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High erythrocyte levels of the n-6 polyunsaturated fatty acid linoleic acid are associated with lower risk of subsequent rheumatoid arthritis in a Southern European nested case-control study.

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ABSTRACT

Objectives

Findings relating to dietary intake of n-3 polyunsaturated fatty acids (PUFA) and risk of rheumatoid arthritis (RA) are mixed. Erythrocyte membrane PUFA are an accurate objective biomarker of PUFA status; however, there is little data on erythrocyte membrane PUFA and risk of RA. The objective was therefore to compare erythrocyte membrane PUFA between pre-RA individuals and matched controls from a population-based sample, and specifically to test the hypothesis that higher levels of longer chain n-3 PUFA are associated with lower risk of RA.

Methods

The European Prospective Investigation into Cancer and Nutrition (EPIC) is a large European prospective cohort study of apparently healthy populations. We undertook a nested case-control study, by identifying RA cases with onset after enrolment (pre-RA) in four EPIC cohorts in Italy and Spain. Confirmed pre-RA cases were matched with controls by age, sex, centre, and date, time and fasting status at blood collection. Conditional logistic regression analysis was used to estimate associations of PUFA with development of RA , adjusting for potential confounders including body mass index (BMI), waist circumference, education level, physical activity, smoking status, and alcohol intake.

Results

The study analysed samples from 96 subjects taken prior to RA onset (pre-RA) and 258 matched controls. In this analysis, the median time to diagnosis (defined as time between date of blood sample and date of diagnosis) was 6.71 years (range 0.8-15).

A significant inverse association was observed with n-6 PUFA linoleic acid (LA) levels and pre-RA in the fully adjusted model (highest tertile : OR 0.29; 95% CI 0.12 to 0.75; p for trend 0.01). No association was observed with any individual n-3 PUFA, total n-3 PUFA, or total n-3/n-6 ratio.

Conclusions

In conclusion, we found erythrocyte levels of the n-6 PUFA LA were inversely associated with risk of RA whereas no associations were observed for other n-6 or n-3 PUFA. Further work is warranted to replicate these findings and to investigate if lower LA levels are a bystander or contributor to the process of RA development.

Keywords

Rheumatoid arthritis (RA), polyunsaturated fatty acids, pre-RA, linoleic acid, n-3 fatty acids, omega-3 fatty acids, n-6 fatty acids, omega-6 fatty acids

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by inflammation of synovial joints with a prevalence worldwide of 0.5-1%. Environmental/non-genetic susceptibility factors account for up to 60% of the risk for developing RA,[1] however only a limited number of such factors have been identified.[2, 3] Long-chain n-3 (also known as omega-3) polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), have long-been considered to have anti-inflammatory and immunomodulatory actions.[4] Neither n-3 nor n-6 (omega 6) series PUFA (each family is determined by the number of carbon atoms between the methyl end of the fatty acyl chain and the first double-bond) can be synthesized *de novo* by large animals and so they are considered essential. The main dietary form of n-3 PUFA in most humans is the plant-derived alpha-linolenic acid (ALA; 18:3n-3).[5] Alpha-linolenic acid is metabolised by a series of desaturation and elongation steps to the longer chain EPA and DHA. This process of conversion into longer-chain PUFA is poor in humans[6] and in direct competition with the desaturation and elongation of the considerably more abundant n-6 PUFA. Longer chain n-3 PUFA can be synthesised by phytoplankton and are passed up the food chain through zooplankton to fish. Because of limited synthesis from ALA in humans, most EPA and DHA in the humam body is of dietary origin, with marine foods such as deep sea fatty fish or fish oil supplements being particularly good sources. The parent n-6 PUFA is linoleic acid (LA; 18:2n-6) which is more commonly obtained in the diet than any n-3 PUFA, with vegetable oils, especially sunflower, corn and soybean, being particulary good sources. Desaturation and elongation of LA gives rise to longer chain n-6 PUFA such as arachidonic acid and gamma-linolenic acid. Although there is a widespread conception that n-6 PUFA are pro-inflammatory, some data suggest an immunomodulatory potential.[7]

