were applied. After QC, 349 known metabolites were identified.

Statistical analyses

Associations between metabolites and RA

For each metabolite, associations with incident RA were identified via conditional logistic regression to account for the matched case–control study design. Adjustment for age at blood draw was made to account for potential residual confounding by age. Each metabolite model was also adjusted for BMI and smoking pack-years, which are risk factors for RA and are also associated with metabolites.

Adjustment for multiple comparisons and signal selection

Because metabolites belonging to the same pathways or classes may demonstrate high correlation, adjustment for multiple comparisons was made using a principal-components analysis-based approach to identify the number of effective tests [17]. Principal-components analysis was applied to determine the number of principal components explaining ∼85% of the total variance within the metabolomics data across the pooled NHS/NHSII subjects for all three metabolomics platforms, jointly. Then, similar to Bonferroni correction procedures, the adjusted *P*-value threshold accounting for multiple comparisons was calculated as α/M, where $\alpha = 0.05$ is the nominal significance threshold, and M is the number of effective tests (i.e. the number of components explaining 85% of the variance). In the NHS, 58 components were required to explain 85% of the variance, and thus a threshold of $P = 8.6 \times 10^{-4}$ was considered statistically significant (Supplementary Fig. S2, available at *Rheumatology* online).

Multiple testing corrections based on *post hoc* statistical estimation of the number of total independent principal components (as in the number of effective tests approach) may nonetheless produce an overly conservative correction given the highly dense correlation structure observed across the metabolome. As such, we also employed a secondary signal filtering approach via second generation *P*-values [18]. Second generation *P*-values (P_δ) are derived by directly comparing the 95% CI of an estimate of interest with a null interval (i.e. an interval containing a range of effect sizes that are not biologically or scientifically meaningful). This approach has been shown to protect type 1 error rates and reduce false discovery rates comparable to standard thresholding false discovery rate procedures [18, 19]. Signal selection proceeds by characterizing compatibility of the data with the null hypothesis ($P_\delta = 1$) or with the alternative ($P_\delta = 0$), or a weighted range when the data are inconclusive ($0 < P_\delta < 1$). The values of P_δ correspond to the proportion of the data-supported hypotheses that fall in the null interval and are consistent with effects that are not clinically meaningful. To obtain P_δ values for this study, we employed a uniform null hypothesis interval of (0.95, 1.05), and a selection threshold of $P_\delta = 0$. Metabolites with P_δ values $= 0$ in NHS analyses were chosen for replication in the DoD dataset.

Stratified analyses: Sero+ RA and time to diagnosis

Subgroup analyses were performed for the following clinical subgroups: (i) pre-sero+ RA; (ii) blood drawn between 1 and 5 years before RA diagnosis; and (iii) blood drawn between 1 and 5 years before sero+ RA diagnosis. All subgroup analyses were conducted using the conditional logistic regression models of the main analysis.

Replication dataset: DoD

A DoD female pre-RA case–control study was conducted to replicate the most strongly associated metabolites in the NHS analyses, both for all RA and for sero+ RA. The mean duration from blood draw to RA diagnosis date among DoD pre-RA cases was 2.6 years (comparable to the 1–5 years prior to RA analyses in NHS). Pre-RA cases and controls were matched on age at index date, race, recruitment region and duration and breadth

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of sample collection. As data for some of the NHS blood draw matching factors were not available, DoD analyses were conducted using unconditional logistic regression, adjusting for age at blood draw, race and time from blood draw to diagnosis/index date. As in the NHS, a multivariable model adjusting for BMI and smoking history (ever/never) was performed to investigate the effects of these factors.

Results

Clinical features of the NHS study sample

Matched case–control sets comprised white, female nurses, and the mean age at blood draw was 51.5 years of age (Table 1). Plasma was drawn in the morning (83.9%) across the majority of matched sets with available time of blood draw information (93.7%). Most matched sets had plasma samples drawn under fasting conditions (68.1%). The proportions of pre-RA cases and controls who were ever-smokers were 53.5 and 48.3%, respectively, and the smoking history among cases was greater [median (interquartile range; IQR) 30 (10–31) pack-years] than in controls [median (IQR) 14 (5–28.8) pack-years]. Among pre-RA cases, the mean age at RA diagnosis was 61.3 years, plasma was drawn <5 years to diagnosis among 26.4%, 15.6% were seropositive at time of blood draw and 62.2% of the total were eventually diagnosed with sero+ RA.

