BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2020-047982
Article Type:	Protocol
Date Submitted by the Author:	14-Dec-2020
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Keywords:	RHEUMATOLOGY, EPIDEMIOLOGY, NUTRITION & DIETETICS

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Intake of marine n-3 polyunsaturated fatty acids and the risk of rheumatoid arthritis: protocol for a cohort study using data from the Danish Diet, Cancer and Health Cohort and Danish health registers

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Word count: 2960

ABSTRACT

Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory joint disease with multifactorial aetiology. Smoking is a well-established lifestyle risk factor, but diet may also have an impact on the risk of RA. Intake of the major marine n-3 polyunsaturated fatty acids in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been hypothesised to lower the risk of RA due to their anti-inflammatory effects, albeit based on limited knowledge. Therefore, we aim to investigate the associations between dietary intake of EPA and DHA and the risk of incident RA.

Methods and analysis: A cohort study. The follow-up design will be based on data from the Danish Diet, Cancer and Health cohort, which was established between 1993 and 1997. The participants will be followed through record linkage using nationwide registers including the Danish Civil Registration System, the Danish National Patient Registry and the Danish National Prescription Registry using the unique Civil Personal Registration number. Time-to-event analyses will be conducted with RA as the outcome of interest. The participants will be followed from inclusion until date of RA diagnosis, death, emigration or end of follow-up. Hazard ratios with 95 % confidence intervals obtained using Cox proportional hazard regression models, with age as underlying time scale and adjustment for established and potential risk factors, will be used as measures of association.

Ethics and dissemination: The study has been approved by the Data Protection Committee of Northern Jutland, Denmark (2019-87) and the North Denmark Region Committee on Health Research Ethics (N-20190031). Study results will be disseminated through peer-reviewed journals and presentations at international conferences.

Keywords: Rheumatology, epidemiology, nutrition & dietetics

STRENGTHS AND LIMITATIONS OF THIS STUDY

- Danish administrative registers ensure nearly complete follow-up of the study population.
- Use of a validated RA case definition with a positive predictive value of 88% for overall RA.
- All exposures will be energy-adjusted.
- Dietary assessment is based on a single food frequency questionnaire, which may not capture changes in dietary habits during follow-up.
- Participants were 50 to 64 years old Caucasians at enrolment and therefore the results may not be valid to other age and ethnic groups.

INTRODUCTION

Rheumatoid arthritis (RA) is the most common chronic autoimmune joint disease. RA is characterised by inflammation of the synovial joints leading to irreversible joint damage and deformity, which may result in severe disability. The overall incidence rate of RA from 1996 to 2016 in Denmark has been reported to be 35.2 per 100,000 person years. The incidence rate was approximately 2-fold higher in women than in men with a peak in incidence rate between ages of 70 to 74 in both sexes.(1)

The aetiology of RA is considered multifactorial and lifestyle factors seem to play an important role for the development of RA. Smoking is the best established lifestyle risk factor for RA,(2–5) whereas the impact of other lifestyle factors such as diet are less clear.

Marine n-3 polyunsaturated fatty acids (PUFAs) are organic compounds that may affect a variety of biological pathways which may in turn influence inflammatory processes. Marine n-3 PUFAs may upon ingestion become incorporated into cellular membranes, pooled for storage or converted into lipid signalling molecules. The marine n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are mainly derived from seafood, especially fatty fish.(6) PUFA seems to play an important role of the immune system. While the more common n-6 PUFAs mainly derived from vegetable oils and meat have proinflammatory actions, the marine n-3 PUFAs, EPA and DHA, has been ascribed anti-inflammatory properties by inhibiting a number of aspects of inflammation including production of cytokines and eicosanoids, leukocyte chemotaxis, adhesion molecule expression, and leukocyte-endothelial adhesive interactions.(7,8) Further, EPA and DHA promote production of inflammation resolving mediators in form of resolvins, protectins and maresins.(8) Previous studies have supported that marine n-3 PUFAs may lower the risk of several inflammatory diseases including cardiovascular disease, inflammatory bowel disease and cancer.(9,10) However, limited data exist regarding the role of fish and marine n-3 PUFAs in relation to development of RA and findings have been conflicting. Thus, a previous cohort study, with a limited number of RA patients, reported that intake of fatty fish was associated with a statistically non-significant lower risk of RA, whereas intake of medium fatty fish was associated with a significantly higher risk of RA compared with participants with a lower intake.(11) A previous cohort study based on the Swedish Mammography Cohort reported that a intake of marine n-3 PUFAs (EPA, DHA and docosapentaenoic acid) of more than 0.21 grams per day was associated with a markedly lower risk of RA compared with a lower intake.(12) In contrast, a recent cohort study conducted among US women found no clear association between total marine n-3 PUFAs from diet and supplements and the risk of RA.(13)

Therefore, whether marine n-3 PUFAs may lower the risk of developing RA remains unclear.