Fish oil ameliorates collagen-induced arthritis (CIA) an animal model of RA,[8, 9] and has shown efficacy in clinical trials.[10-12] Whilst the observed effects of fish oil in established RA are modest, the concept of a ‘window of opportunity’ in early disease suggests a greater potential for protective

immunomodulation in the very earliest stages. Findings on the relationship between dietary intake of fish/fish oil and prevention of RA have been inconsistent.[13-17] It is usually assumed that any observable effects of fish intake are due to their long chain n-3 PUFA content. One study reported that higher intake of n-3 PUFA, determined using food frequency questionnaires data, was associated with lower risk of RA.[18] However estimated dietary intakes of n-3 PUFA often correlate poorly with *in vivo* levels of these fatty acids,[19, 20] which in turn are also influenced by endogenous processes including elongation, desaturation and β-oxidation. Erythrocyte membranes offer an attractive biomarker of PUFA status in vivo, and reflect dietary intake, uptake and endogenous metabolism over a period of at least one month.[19] Recently higher erythrocyte n-3 PUFA levels were found to be associated with a lower risk of RA-related autoantibodies in a population at risk of RA.[21, 22] However, there is little data on the erythrocyte membrane PUFA and risk of RA. We therefore sought to establish whether there were differences in erythrocyte membrane fatty acid profiles, as a biomarker of PUFA status, between individuals with pre-RA and matched controls in a nested case-control study, and to test the hypothesis that higher levels of long-chain n-3 fatty acids are associated with lower risk of RA. As a pro-inflammatory state may exist prior to the clinical onset of RA, and conceivably might alter PUFA metabolism, we also analysed the relationship between PUFA levels and serum cytokines as a secondary outcomes.

Methods

Study sample

The European Prospective Investigation into Cancer and Nutrition (EPIC) is a multicentre, pan-European prospective cohort study designed to investigate the association between diet and cancer, as well as other diseases, in apparently healthy populations.[23] We undertook a nested case-control study to investigate risk factors for RA, by identifying subjects who developed incident RA after enrolment (referred to here as pre-RA). These pre-RA cases were matched with controls amongst subjects enrolled in four EPIC cohorts: Naples, Turin and Ragusa in Italy, and Murcia in

Spain. Potential RA cases were identified as previously described.[24] In brief: in Murcia, RA cases were identified by linkage with primary health care records (International Classification of Primary Care code L88) and prescriptions of disease-modifying anti-rheumatic drugs, and linkage using ICD codes with hospital discharge (ICD9 – 714) and mortality databases (ICD10 – M05 and M06). In Naples, RA cases were identified by linkage with hospital discharge databases and information from systematic telephone follow-up of participants. In Turin, RA cases were identified by linkage with hospital discharge databases and a drug prescription database with a disease-specific code. In Ragusa, cases were identified by linkage with hospital discharge databases. All RA case identification was undertaken in 2011. All cases were then subsequently validated by medical record review to confirm a physician diagnosis of RA and to confirm date of diagnosis, as previously described. [24] Subjects with prevalent RA at the time of the blood sample were excluded from the analyses. The EPIC cohorts included in this study recruited subjects between 1992 and 1998.[23]

Three controls were randomly selected from living cohort members and matched for every individual case by age at blood collection (± 1 year), sex, centre, date (± 2months) and time (± 3 hours) of baseline blood collection, and fasting status at blood collection (<3/3-6/>6 hours). There were erythrocyte samples available for 96 of the 103 pre-RA cases and 258 matched controls.

Data collection

Baseline questionnaires collected detailed data on diet,[23] physical activity, medical history, smoking status, socio-demographic and lifestyle factors (current and lifetime history). Level of education was defined as being (i) no education, (ii) primary school, (iii) technical or professional school, (iv) higher education (including University degrees). Physical activity was estimated using the Cambridge physical activity index and defined as being either inactive, moderately inactive, moderately active, or active.[25] Alcohol intake was estimated as the average alcohol intake (in grams/day) using a 24 hour dietary recall. Smoking status was defined as being (i) never, (ii) former, (iii) current smoker and (iv) unknown. Anthropometric measures were performed using standardised

protocols and Body Mass Index (BMI) was calculated as weight in kilograms divided by squared height in meters (kg/m^2) and modelled as a continuous variable.

In each centre, blood samples were collected at baseline, transferred to a local laboratory at 5-10°C whilst protected from light and, following processing, erythrocytes were stored in 0.5 ml straws at -196°C in liquid nitrogen. Samples for this study were retrieved and sent on dry ice to a central laboratory at University of Southampton where they were analysed blinded to case/control status.