Metabolites associated with risk of RA

After adjustment for age at blood draw, smoking and BMI, one metabolite, spermidine (odds ratio (OR) = 1.27 per S.D., 95% CI: 1.05, 1.53), was associated with increased risk of RA. Metabolites associated with decreased incidence of RA per S.D. included 4-acetamidobutanoate (OR = 0.79, 95% CI: 0.66, 0.95), *N*-acetylputrescine (OR = 0.82, 95% CI: 0.69, 0.96), C16:1 cholesterol ester (CE) (OR = 0.83, 95% CI: 0.70, 0.98), *N*-acetyltryptophan (OR = 0.84, 95% CI: 0.71, 0.99), C5 carnitine (OR = 0.86, 95% CI: 0.71, 0.99) and C5:1 carnitine (OR = 0.81, 95% CI: 0.68, 0.95) (Table 2). Although no metabolites were significantly associated with incidence of RA after adjustment for multiple comparisons using the number of effective tests threshold of $P < 8.6 \times 10^{-4}$, 4-acetamidobutanoate remained significantly associated using the second-generation *P*-value threshold of $P_\delta = 0$. Models adjusting for only age at blood draw revealed similar effect estimates as those reported in the fully adjusted models.

Metabolites associated with risk of sero+RA

After adjustment for age at blood draw, smoking and BMI, metabolites associated with decreased risk of sero+ RA up to a nominal *P*-value threshold of 0.05 per S.D. included 4-acetamidobutanoate (OR = 0.76, 95% CI:0 0.61, 0.94), *N*-acetylputrescine (OR = 0.78, 95% CI: 0.63, 0.97), 3-oxooctadecanoate (OR = 0.76, 95% CI: 0.59, 0.98), C16:1 CE (OR = 0.80, 95% CI: 0.65, 0.99), C36:0 phosphatidylethanolamine (PE) (OR = 0.80, 95% CI: 0.64, 0.99), methionine sulphoxide (OR = 0.78, 95% CI: 0.60, 1.00) and several short chain carnitines: C5 carnitine (OR = 0.73, 95% CI: 0.58, 0.91), C5:1 carnitine (OR = 0.76, 95% CI: 0.61, 0.95), 3-dehydroxycarnitine (OR = 0.77, 95% CI: 0.61, 0.96), C7 carnitine (OR = 0.7, 95% CI: 0.61, 0.96) and C3 carnitine (OR = 0.79, 95% CI: 0.63, 0.98). Three metabolites remained significantly associated with incidence of sero+RA using a second-generation *P*-value threshold of $P_\delta = 0$: 4-acetamidobutanoate, C5 carnitine and C5:1 carnitine—although none were significantly associated after adjustment for

Table 1 Characteristics of the NHS study sample

Characteristic	Pre-RA cases (*n* = 254)	Controls (*n* = 501)	All (*n* = 755)
Age at blood draw[a], mean (S.D.), years	51.3 (8.0)	51.6 (7.9)	51.5 (7.9)
Menopause and PMH at blood draw[a], *n* (%)			
Premenopausal, no PMH	97 (38.2)	192 (38.3)	289 (38.3)
Postmenopausal, no PMH	50 (19.7)	100 (20.0)	150 (20.0)
Postmenopausal, past PMH	76 (28.9)	150 (29.9)	226 (29.9)
Postmenopausal, current PMH	31 (12.2)	59 (11.8)	90 (11.9)
Smoking status, ever, *n* (%)	136 (53.5)	242 (48.3)	378 (50.1)
Pack-years among smokers			
Median (IQR)	30 (10–31)	14 (5–28.8)	17 (6–30)
Range	1–76	1–104	1–104
BMI, mean (S.D.), kg/m^2	25.8 (4.9)	25.1 (4.6)	25.4 (4.7)
Case-only features			
Age at diagnosis[a], mean (S.D.), years	61.2 (10.3)		
Seropositive status[a], *n* (%)			
Seropositive, at blood draw	39 (15.6)		
Seropositive, ever	158 (62.2)		
Time to RA from blood draw[a], *n* (%)			
< 5 years to diagnosis	67 (26.4)		

[a]Match factors. PMH: post-menopausal hormone use.