The aim of the present study is to assess the associations between dietary intake of EPA and DHA and the risk of incident RA. We hypothesise that intake of marine n-3 PUFAs, respectively EPA and DHA is inversely associated with the rate of incident RA.

METHODS AND ANALYSES

Study design

We will conduct a cohort study using data from the Danish Diet, Cancer and Health (DCH) cohort and Danish health registers.

Data sources

We will follow the participants enrolled into the DCH cohort through nationwide Danish health registers including the Danish National Patient Registry (DNPR), the Danish National Prescription Registry (NPR) and the Civil Registration System (CRS). Use of the unique 10-digit personal identifier assigned to all Danish residents at birth or immigration, allows for accurate register-linkage on an individual-based level.

DCH: The DCH cohort was established from December 1993 to May 1997 and includes participants from the area of greater Copenhagen and Aarhus in Denmark who did not have a previous cancer diagnosis registered in the Danish Cancer Registry prior to enrolment. In total, 57,053 subjects between 50 and 64 years of age were included in the study (27,179 men and 29,874 women). At baseline, participants completed a detailed questionnaire on health status, social factors, lifestyle, and a validated 192-item semi-quantitative food frequency questionnaire.(14,15) The questionnaires were checked for reading errors and missing information at baseline by a technician. Furthermore, anthropometric measurements were collected at baseline. A detailed description of the DCH cohort has been published previously.(16)

DNPR: The DNPR holds data on all admissions to somatic hospitals in Denmark since 1977 and all out-patient attendances since 1995. The data includes dates of hospital admissions, ward types, discharge diagnoses, dates of all attendances at out-patient clinics, and diagnoses recorded at each attendance.(17,18) Diseases are classified according to the International Classification of Disease (ICD). The ICD 8th edition was used until January 1994, and thereafter the ICD 10th edition was implemented.

NPR: The NPR provides data about all prescription drugs dispensed at Danish community pharmacies since 1994,(19) however the register does not include information on drugs dispensed by hospital pharmacies directly to in- or out-patients.

CRS: The CRS are continuously updated and ensures complete follow-up regarding vital and migration status of all Danish citizens.(20)

Study population

The study population consists of all participants from the DCH cohort with complete records of data on exposures and covariates and without a diagnosis of RA recorded in the DNPR prior to their enrolment into the cohort. Also, participants with a previous diagnosis of cancer before enrolment will be excluded as well.

Exposures and outcome of interest

Intake of EPA and DHA will be calculated based on the food frequency questionnaire collected at baseline. By multiplying the frequencies of intake by the portion size, the individual average intake

in grams per day of all foods and nutrients will be calculated using the software FoodCalc based on Danish Food composition tables.(21) Exposures of interest include the energy-adjusted intake of EPA and DHA.

The outcome of interest for this study is incident RA, defined as the first occurrence of an ICD-10 code of M05-06 registered in DNPR and a subsequent redeemed prescription of a conventional synthetic DMARD (csDMARD) within 1 year from the date of diagnosis in the DNPR. Redeemed prescriptions of csDMARD are identified in the NPR by ATC codes (Table 1). A previous validation study by Linauskas et al. reported in the same cohort a positive predictive value of 88 % for overall RA based on this case definition.(22)

Table 1. ATC codes for conventional synthetic DMARD

Conventional synthetic DMARD	ATC Code
Methotrexate	L04AX03 / L01BA01
Sulfasalazine	A07EC01
Hydroxychloroquine	P01BA02
Leflunomide	L04AA13
Ciclosporine	L04AD01 / L04AA01
Gold	MC01CB
Azathioprine	L04AX01

DMARD = disease-modifying anti-rheumatic drug

Established risk factors for RA were identified by review of the existing literature prior to data analysis. We identified the following covariates that needed to be adjusted for: age(1), sex(1,23–25), smoking(3–5), level of education(3), alcohol intake(26), waist circumference(27), body fat percentage(27), physical activity(28); and further for women: early menopause(29), breast-feeding(29), hormone replacement therapy(29), oral contraception(29), and parity(29). Data on genetic disposition are not available and therefore not adjusted for.

Statistical analysis plan

Demographic and descriptive data will be presented as proportions (%) for categorical variables and medians values for continuous variables with 95 % central ranges (2.5th; 97.5th percentile). The participants are followed from date of entry into the DCH cohort until date of RA diagnosis, death, emigration or end of follow-up (end of year 2018), whichever comes first.

We will perform time-to-event analyses with RA as the event of interest. Hazard ratios (HRs) with 95 % confidence intervals obtained from Cox proportional hazard regression models will be used as measures of association between dietary intake of EPA and DHA and rate of incident RA. The time-to-event analyses will be conducted by allowing for different baseline hazards among men and women with attained age as the underlying timescale, with adjustment for baseline age to ensure that baseline covariates have same age.

