Study protocol

Microbial dYsbiosis in the pathogenesis of Rheumatoid Arthritis: using metagenomics to predict methotrexate efficacy (MyRA)

Study Team

Non-clinical investigators:

Chief Investigator — Professor Simon Carding, Quadram Institute Bioscience
simon.carding@quadram.ac.uk (01603) 251410

Principal Investigator — Miss Ellie Sayers, University of East Anglia
ellie.sayers@quadram.ac.uk (01603) 255184

Clinical investigators:

Principal Investigator — Professor Alexander MacGregor, University of East Anglia
a.macgregor@uea.ac.uk (01603) 593570

Co-investigator — Mrs Jacqueline Chipping, University of East Anglia
jacqueline.chipping@uea.ac.uk (01603) 597204

Co-investigator — Mrs Tarnya Marshall, Norfolk and Norwich University Hospital
tarnya.marshall@nnuh.nhs.uk (01603) 597204

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Sponsor's approval:

This protocol has been approved by the Quadram Institute Bioscience Human Research Governance Committee (HRGC)

Name Dr Antonietta Melchini Signature .....

Role QIB HRGC chair Date 8th October 2018

I have fully discussed the objectives of this study and the contents of this protocol with the Sponsor's representative. I understand that the information in this protocol is confidential and should not be disclosed other than to those directly involved in the execution or ethical review of the trial.

I agree to conduct this study according to this protocol and to comply with its requirements, subject to ethical and safety considerations and guidelines, and to conduct the study in accordance with International Conference on Harmonisation (ICH) guidelines on Good Clinical Practice (GCP) and with the applicable regulatory requirements.

Chief Investigator name and address:

Prof Simon Carding
Quadram Institute Bioscience
Norwich Research Park
Colney Lane
Norwich
NR4 7UA

Signature ..... Date 08.10.18

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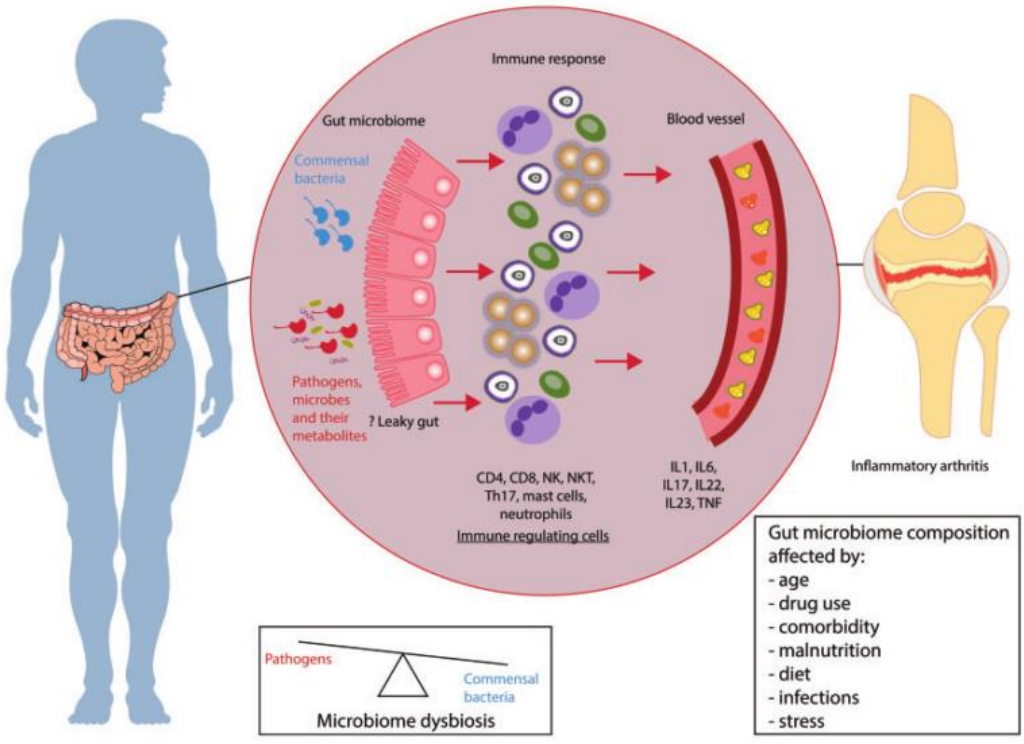
1 Abstract