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Table 2 Metabolites associated with incidence of all RA in the NHS/NHSII

Name	HMDB	Super class	Subpathway	Model 1[a]			Model 2[b]		
				OR (95% CI)	P^c	P_δ	OR (95% CI)	P^c	P_δ
4-Acetamidobutanoate	**03681**	**Amino acid**	**Polyamine metabolism**	**0.814 (0.685, 0.968)**	**0.020**	**0.092**	**0.791 (0.663, 0.945)**	**0.010**	**0**
C5:1 carnitine	02366	Amino acid	Leucine, isoleucine and valine metabolism	0.803 (0.681, 0.947)	0.009	0	0.808 (0.684, 0.954)	0.012	0.019
Spermidine	01257	Amino acid	Polyamine metabolism	1.277 (1.06, 1.538)	0.010	0	1.266 (1.046, 1.533)	0.016	0.021
N-Acetylputrescine	02064	Amino acid	Polyamine metabolism	0.840 (0.713, 0.99)	0.038	0.201	0.815 (0.69, 0.964)	0.017	0.069
C16:1 CE	00658	Lipid	Fatty acid metabolism	0.876 (0.746, 1.03)	0.109	0.4	0.829 (0.702, 0.979)	0.027	0.147
C5 carnitine	00688	Amino acid	Leucine, isoleucine and valine metabolism	0.862 (0.732, 1.016)	0.077	0.33	0.835 (0.705, 0.989)	0.037	0.197
N-Acetyltryptophan	13713	Amino acid	Tryptophan metabolism	0.839 (0.71, 0.992)	0.040	0.21	0.837 (0.707, 0.99)	0.038	0.199

$n = 755$; number of cases/number of controls = 254/501. Significant metabolites based on Model 2 P_δ values are displayed in bold. [a]Model 1: adjusted for age at blood draw. [b]Model 2: adjusted for age at blood draw, BMI and smoking. [c]Number of effective tests significance threshold: $P < 8.6 \times 10^{-4}$. P_δ: second generation *P*-value (i.e. the proportion of the CI for the effect estimate that supports the interval null hypothesis; significance threshold is $P_\delta = 0$). HMDB: Human Metabolome Database; NHS: Nurses' Health Study; NHSII: Nurses' Health Study II; OR: odds ratio.

multiple comparisons using the number of effective tests threshold of $P < 8.6 \times 10^{-4}$ (**Table 3**).

Metabolites associated with risk of RA and sero+ RA within 5 years of diagnosis

In the multivariable models, metabolites associated with increased risk of RA and sero+ RA within 5 years of diagnosis up to a nominal *P*-value threshold of 0.05 can be seen in Table 4. Although no metabolites were significantly associated with either RA or sero+ RA incidence in these subgroup analyses after adjustment for multiple comparisons using the number of effective tests, a second-generation *P*-value threshold of $P_\delta = 0$ revealed associations between homoarginine (OR = 1.71, 95% CI: 1.20, 2.45), N-Monomethyl-L-arginine (NMMA) (OR = 1.58, 95% CI: 1.13, 2.23), spermidine (OR = 1.58, 95% CI: 1.05, 2.35) and incidence of all RA. In sero+ RA, six metabolites were significant at $P_\delta = 0$: homoarginine (OR = 2.25, 95% CI: 1.33, 3.80), *N*-acetyltryptophan (OR = 0.56, 95% CI: 0.35, 0.91), C3 carnitine (OR = 0.57, 95% CI: 0.36, 0.91), C5 carnitine (OR = 0.55, 95% CI: 0.33, 0.93), creatinine (OR = 0.56, 95% CI: 0.34, 0.93) and C38:6 PE plasmalogen (OR = 1.71, 95% CI: 1.05, 2.78). In sero+ RA, the effects among inversely associated metabolites were attenuated with adjustment for BMI and smoking.

External validation in the DoD

Replication sample

The DoD sample consisted of females with a mean age at blood draw of 29.8 years (31.9 years among cases, 27.7 years among controls) and mean age at RA diagnosis was 35.5 years. Similar distributions of ever-smoking history were observed among cases and controls: 24.8% *vs* 25.5%. DoD subjects were racially diverse: 43.1% white, 23.1% black and 33.8% other race/ethnicity. Among RA cases, 83.5% were sero+ at diagnosis. More characteristics of the DoD are shown in Supplementary Table S1, available at *Rheumatology* online.