All exposure variables will be examined in continuous analyses using restricted cubic splines and in categorical analyses in quintiles. Radar plots will be constructed to support the interpretation of our results and to visualize potential confounding from the underlying dietary pattern.

A two-tailed p-value <0.05 will be considered statistically significant.

All exposures will be energy-adjusted using the residual method assuming that intake of EPA and DHA is of most biological relevance relative to total energy intake. Residuals will be estimated

separately among men and women based on linear regression between total energy intake and reported intakes of the exposures of interest. All estimated residuals will be added to the mean intake among men and women within the cohort. By definition residual energy-adjusted intakes of EPA and DHA are independent of the total energy.

Based on the identified risk factors, three models were created (Table 2). Model 1 contains adjustment for demographic risk factors. Model 2 (primary model for interpretation) contains the same covariates as model 1 combined with lifestyle risk factors. Differences in point estimates observed in analyses including adjustment for the covariates included in model 2 compared with model 1 may reflect the importance of confounding. In supplemented analyses, we will conduct sex-specific analyses. In analyses of the associations between EPA and DHA and the rate of RA in women additional adjustment for potential hormonal risk factors (model 3) will be undertaken to investigate residual confounding.

If we have few cases of RA it may be necessary to lower the number of risk factors that we adjust for.

In addition, secondary and sensitivity analyses will be conducted. In supplemental analyses we will investigate our exposure of interest and the risk of seropositive and seronegative RA. Also, we will terminate the follow-up earlier to explore the influence of follow-up time on our associations of interest. Relevant secondary analyses include adjustment for risk factors using other available variables than the ones used in the primary analysis, e.g. changing the anthropometry variable body fat percentage with BMI. For further adjustment for former smokers we will use the age at smoking cessation to estimate duration since smoking cessation in years and adjust for this in the analyses. Model 2 contains adjustment for education and for a secondary analysis we will add a variable for household income. In sensitivity analyses the associations will be investigated by modeling the continuous analyses by moving the knots and with a different number of knots. Further, our main analysis will be conducted with a less strict and a more restrict outcome definition, respectively.

Table 2. Overview of models including adjustment for risk factors in each model

Covariates	**Model 1**	**Model 2***	**Model 3 ****
Demographic	Age Sex	Age Sex	Age Sex
Lifestyle		Smoking Education Alcohol Waist circumference Body fat percentage Physical activity	Smoking Education Alcohol Waist circumference Body fat percentage Physical activity
Hormonal			Early menopause Breast-feeding Hormone replacement therapy Oral contraception Parity

* Model 2 will be considered the main analysis

** Model 3 will be performed as a sex-specific supplemented analyses in women only

Model diagnostics will be performed, the independent censoring assumption will be evaluated by adjustment for recruitment time. For each Cox model the proportional hazards assumption will be investigated for all covariates. The proportional hazard assumption will be evaluated by plotting the scaled Schoenfeld residuals against time since inclusion assessing potential violations, and by visual testing for independence between the residuals and time.

Patient and public involvement

A patient research partner has been included in the development of the protocol and had the opportunity to submit comments to the protocol.

DISCUSSION

This study will explore the association between intake of the major marine n-3 PUFA, EPA and DHA, and the risk of incident RA. We will study a large number of cases, identified within a well-defined cohort (DCH) with nearly complete follow-up. Baseline age in the cohort was between 50 to 64 years and the sex- and age-distribution displays a slightly higher participation among women than among men.(16)

For each of the covariates selected for the models the following arguments apply. Age is an important risk factor for RA. RA can occur in patients of any age, but the peak onset is between the ages of 70 and 74 in both sexes.(1) Sex is a known risk factor for RA, with an approximately 2-fold higher incidence in women compared to men.(1,23–25) Current and previous smoking are associated with higher risk of RA in both sexes, with both duration and intensity of importance.(3,4) Thus, Liu et al. conducted at cohort study among US women and showed that increased duration of smoking cessation was associated with a lower risk of overall RA in women, nevertheless, a higher risk of overall RA was still detectable after 30 years of smoking cessation compared to never smokers.(5) With increasing levels of education the risk of RA decreases, which applies for both sexes.(3) Low to moderate alcohol consumption, but not high alcohol consumption, was associated with a lower rate of incident RA compared to no alcohol consumption.(26) Higher body fat percentage, waist circumference and BMI were associated with higher risk of overall and other RA in women, whereas no clear association was found in men.(27) For anthropometry measurements we selected the variables waist circumference and body fat percentage as a measurement of fat distribution. Leisure-time activity was associated with lower risk of incident RA in women. The lower risk of RA seemed to be dose-response related, as the findings were more pronounced in women who bicycled or walked more than 20 minutes per day and exercised more than 1 hour per week compared to those who did not.(28) Because the highest incidence of RA occur in women after menopause several hormonal risk factors has been suggested. Early menopause is considered associated with higher risk of RA.(29) In contrast, breast-feeding, use of hormone replacement therapy and oral contraception over 7 years has been mentioned as protective factors for RA.(29) The association between parity and risk of RA is less clear, and parity has been suggested both to be a risk and a protective factor.(29)