Time to diagnosis was defined as the time in years that had elapsed between date of blood sample and date of diagnosis, and was categorised in tertiles [years, median (range): first period: 2.77 (0.82-4.77); second period: 6.70 (4.94-7.99); third period: 10.07 (7.99-15)].

Informed consent was given by all participants and the study was approved by the International Agency for Research on Cancer (IARC) review board as well as by the local committees of participating EPIC centres.

Erythrocyte fatty acids

Total red cell lipids were extracted with chloroform and methanol (2:1, vol/vol) and dried under nitrogen. The total lipid extracts were dissolved in toluene, and fatty acid methyl esters (FAME) were synthesised by heating the purified lipids at 50°C in the presence of methanol containing 2% (v/v) sulphuric acid. Fatty acid methyl esters were recovered by extraction with hexane and resolved in a BPX-70 fused silica capillary column (32 m × 0·25 mm × 25 µm; SGE Analytical Science) using an Agilent 6890 gas chromatograph equipped with flame ionisation detection (Agilent Technologies). The relative concentrations (% of total) of individual fatty acids were calculated from the peak area using HPChemStation (Agilent Technologies).

Long-chain n-3 PUFA were considered to be EPA, docosapentaenoic acid (DPA; 22:5n-3) and DHA. Using the fatty acid measurements, we calculated the sums of total n-3 PUFA and total n-6 PUFA.

Activities of desaturase enzymes, and in particular delta-6 desaturase (D6D), are considered to be rate-limiting steps in the conversion of the precursor n-3 PUFA ALA to the longer chain EPA and DHA, as well as in the parallel metabolism of n-6 PUFA. Activity of desaturase enzymes can be inferred from product to precursor ratios: 20:4n-6/20:3n-6 for delta-5 desaturase (D5D) and 18:3n-6/18:2n-6 for for delta-6 desaturase (D6D).[26, 27]

Serum cytokines

The following cytokines were measured in serum by multi-spot assay following the manufacturer's instructions (Meso Scale Diagnostics, Rockville, USA): TNFα, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13 and IFNγ.

HLA-DRB1 and autoantibodies

Human Leucocyte Antigen (HLA)-DRB1 gene full exon 2 DNA sequence was established using the Sanger method and SSOP hybridization used for quality assurance. Human Leucocyte Antigen (HLA)-DRB1 alleles considered to be shared epitope (SE) positive, a major genetic risk factor for RA, were*0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001, *1402 and *1406. Immunoglobulin M rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA; measured with an anti-CCP2 ELISA) were measured in baseline samples as previously described.[24]

Statistical analysis

Fatty acids were categorised in tertiles based on their distribution in the control group, with the lowest category chosen as the referent. Variables with a non-normal distribution were log-transformed for analyses.

Fatty acid levels across the three time periods before RA diagnosis were compared using linear regression. Only one pre-RA subject had a diagnosis <1 year after blood sampling and exclusion of this case did not substantially change the results.

Confirmed pre-RA cases were matched with controls by age at blood collection (± 1 year), sex, centre, date (± 2months) and time (± 3 hours) of baseline blood collection, and fasting status at blood collection (<3/3-6/>6 hours).

We estimated the association with incident RA of each of the individual n-3 PUFA and n-6 PUFA, total n-3 PUFA, total n-6 PUFA and pre-defined ratios of PUFA variables.

Conditional logistic regression was conducted to estimate associations of PUFA with incident RA and obtain odds ratios and 95% confidence interval, adjusting for potential confounders including BMI, waist circumference, education level, physical activity, smoking status, and alcohol intake. We performed a complete case analyses. The proportion of subjects with missing data for covariates was <5%. In a sensitivity analyses, the model was further adjusted for the presence of the HLA DRB1 shared epitope. The interaction between SE and linoleic acid (LA) was tested using multiplicative terms.

As a pro-inflammatory state may exist prior to the clinical onset of RA, and conceivably might alter PUFA metabolism, the relationship between PUFA levels and serum cytokines was analysed in the pre-RA and control groups separately. We also compared RF positive pre-RA subjects to RF negative matched controls, using matched conditional logistic regression models further adjusting for fewer potential confounders (i.e. smoking status, alcohol intake, education level and shared epitope) given the smaller sample size.