Replication of metabolites associated with RA and sero+RA within 5 years of diagnosis

Three metabolites from NHS analyses of incidence of sero+ RA within 5 years were replicated in DoD at $P < 0.05$: C3 carnitine (OR = 0.57, 95% CI: 0.36, 0.89; $P = 0.015$), C5 carnitine (OR = 0.68, 95% CI: 0.49, 0.94; $P = 0.019$) and C5:1 carnitine (OR = 0.65, 95% CI: 0.45, 0.95; $P = 0.027$) (Table 5). For all RA, however, no metabolites found in the NHS case–control study replicated in the DoD at this significance level. The list of replication results for top NHS metabolites is shown in Supplementary Tables S2 and 3, available at *Rheumatology* online.

Discussion

In this large case–control study nested within a cohort of women with banked blood samples who were followed prospectively for the development of RA, we identified several metabolites associated with incidence of RA. Within the NHS cohort study, 4-acetomidobutanoate and

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Table 3 Metabolites associated with incidence of seropositive RA in the NHS/NHSII

Name	HMDB	Super class	Subpathway	Model 1[a]			Model 2[b]		
				OR (95% CI)	P^c	P_δ	OR (95% CI)	P^c	P_δ
C5 carnitine	**00688**	**Amino acid**	**Leucine, isoleucine and valine metabolism**	**0.768 (0.618, 0.955)**	**0.018**	**0.025**	**0.725 (0.577, 0.912)**	**0.006**	**0**
4-Acetamidobutanoate	**03681**	**Amino acid**	**Polyamine metabolism**	**0.791 (0.640, 0.979)**	**0.031**	**0.143**	**0.755 (0.605, 0.943)**	**0.013**	**0**
C5:1 carnitine	**02366**	**Amino acid**	**Leucine, isoleucine and valine metabolism**	**0.749 (0.602, 0.931)**	**0.009**	**0**	**0.758 (0.608, 0.946)**	**0.014**	**0**
3-Dehydroxycarnitine	06831	Lipid	Fatty acid metabolism	0.743 (0.599, 0.923)	0.007	0	0.766 (0.614, 0.956)	0.018	0.031
C7 carnitine	13238	Lipid	Fatty acid metabolism	0.756 (0.604, 0.946)	0.015	0	0.766 (0.609, 0.964)	0.023	0.070
N-Acetylputrescine	02064	Amino acid	Polyamine metabolism	0.841 (0.685, 1.034)	0.100	0.419	0.784 (0.631, 0.973)	0.027	0.117
3-Oxooctadecanoate	10736	Lipid	Fatty acid metabolism	0.812 (0.639, 1.033)	0.090	0.415	0.759 (0.59, 0.978)	0.033	0.140
C3 carnitine	00824	Lipid	Fatty acid metabolism (also BCAA metabolism)	0.818 (0.662, 1.010)	0.062	0.302	0.787 (0.631, 0.981)	0.033	0.156
C16:1 CE	00658	Lipid	Fatty acid metabolism, steroid esters	0.863 (0.703, 1.060)	0.160	0.500	0.800 (0.647, 0.989)	0.039	0.196
C36:0 PE	08991	Lipid	Fatty acid/phospholipid metabolism	0.811 (0.657, 1.001)	0.051	0.255	0.800 (0.644, 0.994)	0.044	0.221

$n = 468$; number of cases/number of controls = 158/310. Significant metabolites based on Model 2 P_δ values are displayed in bold. [a]Model 1: adjusted for age at blood draw. [b]Model 2: adjusted for age at blood draw, BMI and smoking. [c]Number of effective tests significance threshold: $P < 8.6 \times 10^{-4}$. P_δ: second generation *P*-value (i.e. the proportion of the CI for the effect estimate that supports the interval null hypothesis; significance threshold is $P_\delta = 0$). BCAA: branched-chain amino acid; CE: cholesterol ester; HMDB: Human Metabolome Database; NHS: Nurses' Health Study; NHSII: Nurses' Health Study II; OR: odds ratio; PE: phosphatidylethanolamine.

short-chain acylcarnitines were reduced in women who later developed RA, while spermidine levels were elevated. These findings were stronger among the women who later developed sero+ RA and those with blood drawn in the 5 years prior to developing RA, compared with their matched controls. Homoarginine and NMMA were statistically elevated among women who were within 5 years of developing RA, and concentrations of short chain acylcarnitines were lower among women who later developed sero+ RA, in particular among those within 5 years of diagnosis. Several of the short-chain acylcarnitines (C3, C5 and C5:1 carnitine) were similarly significantly inversely associated with risk of sero+ RA occurring within a mean of 2.6 years in our replication pre-RA case–control study based in the DoD. We further identified *N*-acetyltryptophan, involved in polyamine and tryptophan metabolism, as well as a CE, C16:1 CE, as being related to incidence of RA.