This study will not likely be prone to systematic problems with misclassification on exposure level and selection bias, since all exposure variables and covariates were collected before and independently of the outcome of interest. However, it has previously been shown that the case definition of RA affects the estimates of its incidence and prevalence.(1,24) Therefore, a strength

of this study is that cases are identified using a validated case definition of RA that rely on linkage between Danish health registers. Furthermore, we will conduct sensitivity analyses based on a less and more restrictive case definition of RA, respectively.
Measurements of both our exposure (EPA and DHA) and outcome (incident RA) variables are believed to be of high quality,(15,22) and the intake of n-3 PUFA in the DCH cohort were higher than in other western population-based studies. However, the information on dietary intake of EPA and DHA will be based on a single food frequency questionnaire and repeated dietary measurements would have been preferable in order to capture changes in dietary habits over time and to reduce random measurement errors. We believe the intake of EPA and DHA are of most biological relevance relative to the total energy intake, and therefore we will use the residual method to energy-adjust all exposures. Adjustment for total energy is obtained by this method. By applying an analytical method as energy adjustment the risk of measurement error in dietary exposures is reduced.(30)
Potential confounders identified prior to data analysis are included in our statistical models. The underlying dietary pattern will be explored using radar plots to identify any potential confounding from the underlying dietary pattern in the study population. Based on the design and available data, potential residual confounding can occur due to inaccurate self-reported information from the DCH questionnaires, insufficient adjustment or risk factors not identified and incorporated into the statistical model.
RA patients from private practising rheumatologists in primary care will not be included in our data, unless they have been transferred to a hospital-based rheumatologist or admitted to a hospital for any reason. Therefore, our findings might not be generalised to mild RA cases treated out of hospital. The DCH data were obtained from the area of greater Copenhagen and Aarhus and finding may therefore not be generalised to the entire Danish population. Of the invited subjects in the DCH cohort study, only 35 % accepted to participate in the study.(16) Previous work indicates that participants are socio-economically more affluent than non-participants, suggesting that our study sample may be healthier than the general population.(16) Moreover, all participants were Caucasians and at least 50 years of age at inclusion, so our conclusions about associations may not be valid in younger age or other ethnicity groups.

This study is expected to bring new insight into the association between dietary intake of marine n-3 PUFAs and the risk of development of RA. The study may potentially bring new insights into the pathogenesis of RA, and it may redefine our understanding of how dietary intake of marine n-3 PUFA, EPA and DHA, may influence the risk of RA.

ETHICS AND DISSEMINATION

The study has been approved by the North Denmark Region Committee on Health Research Ethics (N-20190031) and the Data Protection Committee of Northern Jutland, Denmark (2019-87).
The results of this study will be published in an international peer-reviewed journal and disseminated at international conferences.

ACKNOGLEDGEMENTS

The authors will like to thank the patient research partner for the contribution to the protocol.

PROJECT ORGANISATION

This study is a collaboration that includes clinical specialists within rheumatology, nutrition and biostatisticians.

AUTHORS' CONSTRIBUTION

The study idea was established by BS, LD, SK, ES and JC and the protocol was finalised in collaboration with CB. The first draft of the manuscript was written by BS. All authors were involved in methodological considerations and contributed to the final manuscript.

FUNDING STATEMENT

This work was supported by the Danish Rheumatism Association (R172-A6090; R175-A6091; R186-A6573, Bolette Gylden Soussi) and Aase and Ejnar Danielsens Foundation (19-10-0295, Bolette Gylden Soussi).

COMPETING INTERESTS STATEMENT

Dr. Soussi reports grants from The Danish Rheumatism Association, grants from Aase and Ejnar Danielsens Foundation, during the conduct of the study. Dr. Bork has nothing to disclose. Dr. Kristensen has nothing to disclose. Dr. Lundbye-Christensen has nothing to disclose. Dr. Duch has nothing to disclose. Dr. Cordtz has nothing to disclose. Dr. Christensen has nothing to disclose. Dr. Schmidt has nothing to disclose. Dr. Dreyer reports grants from BMS, other from Galderma, other from Eli Lilly, outside the submitted work.

DATA SHARING STATEMENT

No data are available.

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BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2020-047982.R1
Article Type:	Protocol
Date Submitted by the Author:	27-May-2021
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Primary Subject Heading:	Rheumatology
Secondary Subject Heading:	Epidemiology, Nutrition and metabolism
Keywords:	RHEUMATOLOGY, EPIDEMIOLOGY, NUTRITION & DIETETICS

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Intake of marine n-3 polyunsaturated fatty acids and the risk of rheumatoid arthritis: protocol for a cohort study using data from the Danish Diet, Cancer and Health Cohort and Danish health registers

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Word count: 3435

ABSTRACT

Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory joint disease with multifactorial aetiology. Smoking is a well-established lifestyle risk factor, but diet may also have an impact on the risk of RA. Intake of the major marine n-3 polyunsaturated fatty acids in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been hypothesised to lower the risk of RA due to their anti-inflammatory effects, albeit based on limited knowledge. Therefore, we aim to investigate the associations between dietary intake of EPA and DHA and the risk of incident RA.