Given that the cytokine data were over-dispersed, negative binomial regression was used to test the relationship between fatty acids (LA, total n-3 and n-6 PUFA, and the n-3/n-6 ratio) and serum cytokine levels, stratified by incident RA, adjusting for age, sex, country of origin, BMI, and smoking status. The exponentiated regression coefficients (relative risk [RR]) were interpreted as a ratio of means. The RR indicates a positive or negative association between PUFA and cytokines. We used

Stata software (StataCorp, College Station, TX) for statistical analyses. A *P* value less than 0.05 was considered as statistically significant. Formal alpha adjustments were not performed.[28-30]

Results

Baseline characteristics of pre-RA cases and controls are shown in Table 1. The study sample included 354 individuals, of whom 96 had pre-RA. There were no differences in age, sex, BMI, WHR, percentage of energy provided by protein, carbohydrate and fat, and dietary fibre intake between pre-RA cases and controls. Pre-RA individuals were more likely to be former smokers (OR 1.91, 95% CI 1.03-3.52) and positive for RF (OR 22.89; 95% CI 9.83-53.33) and ACPA (OR 5.22; 95%CI 1.74-15.69).

The relationship between tertiles of n-3 and n-6 PUFA and risk of RA is shown in Table 2. A significant inverse association was observed with the n-6 PUFA LA (highest tertile: OR 0.29; 95% CI 0.12 to 0.75; p for trend 0.01) and pre-RA in the fully adjusted model. However, no association was observed with any of the individual n-3 PUFA, total n-3 PUFA or total long chain n-3 PUFA, or with the total n-3/n-6 ratio. In a sensitivity analyses, a model with further adjustment for the presence of the HLA DRB1 shared epitope, confirmed the significant inverse association with the n-6 PUFA LA and pre-RA (second tertile: OR 0.32; 95% CI 0.13 to 0.79; highest tertile: 0.21; 95% CI 0.07 to 0.67; p for trend 0.008). No interaction was observed between LA and the presence of the HLA-DRB1 shared epitope (SE; 1 or 2 copies) in relation to risk for RA (p=0.68).

Longitudinal samples were not available but we did compare pre-RA subjects across different time periods before diagnosis, and found a significant difference in the median PUFA membrane content of 20:3n-6 and 22:5n-3 with increasing levels closer to diagnosis (Table 3). Compared with the period of time immediate before diagnosis (period 1), 20:3n-6 and 22:5n-3 were significantly lower in the period of time furthest away from diagnosis (period 3) (β -0.14; 95% CI -0.25 to -0.02; p=0.02, and β -

0.25; 95% CI-0.46 to -0.03; p=0.02, for 20:3n-6 and 22:5n-3 respectively). Conversely there was a trend for decreasing levels of LA closer to diagnosis (data for other PUFA not shown).

Because final autoantibody status was not known for all subjects, we did not to compare the effects of PUFA on risk for autoantibody positive versus autoantibody negative RA. However, when restricting the analysis to pre-RA subjects who had documented RF post-diagnosis or a positive RF baseline sample (n=56 (58%)), compared with RF negative matched controls (n=245 (96%)), an inverse association was also observed with LA for RF positive pre-RA [lowest tertile OR 1 (reference); second tertile OR 0.18; 95% CI 0.03 to 0.96; highest tertile OR 0.13; 95% CI 0.02 to 0.90; p for trend=0.04] in a multivariable-adjusted model.

The relationship between levels of LA, total n-3 and n-6 PUFA and the n-3/n-6 ratio and serum cytokines are displayed in online Supplementary Table 1 for the control population, and online Supplementary Table 2 for the pre-RA population. In the control population, LA was positively associated with levels of TNFα and IL-6 but negatively associated with IL-4, IL-5, IL-10, IL-12, IL-13 and IFNγ in fully adjusted models. In the pre-RA population, LA remained positively associated with TNFα and also IL-1, but was now negatively associated with IL-6. Negative associations between LA and IL-5, IL-12 and IL-13 persisted in the pre-RA group. The n-3/n-6 ratio was inversely associated with TNFα and IL-1 in both groups.