The finding of metabolites associated with all RA and sero+ RA involved in polyamine metabolism among samples collected prior to RA diagnosis supports prior studies, including one study that reported increased polyamine catabolism and recycling in the synovial fibroblasts of prevalent RA cases relative to controls [20]. Our study adds to accumulating literature concerning the role of polyamines in chronic diseases associated with ageing and oxidative stress [21, 22]. Increasing concentrations of spermine and spermidine have been shown to protect spermine-deficient mammalian embryonic fibroblasts from cytotoxic damage [23], while polyamine catabolism via spermine oxidase has been shown to produce reactive oxygen species [24]. Oxidative stress has been associated with immunosenescence, premature ageing of the immune system with loss of immune regulation and abnormal T- and B-cell function, observed in RA [25, 26]. Anti-oxidative metabolite depletion as RA brews pre-diagnosis is interesting in this light.

Three metabolites inversely associated with risk of sero+ RA and sero+ RA within 5 years in NHS replicated in the DoD: C5, C5:1 and C3 carnitine. All are short chain acylcarnitines, involved in intracellular fatty acid metabolism. A Swedish blood bank study of 30 pre-RA cases and 19 non-RA controls reported a similar

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Table 4 Metabolites associated with incidence of RA within 5 years of diagnosis in the NHS/NHSII

Name	HMDB	Super class	Subpathway	Model 1[a]			Model 2[b]		
				OR (95% CI)	P^c	P_δ	OR (95% CI)	P^c	P_δ
All RA, <5 years to Dx									
Homoarginine	**00670**	**Amino acid**	**Urea cycle; arginine and proline metabolism**	**2.057 (1.24, 3.413)**	**0.005**	**0**	**1.712 (1.197, 2.45)**	**0.003**	**0**
NMMA	**29416**	**Amino acid**	**Urea cycle; arginine and proline metabolism**	**1.563 (1.08, 2.261)**	**0.018**	**0**	**1.583 (1.127, 2.225)**	**0.008**	**0**
Spermidine	**01257**	**Amino acid**	**Polyamine metabolism**	**1.701 (1.06, 2.728)**	**0.028**	**0**	**1.575 (1.054, 2.354)**	**0.027**	**0**
C18:0 SM-minor	01348	Lipid	Sphingolipids, fatty acid metabolism	1.104 (0.775, 1.572)	0.585	0.5	0.683 (0.476, 0.979)	0.038	0.144
C14:0 SM	12097	Lipid	Sphingolipids, fatty acid metabolism	0.913 (0.616, 1.355)	0.653	0.5	0.674 (0.463, 0.981)	0.040	0.157
5-HETE	11134	Lipid	Eicasanoid	1.460 (1.007, 2.116)	0.046	0.217	1.437 (1.016, 2.034)	0.041	0.172
4-Guanidinobutanoic acid	03464	Amino acid	Guanidino and acetamido metabolism	1.325 (0.788, 2.226)	0.288	0.5	1.647 (1.003, 2.705)	0.048	0.233
ADMA	01539	Amino acid	Urea cycle; arginine and proline metabolism	1.431 (0.972, 2.106)	0.069	0.39	1.448 (1.002, 2.091)	0.048	0.238
Sero+RA, <5 years to Dx									
Homoarginine	**00670**	**Amino acid**	**Urea cycle; arginine and proline metabolism**	**1.920 (1.071, 3.442)**	**0.029**	**0**	**2.246 (1.327, 3.8)**	**0.003**	**0**
C3 carnitine	**00824**	**Lipid**	**Fatty acid/BCAA metabolism**	**0.814 (0.516, 1.285)**	**0.377**	**0.5**	**0.570 (0.357, 0.911)**	**0.019**	**0**
***N*-Acetyltryptophan**	**13713**	**Amino acid**	**Tryptophan metabolism**	**0.626 (0.392, 1.001)**	**0.050**	**0.254**	**0.563 (0.347, 0.914)**	**0.020**	**0**
Creatinine	**00562**	**Amino acid**	**Creatine metabolism**	**0.553 (0.317, 0.965)**	**0.037**	**0.073**	**0.559 (0.337, 0.926)**	**0.024**	**0**
C5 carnitine	**00688**	**Amino acid**	**Leucine, isoleucine and valine metabolism**	**0.694 (0.426, 1.13)**	**0.142**	**0.5**	**0.552 (0.329, 0.928)**	**0.025**	**0**
NMMA	29416	Amino acid	Urea cycle; arginine and proline metabolism	1.562 (0.991, 2.462)	0.055	0.294	1.634 (1.048, 2.548)	0.030	0.008
C38:6 PE plasmalogen	**11387**	**Lipid**	**Fatty acid/phospholipid metabolism**	**1.637 (1.012, 2.65)**	**0.045**	**0.191**	**1.709 (1.053, 2.775)**	**0.030**	**0**
3-Dehydroxy carnitine	06831	Lipid	Fatty acid metabolism	0.624 (0.39, 0.999)	0.050	0.246	0.625 (0.406, 0.961)	0.032	0.057
Acetaminophen	01859	Xenobiotics	Drug—analgesics, anaesthetics	1.344 (0.841, 2.149)	0.217	0.5	1.688 (1.033, 2.759)	0.037	0.087
C5:1 carnitine	02366	Amino acid	Leucine, isoleucine and valine metabolism	0.655 (0.396, 1.084)	0.100	0.5	0.617 (0.385, 0.987)	0.044	0.186
Cytosine	00630	Nucleotide	Pyrimidine metabolism	0.573 (0.331, 0.992)	0.047	0.209	0.582 (0.34, 0.994)	0.048	0.22