Methods and analysis: A cohort study. The follow-up design will be based on data from the Danish Diet, Cancer and Health cohort, which was established between 1993 and 1997. The participants will be followed through record linkage using nationwide registers including the Danish Civil Registration System, the Danish National Patient Registry and the Danish National Prescription Registry using the unique Civil Personal Registration number. Time-to-event analyses will be conducted with RA as the outcome of interest. The participants will be followed from inclusion until date of RA diagnosis, death, emigration or end of follow-up. Hazard ratios with 95 % confidence intervals obtained using Cox proportional hazard regression models, with age as underlying time scale and adjustment for established and potential risk factors, will be used as measures of association.

Ethics and dissemination: The study has been approved by the Data Protection Committee of Northern Jutland, Denmark (2019-87) and the North Denmark Region Committee on Health Research Ethics (N-20190031). Study results will be disseminated through peer-reviewed journals and presentations at international conferences.

Keywords: Rheumatology, epidemiology, nutrition & dietetics

STRENGTHS AND LIMITATIONS OF THIS STUDY

- Danish administrative registers ensure nearly complete follow-up of the study population.
- Use of a validated rheumatoid arthritis (RA) case definition with a positive predictive value of 88 % for overall RA.
- All exposures will be energy-adjusted.
- Dietary assessment is based on a single food frequency questionnaire, which may not capture changes in dietary habits during follow-up.
- Participants were 50 to 65 years old Caucasians at enrolment and therefore the results may not be valid to other age and ethnic groups.

INTRODUCTION

Rheumatoid arthritis (RA) is the most common chronic autoimmune joint disease. RA is characterised by inflammation of the synovial joints leading to irreversible joint damage and deformity, which may result in severe disability. The overall incidence rate of RA from 1996 to 2016 in Denmark has been reported to be 35 per 100,000 person years. The incidence rate was approximately 2-fold higher in women than in men, and with a peak in incidence rate between ages of 70 to 74 in both sexes.(1)

The aetiology of RA is considered multifactorial and lifestyle factors seem to play an important role for the development of RA. Smoking is the best established lifestyle risk factor,(2–5) whereas the impact of other lifestyle factors such as diet are less clear.

Marine n-3 polyunsaturated fatty acids (PUFAs) are organic compounds that may affect a variety of biological pathways which may in turn influence inflammatory processes. Marine n-3 PUFAs may, upon ingestion, become incorporated into cellular membranes, pooled for storage, or converted into lipid signalling molecules. The marine n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are mainly derived from seafood, especially fatty fish.(6) PUFA seems to play an important role in the immune system. While the more common n-6 PUFAs mainly derived from vegetable oils and meat have proinflammatory actions, the marine n-3 PUFAs, EPA and DHA, has been ascribed anti-inflammatory properties by inhibiting a number of aspects of inflammation including production of cytokines and eicosanoids, leukocyte chemotaxis, adhesion molecule expression, and leukocyte-endothelial adhesive interactions.(7,8) Further, EPA and DHA promote production of inflammation resolving mediators in form of resolvins, protectins and maresins.(8) Previous studies have supported that marine n-3 PUFAs may lower the risk of several inflammatory diseases including cardiovascular disease, inflammatory bowel disease and cancer.(9,10) However, limited data exist regarding the role of fish and marine n-3 PUFAs in relation to development of RA and findings have been conflicting. Thus, a previous cohort study, with a limited number of RA patients, reported that intake of fatty fish was associated with a statistically non-significant lower risk of RA, whereas intake of medium fatty fish was associated with a significantly higher risk of RA compared with participants with a lower intake.(11) A previous cohort study based on the Swedish Mammography Cohort reported that a intake of marine n-3 PUFAs (EPA, DHA and docosapentaenoic acid) of more than 0.21 grams per day was associated with a markedly lower risk of RA compared with a lower intake.(12) In contrast, a recent cohort study conducted among US women found no clear association between total marine n-3 PUFAs from diet and supplements and the risk of RA.(13) Therefore, whether marine n-3 PUFAs may lower the risk of developing RA remains unclear.

The aim of the present study is to assess the associations between dietary intake of EPA and DHA and the risk of incident RA. We hypothesise that intake of marine n-3 PUFAs, respectively EPA and DHA is inversely associated with the rate of incident RA.

METHODS AND ANALYSES

Study design

We will conduct a cohort study using data from the Danish Diet, Cancer and Health (DCH) cohort and Danish health registers.