Discussion

We observed that erythrocyte membrane levels of the n-6 PUFA LA were inversely proportional to the risk of developing RA, whereas no protective effect was observed with n-3 PUFA. Previous studies have reported a higher estimated intake of n-3 fatty acids may be protective against development of RA in Swedish women,[18] and that erythrocyte n-3 levels were inversely related to

the risk of RA-related autoimmunity in a population at risk of RA.[21, 22] This discrepancy with our findings may firstly be explained by n-3 bioavailability, since previous research has shown lower long-chain n-3 PUFA blood levels in southern European populations compared with those from Scandinavia,[31] despite a similar estimated dietary intake of seafood n-3 PUFA.[32] In line with this, we observed mean erythrocyte levels of n-3 fatty acids in the control group that were lower than those observed in Northern Sweden, with levels of EPA almost half (0.75% vs 1.4%).[33, 34] Secondly, Gan et al found that higher erythrocyte n-3 fatty acid levels were protective against RA-related autoimmunity only in patients who were HLA-DRB1 SE-positive, and hypothesised that this might be due to the ability of the n-3 PUFA DHA to alter lipid rafts and so influence antigen presentation by HLA molecules, or regulatory T cell function.[21] Notably, the prevalence of the SE is considerably higher in Northern European RA populations compared to those from Southern Europe (approaching 80% in some population based studies [35] vs 44% in the pre-RA group studied here).

We did, however, observe that higher levels of the n-6 PUFA LA were associated with lower risk for RA. Linoleic acid was also negatively associated with RF positivity, which overlaps considerably with the presence of ACPA, but no interaction was observed with the SE. A key question is whether LA is protective against RA, or whether lower LA levels in the pre-RA group are secondary to metabolic and inflammatory changes occurring before the clinical onset of RA. Interestingly, previous small studies in established RA have identified lower plasma phospholipid and adipose tissue LA relative to controls.[36, 37] This was thought to be reverse causation with a metabolic state in RA driving increased desaturase enzyme activity for which linoleic acid is a substrate. Indeed, we have previously suggested that D5D activity may be reduced following treatment with anti-TNF.[38] In this cohort we did not find D5D or D6D product/precursor ratios to be associated with risk of RA, or to vary with time to diagnosis, but we did observe a non-statistically significant trend for lower LA levels in subjects closest in time to RA diagnosis. Furthermore, we found increasing levels of 20:3n-6 and 22:5n-3 in subjects closest to diagnosis, compatible with increased production of downstream products from the metabolism of n-6 LA and n-3 ALA. Other published reports point to metabolic

alterations occurring in the years before clinical onset of RA including a reduction in total cholesterol and LDL and alterations in tryptophan metabolism.[39, 40]

Certainly reverse causation as an explanation of our LA findings would be more compatible with the widespread conception that n-6 PUFA are pro-inflammatory. However the evidence to support this conception in humans *in vivo* is sparse.[41] Indeed, data from non-RA population based studies have suggested that intake of n-6 PUFA are inversely associated with CRP and certain pro-inflammatory cytokines.[42-45] Furthermore, n-6 PUFA intake has been positively associated with levels of the anti-inflammatory cytokine TGFβ,[42] and n-6 PUFA supplementation in healthy volunteers increases TGFβ production by peripheral blood mononuclear cells *ex vivo*.[46] The n-6 PUFA arachidonic acid gives rise to prostaglandins and leukotrienes that are proinflammatory in a context-dependent fashion, but n-6 PUFA can also give rise to potent anti-inflammatory mediators such as lipoxins,[47] 13-hydroxyoctadecadienoic acid (13-HODE),[48] and nitrated-LA.[49] Higher doses of the longer chain n-6 PUFA gamm-alinolenic acid were found in a small study to improve the signs and symptoms of RA.[50]

Regarding other health outcomes, a meta-analysis of prospective studies on fatty acids and risk of coronary heart disease (CHD), observed an independent inverse association between LA and CHD risk [relative risk of CHD: 0.91; 95% CI 0.84–0.98]. Other fatty acids were not associated with CHD.[51] More recently, the Cardiovascular Health Study, a community-based US cohort of 2,792 participants aged 65 years and older free of CVD at baseline, observed that high circulating LA, but not other n-6 PUFA, was inversely associated with total and CHD mortality.[52] A recent large cohort study including over 27,000 participants in the EPIC-Interact study across European countries observed that LA is inversely associated with type 2 diabetes (T2D). Theresults also suggested an inverse association of ALA but no convincing association of marine-derived n-3 PUFA with T2D.[27] Also a recent large pooled analysis of individual-level data for nearly 40,000 adults from 20 prospective studies has shown that higher levels of LA biomarkers were independently associated with a lower risk of type 2 diabetes.[53]