All RA: $n = 200$; number of cases/number of controls=67/133. Sero+RA: $n = 98$, number of cases/number of controls=35/63. Significant metabolites based on Model 2 P_δ values are displayed in bold. [a]Model 1: Adjusted for age at blood draw. [b]Model 2: Adjusted for age at blood draw, BMI and smoking. [c]Number of effective tests significance threshold: $P < 8.6 \times 10^{-4}$. P_δ: second generation P-value (i.e. the proportion of the CI for the effect estimate that supports the interval null hypothesis; significance threshold is $P_\delta = 0$). 5-HETE: 5-Hydroxyeicosatetraenoic acid; ADMA: asymmetric dimethylarginine; BCAA: branched-chain amino acid; Dx: disease; HMDB: Human Metabolome Database; NHS: Nurses' Health Study; NHSII: Nurses' Health Study II; NMMA: N-Monomethyl-L-arginine; OR: odds ratio; PE: phosphatidylethanolamine; Sero+: seropositive; SM: sphingomyelin.

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Table 5 DoD replication of NHS/NHSII metabolites associated with incidence of RA within 5 years of diagnosis

Name	HMDB	Super class	Subpathway	Model 1[a]		Model 2[b]		
				OR (95% CI)	*P*	OR (95% CI)		*P*
All RA, <5 years to diagnosis								
NMMA	29416	Amino acid	Urea cycle; arginine and proline metabolism	0.878 (0.718, 1.074)	0.205	0.963 (0.764, 1.214)		0.752
Homoarginine	00670	Amino acid	Urea cycle; arginine and proline metabolism	1.090 (0.850, 1.398)	0.495	1.067 (0.803, 1.418)		0.654
Spermidine	01257	Amino acid	Polyamine metabolism	NM		NM		
Sero+ RA, <5 years to diagnosis								
C3 carnitine	**00824**	**Lipid**	**Fatty acid/BCAA metabolism**	**0.723 (0.500, 1.043)**	**0.083**	**0.568**	**(0.361, 0.896)**	**0.015**
C5 carnitine	**00688**	**Amino acid**	**Leucine, isoleucine and valine metabolism**	**0.736 (0.557, 0.973)**	**0.031**	**0.678**	**(0.489, 0.938)**	**0.019**
C5:1 carnitine	**02366**	**Amino acid**	**Leucine, isoleucine and valine metabolism**	**0.722 (0.525, 0.993)**	**0.045**	**0.652**	**(0.447, 0.953)**	**0.027**
Homoarginine	00670	Amino acid	Urea cycle; arginine and proline metabolism	1.095 (0.837, 1.431)	0.507	1.072	(0.783, 1.467)	0.666
N-Acetyltryptophan	13713	Amino acid	Tryptophan metabolism	0.944 (0.724, 1.232)	0.673	0.932	(0.691, 1.257)	0.645
Creatinine	00562	Amino acid	Creatine metabolism	0.772 (0.468, 1.276)	0.313	0.778	(0.44, 1.376)	0.388
C38:6 PE plasmalogen	01257	Lipid	Fatty acid/phospholipid metabolism	NM		NM		