Data sources

We will follow the participants enrolled into the DCH cohort through nationwide Danish health registers including the Danish National Patient Registry (DNPR), the Danish National Prescription Registry (NPR) and the Civil Registration System (CRS). Use of the unique 10-digit personal identifier assigned to all Danish residents at birth or immigration, allows for accurate register-linkage on an individual-based level.

DCH: The DCH cohort was established from December 1993 to May 1997 and includes participants from the area of greater Copenhagen and Aarhus in Denmark who did not have a previous cancer diagnosis registered in the Danish Cancer Registry prior to enrolment. In total, 57,053 subjects between 50 and 65 years of age were included in the study (27,179 men and 29,874 women). At baseline, participants completed a detailed questionnaire on health status, social factors, lifestyle, and a validated 192-item semi-quantitative food frequency questionnaire.(14,15) The food frequency questionnaire covered a total of 24 questions regarding intake of fish. The food frequency questionnaire has previously been validated against two times 7-days weighted diet records and found useful for categorising according to their intake of total energy and PUFA intake.(15) The questionnaires were checked for reading errors and missing information at baseline by a technician. Furthermore, anthropometric measurements were collected at baseline. A detailed description of the DCH cohort has been published previously.(16)

DNPR: The DNPR holds data on all admissions to somatic hospitals in Denmark since 1977 and all out-patient attendances since 1995. The data includes dates of hospital admissions, ward types, discharge diagnoses, dates of all attendances at out-patient clinics, and diagnoses recorded at each attendance.(17,18) Diseases are classified according to the International Classification of Disease (ICD). The ICD 8th edition was used until January 1994, and thereafter the ICD 10th edition was implemented.

NPR: The NPR provides data about all prescription drugs dispensed at Danish community pharmacies since 1994,(19) however the register does not include information on drugs dispensed by hospital pharmacies directly to in- or out-patients.

CRS: The CRS are continuously updated and ensures complete follow-up regarding vital and migration status of all Danish citizens.(20)

Study population

The study population consists of all participants enrolled into the DCH cohort with complete records of data on exposures and covariates and without a diagnosis of RA recorded in the DNPR prior to their enrolment into the cohort. Also, participants with a previous diagnosis of cancer before enrolment will be excluded as well.

Exposures and outcome of interest

Intake of EPA and DHA will be calculated based on the food frequency questionnaire collected at baseline. By multiplying the frequencies of intake by the portion size, the individual average intake in grams per day of all foods and nutrients will be calculated using the software FoodCalc version 1.3 based on Danish Food composition tables used in Denmark during enrolment of the DCH participants.(15,21) Exposures of interest include the energy-adjusted intake of EPA and DHA.

The outcome of interest for this study is incident RA, defined as the first occurrence of an ICD-10 code of M05-06 registered in DNPR and a subsequent redeemed prescription of a conventional synthetic Disease-Modifying Anti-Rheumatic Drug (csDMARD) within 1 year from the date of diagnosis in the DNPR. Redeemed prescriptions of csDMARD are identified in the NPR by Anatomical Therapeutic Chemical (ATC) codes (Table 1). A previous validation study by Linauskas et al. reported in the same cohort a positive predictive value of 88 % for overall RA based on this case definition.(22)

Table 1. ATC codes for conventional synthetic DMARD

Conventional synthetic DMARD	ATC Code
Methotrexate	L04AX03 / L01BA01
Sulfasalazine	A07EC01
Hydroxychloroquine	P01BA02
Leflunomide	L04AA13
Ciclosporine	L04AD01 / L04AA01
Gold	MC01CB
Azathioprine	L04AX01

DMARD = disease-modifying anti-rheumatic drug
ATC = anatomical therapeutic chemical

Established risk factors for RA were identified by review of the existing literature prior to data analysis. We identified the following covariates that needed to be adjusted for: age (years),(1) sex (women, men),(1,23–25) smoking (smoking status (never, former or current smoker), and pack years of smoking (years)),(3–5) level of education (basic school, higher education 1 to 2 year, higher education 3 to 4 year or higher education >4 years),(3) alcohol (alcohol consumption (grams per day), and alcohol abstinence (yes, no)),(26) waist circumference (cm),(27) body fat percentage (%),(27) and physical activity (hours per week);(28) and further for women: early menopause (yes, no),(29) breast-feeding (months),(29) hormone replacement therapy (<7, ≥7 years of use),(29) oral contraception (<7, ≥7 years of use),(29) and parity (number of pregnancies).(29) Data on genetic disposition are not available and therefore not adjusted for.