Given our findings on LA and risk of RA, we explored the relationship of LA and serum cytokines in this population. The observed positive association between LA and TNFα and IL-6 in our control group is consistent with the hypothesis that high n-6 PUFA and LA are pro-inflammatory, and, the n-3/n-6 ratio was inversely associated with TNFα and IL-1 levels in both the controls and the pre-RA group. Also in support of inflammation driving a reduction in LA is the shift from a positive association between LA and IL-6 in the control population, to a negative association in the pre-RA group, although interestingly both TNFα and IL-1 remain positively associated with LA in pre-RA. Also consistent with prevailing notions is our finding of a negative association between LA and Th2 cytokines IL-4, IL-5 and IL-13, and also IL-10, which is produced by monocytes and to a lesser extent Th2 cells and has well-established anti-inflammatory functions. However LA was also negatively associated with IL-12, which induces Th1 responses, and also the Th1 cytokine IFNγ, implying more generally downregulated T cell function. The important role of T cells in the pathogenesis of RA, and the observation that high levels of Th2 cytokines have been shown in the earliest phase of disease,[54] could support a hypothesis of high LA levels being protective in this population. Furthermore, polymorphisms in the fatty acid desaturase (FADS) genes which encode the rate-limiting enzymes for the biosynthesis of longer-chain n-6 and n-3 PUFA, have been identified as being risk factors for RA, suggesting that PUFA status may contribute to RA pathogenesis.[55]

The strengths of the present study are the large number of matched controls and pre-RA cases from a population-based cohort and measurement of erythrocyte PUFA and cytokines in samples taken prior to clinical onset of RA. The measure of objective biomarkers allowed investigation of individual PUFA while avoiding potential errors and recall bias related to self-reported dietary intake. Indeed total erythrocyte n-3 PUFA status did not correlate with fish intake in this cohort (data not shown). We also took careful control of important potential confounding factors. However, residual and unmeasured confounding remain important limitations; this may be particularly relevant to studies of specific nutrients which themselves are part of more complex dietary intake.

The analyses of associations between PUFA and incident RA involved multiple tests of significance. A potential limitation relates to multiple testing, because multiple comparisons are associated with an increased chance for a Type I error. However, the utility of formal adjustments for multiple comparisons in an epidemiologic context has been questioned.[28-30] We therefore chose not to perform any formal alpha adjustments in the present study and acknowledge the need to consider with caution the statistical significance of such associations.

In conclusion, we found erythrocyte levels of LA, the major dietary n-6 PUFA, were inversely associated with risk of pre-RA. Other n-6 PUFA and n-3 PUFA were not significantly associated with risk of RA. Further work is warranted to replicate these findings and to investigate if LA is protective against RA development or if lower levels are secondary to metabolic and inflammatory changes occurring before the clinical onset of RA.

Competing interests

None declared.

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Table 1. Baseline characteristics

	Pre-RA cases	Controls
Characteristics		
N (%)	96 (27)	258 (73)
Age (years), mean (SD)	51 (7.56)	51 (7.30)
Female, n (%)	74 (77)	198 (78)
BMI (kg/m^2), mean (SD)	26.60 (3.70)	26.22 (4.11)
WHR, mean (SD)	0.85 (0.08)	0.84 (0.08)
Smoking status, n (%)		
Never	39 (41)	133 (52)
Former	26 (27)	51 (20)
Current	29 (30)	68 (26)
Unknown	2 (2)	6 (2)
Education, n (%)		
None	18 (19)	30 (12)
Primary school completed	43 (46)	105 (42)
Technical/professional school	10 (11)	26 (10)
Secondary school	17 (18)	47 (19)
Longer education	6 (6)	44 (17)
Physical activity, n (%)		
Inactive	44 (46)	102 (40)
Moderately inactive	29 (30)	85 (33)
Moderately active	11 (11)	44 (17)
Active	10 (10)	21 (8)
Missing	2 (2)	6 (2)
Macronutrients dietary intake, mean (SD)		
Carbohydrate intake*	44.17 (6.81)	44.14 (6.62)
Protein intake*	17.30 (2.37)	17.48 (2.44)
Fat intake*	35.16 (5.19)	34.71 (5.58)
Fibre intake§	23.01 (7.48)	24.06 (7.87)
Alcohol intake**	22.37 (17.87-28.19)	23.42 (17.98-28.30)
Shared epitope (%)		
No copies	45 (56)	133 (69)
One copy	26 (33)	56 (29)
Two copies	9 (11)	4 (2)
Shared epitope, >1 copies (%)	35 (44)	60 (31)
ACPA positive (%)†	23 (24)	9 (3.5)
Rheumatoid factor positive (%)†	56 (58)	11 (4)
Time to diagnosis§§, mean (SD)	6.71 (3.43)	-

BMI: body-mass index; WHR: waist-hip ratio; ACPA determined from baseline samples and not post-diagnosis.