All RA: model 1 $n = 290$, $n_{Ca/Co} = 145/145$; model 2: $n = 166$ (decrease in count due to missing data in BMI and smoking status), $n_{Ca/Co} = 83/83$. Seo+ RA: model 1: $n=242$, $n_{Ca/Co} = 121/121$; model 2: $n=136$ (decrease in count due to missing data in BMI and smoking status), $n_{Ca/Co} = 68/68$. Significant replication metabolites at $P<0.05$ are displayed in bold. [a]Adjusted for age, race, time to diagnosis from blood draw. [b]Adjusted for Model 1 covariates, smoking (ever/never) and BMI among those with available data. NM: not measured in DoD—metabolites were not assessed due to (i) non-detection of metabolite in mass spectrometry or (ii) failing quality control. HMDB: Human Metabolome Database; BCAA: branched-chain amino acid; DoD: Department of Defense; $n_{Ca/Co}$: number of cases/number of controls; NHS: Nurses' Health Study; NHSII: Nurses' Health Study II; NMMA: N-Monomethyl-L-arginine; OR: odds ratio; PE: phosphatidylethanolamine; Sero+: seropositive.

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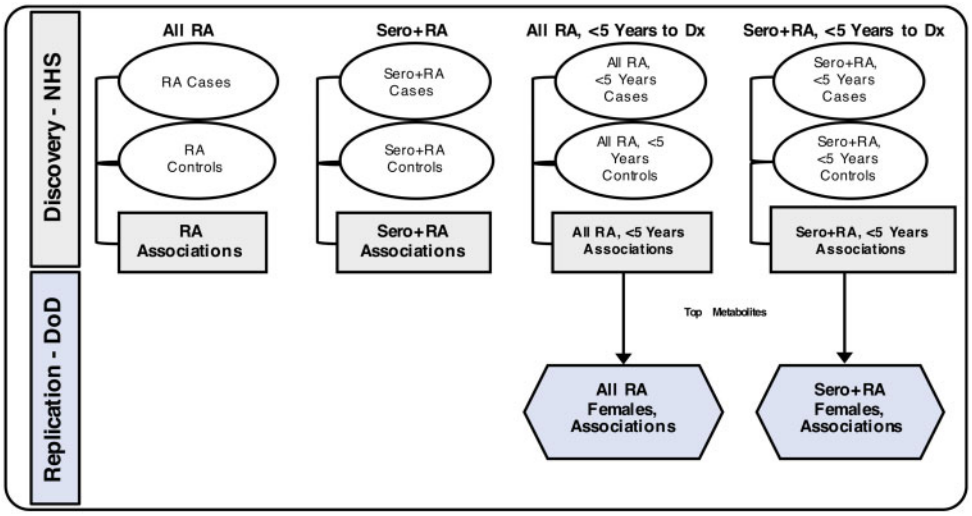
FIG. 1 An overview of the study design



DoD: Department of Defense; Dx: diagnosis; sero+: seropositive; NHS: Nurses' Health Study.

inverse association of acylcarnitines with RA risk [27]. Carnitine is both endogenously synthesized and obtained through the diet in humans. Acyl-carnitines are generated by carnitine acyltransferases that convert carnitine to acyl-carnitines and acyl-coA to coA, transporting acyl groups into the mitochondrial matrix for β-oxidation and production of adenosine triphosphate (ATP) for powering cellular reactions and function in humans [28, 29]. Secondary carnitine deficiencies result from chronic diseases, such as chronic renal failure, malnutrition and cachexia [30, 31]. Low circulating acylcarnitine levels have been observed among RA patients and attributed to decreases in carnitine biosynthesis [32]. Ageing and chronic inflammation are associated with carnitine depletion and trials have tested L-carnitine supplementation to reverse frailty, improve muscular strength and decrease systemic inflammation biomarkers, with mixed results [33–35]. Enhanced cellular oxidation due to inflammation and muscular wasting, well known to be associated with RA, may contribute to acylcarnitine depletion in brewing RA [36–38]. The stronger inverse associations of reduced acylcarnitines in the years closest to RA diagnosis and for sero+ RA risk support this hypothesis.