Statistical analysis plan

Demographic and descriptive data will be presented as proportions (%) for categorical variables and median values for continuous variables with 95 % central ranges (2.5th; 97.5th percentile). The participants will be followed from date of entry into the DCH cohort until date of RA diagnosis, death, emigration or end of follow-up (end of year 2018), whichever comes first.
We will perform time-to-event analyses with RA as the event of interest. Hazard ratios (HRs) with 95 % confidence intervals obtained from Cox proportional hazard regression models will be used as measures of association between dietary intake of EPA and DHA and rate of incident RA. The

time-to-event analyses will be conducted by allowing for different baseline hazards among men and women with attained age as the underlying timescale, with adjustment for baseline age to ensure that baseline covariates have same age.
All exposure variables will be examined in continuous analyses using restricted cubic splines and in categorical analyses in quintiles. Radar plots will be constructed to support the interpretation of our results and to visualize potential confounding from the underlying dietary pattern.
A two-tailed p-value <0.05 will be considered statistically significant.

Intake of EPA and DHA will be energy-adjusted using the residual method assuming that intake of our exposures of interest are of most biological relevance relative to total energy intake. Residuals will be estimated separately among men and women based on linear regression between total energy intake and reported intakes of EPA and DHA. All estimated residuals will be added to the mean intake among men and women within the cohort. By definition, residual energy-adjusted intakes of EPA and DHA are independent of the total energy.

Based on the identified risk factors, three models were created (Table 2). Model 1 contains adjustment for demographic risk factors. Model 2 (primary model for interpretation) contains the same covariates as model 1 combined with lifestyle risk factors. Differences in point estimates observed in analyses including adjustment for the covariates included in model 2 compared with model 1 may reflect the importance of confounding. In supplemented analyses, we will conduct sex-specific analyses. In analyses of the associations between EPA and DHA and the rate of RA in women additional adjustment for potential hormonal risk factors (model 3) will be undertaken to investigate residual confounding.
If we have few cases of RA it may be necessary to lower the number of risk factors that we adjust for.
In addition, secondary and sensitivity analyses will be conducted. In supplemental analyses we will investigate our exposure of interest and the risk of seropositive and seronegative RA. Also, we will terminate the follow-up earlier to explore the influence of follow-up time on our associations of interest. Relevant sensitivity analyses include adjustment for risk factors using other available variables than the ones used in the primary analysis, e.g. adjustment for body mass index (BMI) instead of body fat percentage. Also, we will adjust for duration (years) since smoking cessation in former smokers. Model 2 contains adjustment for education and we will add a variable for household income. In sensitivity analyses we will examine whether the spline curves are robust when the number and location of the knots are modified. Further, our main analysis will be conducted with a less strict and a more restrict outcome definition, respectively.

Table 2. Overview of models including adjustment for risk factors in each model

Covariates	**Model 1**	**Model 2***	**Model 3 ****
Demographic	Age	Age	Age
	Sex	Sex	Sex
Lifestyle		Smoking	Smoking
		Education	Education
		Alcohol	Alcohol
		Waist circumference	Waist circumference
		Body fat percentage	Body fat percentage
		Physical activity	Physical activity

Hormonal	Early menopause Breast-feeding Hormone replacement therapy Oral contraception Parity

* Model 2 will be considered the main analysis
** Model 3 will be performed as a sex-specific supplemented analyses in women only

The proportional hazards assumption will be evaluated by plotting the scaled Schoenfeld residuals against age. The independent censoring assumption will be evaluated by adjustment for recruitment time.

Patient and public involvement

A patient research partner has been included in the development of the protocol and had the opportunity to submit comments to the protocol.

DISCUSSION

This study will explore the association between intake of the major marine n-3 PUFA, EPA and DHA, and the risk of incident RA. We will study a large number of cases, identified within a well-defined cohort (DCH) with nearly complete follow-up. Baseline age in the cohort was between 50 to 65 years and the sex- and age-distribution displays a slightly higher participation among women than among men.(16)

For each of the covariates selected for the models the following arguments apply. Age was an important risk factor for RA. RA can occur in patients of any age, but the peak onset was between the ages of 70 and 74 in both sexes.(1) Sex was a known risk factor for RA, with an approximately 2-fold higher incidence in women compared to men.(1,23–25) Current and previous smoking were associated with a higher risk of RA in both sexes, with both duration and intensity of importance.(3,4) Thus, Liu et al. conducted at cohort study among US women and showed that increased duration of smoking cessation was associated with a lower risk of overall RA in women, nevertheless, a higher risk of overall RA was still detectable after 30 years of smoking cessation compared to never smokers.(5) With increasing levels of education the risk of RA decreased, which applied for both sexes.(3) Low to moderate alcohol consumption, but not high alcohol consumption, was associated with a lower rate of incident RA compared to no alcohol consumption.(26) A higher body fat percentage, waist circumference and BMI were associated with higher risk of overall and other RA in women, whereas no clear association was found in men.(27) For anthropometry measurements we selected the variables waist circumference and body fat percentage as a measurement of fat distribution. Leisure-time activity was associated with lower risk of incident RA in women. The lower risk of RA seemed to be dose-response related, as the findings were more pronounced in women who bicycled or walked more than 20 minutes per day and exercised more than 1 hour per week compared to those who did not.(28) Because the highest incidence of RA occur in women after menopause several hormonal risk factors has been suggested. Early menopause is considered associated with higher risk of RA.(29) In contrast, breast-feeding, use of hormone replacement therapy and oral contraception over 7 years has

been mentioned as protective factors for RA.(29) The association between parity and risk of RA is less clear, and parity has been suggested both to be a risk and a protective factor.(29)