*Macronutrients dietary intake expressed as % of energy. **Median (IQR)

§Fibre intake in grams per day

§§ Time to diagnosis was defined as time (years) elapsed between date of blood sample and date of diagnosis.

†Analysis done on samples taken at enrolment.

Table 2. Risk of RA by polyunsaturated fatty acid (PUFA) tertiles in the EPIC study.

	Tertiles of polyunsaturated fatty acids			
	1 (reference)	2	3	
	OR 95% CI	OR 95% CI	OR 95% CI	P for trend[§]
PUFA				
18:2n-6* LINOLEIC ACID	10.73 (9.69-11.61)	14.15 (13.33-14.74)	16.73 (15.93-17.92)	
Model 1	1	0.66 (0.33, 1.31)	0.37 (0.16, 0.84)	0.018
Model 2	1	0.52 (0.24, 1.11)	0.29 (0.12, 0.75)	0.010
18:3n-6* GAMMA LINOLENIC ACID	0.004 (0.003-0.004)	0.006 (0.005-0.006)	0.009 (0.008-0.011)	
Model 1	1	1.64 (0.86,3.13)	1.16 (0.59, 2.28)	0.782
Model 2	1	1.73 (0.85, 3.51)	1.18 (0.57,2.44)	0.754
18:3n-3* (=ALPHA LINOLENIC ACID	0.10 (0.06-0.11)	0.20 (0.16-0.24)	0.48 (0.34-0.68)	
Model 1	1	1.33 (0.72, 2.45)	0.97 (0.52, 1.82)	0.928
Model 2	1	1.47 (0.74,2.93)	1.02 (0.52,1.99)	0.981
20:2n-6*	0.29 (0.25-0.31)	0.36 (0.34-0.37)	0.45 (0.41-0.50)	
Model 1	1	0.93 (0.49, 1.76)	0.76 (0.40, 1.44)	0.393
Model 2	1	1.13 (0.57, 2.25)	0.80 (0.39 ,1.63)	0.538
20:3n-6*	1.55 (1.38-1.69)	1.97 (1.87-2.05)	2.51 (2.34-2.81)	
Model 1	1	1.23 (0.66,2.30)	1.45 (0.78, 2.68)	0.238
Model 2	1	1.38 (0.69, 2.74)	1.54 (0.77, 3.09)	0.218
20:4n-6* (AA)[§§]	7.20 (6.47-7.95)	9.53 (9.06-9.97)	12.14 (11.23-13.16)	
Model 1	1	0.68 (0.36, 1.28)	1.04 (0.57, 1.90)	0.777
Model 2	1	0.671 (0.337,1.337)	0.969 (0.500,1.879)	0.994
20:4n-3*	0.03 (0.02-0.03)	0.05 (0.04-0.05)	0.08 (0.07-0.09)	
Model 1	1	2.01 (1.04, 3.86)	1.78 (0.89,3.54)	0.111
Model 2	1	1.82 (0.87, 3.77)	1.82 (0.86,3.83)	0.138
20:5n-3 (EPA)*	0.43 (0.32-0.48)	0.68 (0.63-0.76)	1.07 (0.93-1.27)	
Model 1	1	1.85 (0.98, 3.49)	1.06 (0.51,2.21)	0.977
Model 2	1	1.92 (0.93, 3.88)	1.26 (0.55 ,2.88)	0.661
22:4n-6*	0.76 (0.55, 1.05)	0.64 (0.49-0.89)	0.68 (0.49-0.88)	
Model 1	1	1.49 (0.81, 2.76)	1.646 (0.814,3.329)	0.167
Model 2	1	1.38 (0.72,2.67)	1.59 (0.75, 3.33)	0.225
22:5n-3*	0.01 (0.009-0.02)	0.01 (0.008-0.01)	0.01 (0.01-0.02)	
Model 1	1	1.01 (0.56, 1.82)	1.66 (0.86, 3.19)	0.157
Model 2	1	1.04 (0.55, 1.99)	1.70 (0.84, 3.45)	0.150
22:6n-3 (DHA)*	1.58 (1.37-1.890	1.75 (1.53-2.03)	1.97 (1.66 (2.24)	
Model 1	1	1.92 (0.98, 3.75)	1.58 (0.79, 3.12)	0.226
Model 2	1	1.80 (0.87, 3.73)	1.54 (0.73, 3.26)	0.301
Total n-3 PUFA*	4.89 (4.49-5.33)	6.09 (5.79-6.35)	7.33 (6.93-7.95)	
Model 1	1	2.05 (1.10,3.80)	1.44 (0.72,2.86)	0.368
Model 2	1	1.82 (0.90,3.67)	1.64 (0.77, 3.52)	0.252
Total n-6 PUFA*	22.26 (20.96-23.44)	25.82 (25.17-26.61)	29.88 (28.65-31.61)	
Model 1	1	0.80 (0.43,1.47)	0.63 (0.32,1.24)	0.181
Model 2	1	0.78 (0.39,1.54)	0.58 (0.28,1.21)	0.149