We found that C16:1 CE, a CE, was reduced among pre-RA NHS cases relative to controls. This may be explained by increased lipid catabolism and enhanced cellular β-oxidation, stimulated by early RA's upregulated inflammatory response. Prior RA studies demonstrated reduced cholesterol levels among prevalent RA subjects, and a few studies have observed a similar pattern of reduced cholesterol prior to RA onset [39–41]. Clinical trials of tofacitinib and tofalizumab, biologic therapies that reduce inflammation in RA, have demonstrated increased cholesterol and low density lipoprotein cholesterol catabolism in active disease RA compared with controls at baseline, and a resulting reduction in inflammation after biologic therapies for RA, associated with increasing concentrations of total cholesterol and low density lipoprotein cholesterol [42, 43]. Gradients of systemic inflammation in both pre-RA and active RA may influence the catabolism of multiple lipid metabolites and this is a subject of ongoing investigation.

Our study has many strengths. RA status was rigorously ascertained and samples were obtained for analysis prior to disease onset, during the pre-RA time period when inflammatory markers have been observed to be elevated. To maximize the efficiency of inference, a nested case–control design was implemented. Cases and controls were closely matched across many covariates, and we adjusted for RA risk factors including BMI and smoking. We stratified by RA seropositivity and time to RA diagnosis, finding stronger results for the risk of sero+ RA and within 5 years of RA onset, supporting the hypothesis these metabolites are involved in or influenced by RA pathogenesis. Our study was designed with an independent external replication dataset. To our knowledge, only one other study has employed non-targeted metabolomics to assay blood samples prior to RA incidence. The prior pre-RA metabolomics study of 30 cases and 19 controls found elevated relative concentrations of lysophosphatidylcholines, as well as tryptophan metabolites and reduced concentrations of short chain acylcarnitines in the plasma of pre-RA cases compared with controls using plasma samples drawn up to 7 years prior to onset [27].

The current study is not without limitations. While no findings were significant after adjusting for multiple comparisons using a stricter number of effective tests, three metabolites, C5, C5:1 and C3 carnitine, identified in the NHS were replicated in the external DoD validation study based on selection by second generation *P*-values. In our calculations of P_δ, a null interval of (0.95, 1.05) was applied uniformly in all models, a conservative

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specification of 'null effects' in the context of any single metabolite. Thus, the current findings are promising as replication may have been limited in part due to differences in NHS and DoD study sample compositions. Despite the implementation of an efficient, well-matched case–control study design, the power to detect robust but small effects may have been limited by the sample size, which nonetheless reflects the largest metabolomic interrogation of RA to our knowledge. Additionally, protocols for handling blood samples and the collected sample type (serum *vs* plasma) also differed [44], and our analyses were limited only to those metabolites that were identified and measurable. Thus, measured but unnamed metabolites also may be important for RA risk. Finally, the analytical method we implemented did not account for complex associations within and between interrelated metabolic pathways when estimating metabolite associations with RA risk; subsequent studies that directly model and adjust for these dependencies may reveal other associations with incident RA.

In these large and novel untargeted metabolomic analyses in the NHS cohorts, strong supportive evidence for associations between plasma metabolites, particularly polyamines and acylcarnitines, and incidence of RA was found. Biologically plausible associations with sero+ RA and in the years before RA diagnosis were validated in an independent cohort for C3, C5 and C5:1 carnitines, related to fatty acid metabolism and cellular oxidation. While this study is the largest metabolomic interrogation of pre-RA to date, future studies will be necessary for deeper mechanistic insight into RA pathogenesis.

Acknowledgements

The identification of specific products or scientific instrumentation is considered an integral part of the scientific endeavour and does not constitute endorsement or implied endorsement on the part of the author, DoD or any component agency. The views expressed in this presentation are those of the authors and do not reflect the official policy of the Department of Army/Navy/Air Force, Department of Defense or US Government.

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Funding: This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases [grant numbers R01 AR049880, R01 AR057327, K24 AR066109, K23 AR075070, K23 AR069688, L30 AR066953, P30 AR070253, P30 AR072577, R01 AR071326], National Human Genome Research Institute [grant number U01 HG008685] and the National Heart, Lung, and Blood Institute [grant number R01 HL119718] at the National Institutes of Health, and the Congressionally Directed Medical Research Program [grant number PR120839 (W81XWH-13-1-0408)]. NHS is supported by NIH grants UM1 CA186107, UM1 CA17626, R01 CA049449 and R01 CA067262.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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