This study will not likely be prone to systematic problems with misclassification on exposure level and selection bias, since all exposure variables and covariates were collected before and independently of the outcome of interest. However, it has previously been shown that the case definition of RA affects the estimates of its incidence and prevalence.(1,24) Therefore, a strength of this study is that cases will be identified using a validated case definition of RA that rely on linkage between Danish health registers. Furthermore, we will conduct sensitivity analyses based on a less and more restrictive case definition of RA, respectively.
Measurements of both our exposure (EPA and DHA) and outcome (incident RA) variables are believed to be of high quality,(15,22) and the intake of n-3 PUFA in the DCH cohort were higher than in other western population-based studies. However, the information on dietary intake of EPA and DHA will be based on a single food frequency questionnaire and repeated dietary measurements would have been preferable in order to capture changes in dietary habits over time and to reduce random measurement errors. We believe the intake of EPA and DHA are of most biological relevance relative to the total energy intake, and therefore we will use the residual method to energy-adjust all exposures. Adjustment for total energy is obtained by this method. By applying an analytical method as energy adjustment the risk of measurement error in dietary exposures is reduced.(30)
Potential confounders identified prior to data analysis are included in our statistical models. The underlying dietary pattern will be explored using radar plots to identify any potential confounding from the underlying dietary pattern in the study population. Based on the design and available data, potential residual confounding can occur due to inaccurate self-reported information from the DCH questionnaires, insufficient adjustment or risk factors not identified and incorporated into the statistical model.
RA patients from private practising rheumatologists in primary care will not be included in our study, unless they have been transferred to a hospital-based rheumatologist or admitted to a hospital for any reason. Therefore, our findings might not be generalised to mild RA cases treated out of hospital. The DCH data were obtained from the area of greater Copenhagen and Aarhus and finding may therefore not be generalised to the entire Danish population. Of the invited subjects in the DCH cohort study, only 35 % accepted to participate in the study.(16) A previous study have indicated that participants within the DCH cohort was socio-economically more affluent than non-participants, suggesting that our study sample may be healthier than the general population.(16) Moreover, all participants were Caucasians and at least 50 years of age at inclusion, and therefore may our findings not be valid in younger age or other ethnicity groups.

This study is expected to bring new insight into the association between dietary intake of marine n-3 PUFAs and the risk of development of RA. The study may potentially bring new insights into the pathogenesis of RA, and it may redefine our understanding of how dietary intake of marine n-3 PUFA, EPA and DHA, may influence the risk of RA.

ETHICS AND DISSEMINATION

The study has been approved by the North Denmark Region Committee on Health Research Ethics (N-20190031) and the Data Protection Committee of Northern Jutland, Denmark (2019-87). The

DCH cohort was approved by the relevant scientific Ethic Committees and the Data Protection Agency and all participants gave written informed consent at enrolment.
The results of this study will be published in an international peer-reviewed journal and disseminated at international conferences.

ACKNOGLEDGEMENTS
The authors will like to thank the patient research partner for the contribution to the protocol.

PROJECT ORGANISATION
This study is a collaboration that includes clinical specialists within rheumatology, nutrition and biostatisticians.

AUTHORS' CONSTRIBUTION
The study idea was established by BGS, LD, SK, EBS and JHC and the protocol was finalised in collaboration with CSB. All authors (BGS, CSB, SK, SLC, KD, RLC, JHC, EBS, LD) contributed to methodological considerations. BGS, CSB, SLC, KD and LD were involved in the statistical analysis plan. The first draft of the manuscript was written by BGS. All authors (BGS, CSB, SK, SLC, KD, RLC, JHC, EBS, LD) contributed to the final manuscript, critically reviewed the manuscript and agreed on submission.

FUNDING STATEMENT
This work was supported by the Danish Rheumatism Association (R172-A6090; R175-A6091; R186-A6573, Bolette Gylden Soussi) and Aase and Ejnar Danielsens Foundation (19-10-0295, Bolette Gylden Soussi).

COMPETING INTERESTS STATEMENT
Dr. Soussi reports grants from The Danish Rheumatism Association, grants from Aase and Ejnar Danielsens Foundation, during the conduct of the study. Dr. Bork has nothing to disclose. Dr. Kristensen has nothing to disclose. Mr. Lundbye-Christensen has nothing to disclose. Ms. Duch has nothing to disclose. Dr. Cordtz has nothing to disclose. Dr. Christensen has nothing to disclose. Dr. Schmidt has nothing to disclose. Dr. Dreyer reports grants from BMS, other from Galderma, other from Eli Lilly, outside the submitted work.

DATA SHARING STATEMENT
No data are available.

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