n-3/n-6 ratio*	0.18 (0.16-0.19)	0.23 (0.21-0.25)	9.29 (0.27-0.32)	
Model 1	1	1.80 (0.97, 3.35)	1.56 (0.80, 3.05)	0.216
Model 2	1	1.61 (0.81, 3.19)	1.86 (0.88, 3.95)	0.108
D6D*	0.0002 (0.0002-0.0003)	0.0004 (0.0004-0.0005)	0.0008 (0.0006-0.001)	
Model 1	1	1.59 (0.84,2.99)	1.42 (0.66,3.04)	0.360
Model 2	1	1.57 (0.79, 3.12)	1.52 (0.66,3.47)	0.306
D5D*	3.37 (2.92-3.75)	4.68 (4.30-5.04)	6.69 (6.01-7.96)	
Model 1	1	0.64 (0.35,1.17)	0.81 (0.43,1.51)	0.473
Model 2	1	0.64 (0.33,1.25)	0.82 (0.42,1.62)	0.556
LC n-3 PUFA*	4.60 (4.01-4.89)	5.78 (5.48-6.04)	6.94 (6.63-7.58)	
Model 1	1	1.94 (1.04, 3.62)	1.60 (0.82, 3.12)	0.036
Model 2	1	1.63 (0.81, 3.25)	1.66 (0.80, 3.45)	0.197

* Fatty acids are expressed as a percentage, median (IQR) of total erythrocyte membrane fatty acids.
[§] The significance of linear trends across tertiles was tested with linear regression models.
OR, 95% CI: Odds ratio, 95% confidence intervals.
Model 1: Conditional logistic regression model matched by age at blood collection (± 1 year), sex, centre, date (± 2months) and time (± 3 hours) of baseline blood collection, and fasting status at blood collection (<3/3-6/>6 hours). There were erythrocyte samples available for 96 of the 103 pre-RA cases and 258 matched controls.
Model 2 is model 1 with further adjustment for BMI, waist circumference, education level, physical activity, smoking status, and alcohol intake.
AA: arachidonic acid; D5D: delta-5 desaturase activity inferred form product-precursor ratios. D6D: delta-6 desaturase activity inferred from product-precursor ratios. LC PUFA: total longer chain n-3 PUFA.

Table 3.Fatty acids levels across three time periods (tertiles) before the diagnosis of RA

	Pre-RA time periods			
	1	**2**	**3**	**p-value**
Time*	2.77 (0.82-4.78)	6.70 (4.94-7.99)	10.07 (7.99-15)	
FA levels**				
Linoleic acid	13.00 (3.19)	13.43 (2.95)	14.45 (3.21)	0.06
20:3n-6	2.18 (0.53)	2.12 (0.51)	1.89 (0.39)	0.02
22:5n-3	1.95 (0.42)	1.85 (0.43)	1.71 (0.42)	0.02

* Time in years, median (range) Median time to diagnosis, defined as time in years elapsed between date of blood sample and date of diagnosis, was 6.7 years (range 0.8-15). For this analyses the time to diagnosis was categorised into 3 periods based on tertiles [years, median (range): first period: 2.77 (0.82-4.77); second period: 6.70 (4.94-7.99); and third period: 10.07 (7.99-15)].

**Mean (±SD)