Rheumatoid arthritis (RA) is a common autoimmune disease which causes inflammation in the joints and resulting disability. RA has a significant impact on a person's quality of life and carries a heavy financial burden both to the individual and to society. Its' causes remain unknown, however the discovery of alterations in the populations of microbes that normally reside in the gut in RA patients has highlighted the potential of gut microbes being involved in the development of RA. This study will investigate this possibility further by examining the bacteria and viruses resident in the gut of 30 patients with early rheumatoid arthritis. To evaluate the importance of gut bacteria and viruses in the development and successful treatment of RA with methotrexate, stool samples will be collected at 3 time points: a) before treatment, b) during treatment and c) following maintenance or escalation of treatment based on a successful or unsuccessful response, respectively. We will also explore if patients with RA have a leaky gut (increased intestinal permeability) and develop an aberrant immune response to their own gut microbes as a result of them gaining access to the bloodstream, and whether this is altered with methotrexate treatment. Furthermore, as diet and lifestyle affect RA activity and progression and can influence the activity of gut microbes, dietary and lifestyle data will be collected to identify potential covariants with disease onset or progression. The outcomes of this project will help identify microbiome-based predictive factors associated with response to methotrexate, and a clearer understanding of the interplay between gut microbes, intestinal permeability and environmental factors in the development of RA.

Figure 1. Proposed mechanism of the gut-joint axis in inflammatory arthritis (Jethwa, 2017[1])



2 Scientific Background

2.1 RA epidemiology and clinical treatment

Rheumatoid arthritis (RA) is a chronic autoimmune condition characterized by inflammation of the joints that affects approximately 0.3-1% of the world population[2]. Its causes are unclear. Although linked with genetic predisposition, low concordance of RA among genetically identical twins suggests a significant contribution of environmental factors[3]. RA may be either seropositive or seronegative, based on the respective presence or absence of the autoantibodies anti-cyclic citrullinated peptide (anti-CCP; directed against various joint components) and/or rheumatoid factor (RF; directed against immunoglobulin G).

Oral methotrexate (MTX) is the standard initial treatment provided for early RA. It is one of several disease-modifying anti-rheumatic drugs (DMARDs) which may be used in combination if MTX monotherapy is ineffective. The disease-activity lowering effects of MTX are only seen at a clinically important level (i.e. a reduction of ≥1.2 in the Disease Activity Score [DAS]) in approximately 34% of patients[4]. MTX; which has various side effects including increased risk of infection, is therefore sometimes used with little or no clinical benefit to the patient. There are no reliable predictors of MTX response, although those concomitantly using steroid injections (prednisolone) and of the male sex are more likely to respond well[4]. As some gut bacteria are able to assimilate and chemically alter MTX *in vitro*, it is possible that the composition of the gut microbiome might be involved in MTX response[5–7].

2.2 Microbial dysbiosis and “leaky gut” in RA

The vast population of microbes that inhabit the human gut, i.e. the microbiota, includes a wide variety of bacteria, fungi and viruses. The composition of these microbes is altered (microbial dysbiosis) in some chronic conditions including RA. A gut-joint axis (Figure 1) has been proposed for RA pathogenesis whereby microorganisms in the gut are able to influence disease initiation and progression, potentially via increased intestinal permeability (“leaky gut”) and microbial dysbiosis[1]. However, although several studies have identified microbial dysbiosis in RA patients[8–10], it is not clear how these changes contribute to disease outcomes.

The microbiota alterations identified so far in RA have generally consisted of a reduced diversity of bacteria, coupled with increased or decreased abundance of individual microbial taxa. For instance, abundance of *Prevotella copri* is associated with new-onset RA, and *Collinsella*; which has the ability to alter intestinal permeability possibly allowing microbial/dietary components in the gut lumen to access host immune structures, is also increased in abundance RA[8–11]. On the contrary, potentially beneficial bacteria such as *Faecalibacterium* are depleted in some RA patients[10]. The relevance of these alterations to RA treatment is unknown, although some differences in the abundance of specific bacteria between responders and non-responders to MTX and herbal medications have been observed[9]. This suggests the involvement of gut microbes in response to MTX, potentiating the use of gut microbiota profiles as a predictive tool for response, or the use of microbiota-altering strategies to improve treatment outcome.

The gut-joint axis theory encompasses the possibility that the autoimmune response seen in RA may arise as a result of immune responses against antigens in the gut (microbial or dietary), which are cross-reactive with antigens expressed by human cells (molecular mimicry)[9,12]. These immune responses may be enabled by an increase in intestinal permeability. There is variable evidence of increased intestinal permeability in RA patients, which may also be influenced by gut microbes such as *Collinsella*[10,13,14]. However, little is known about any specific immune responses against gut microbes, or how immune cells respond to the expanded subsets of microbes in RA. Elucidating if

microbial dysbiosis and/or an immune response against gut microbes effects disease severity and response to treatment is important to make microbiome research translatable and identify targets for the improvement of treatment strategies.

2.2.1 The gut virome in RA

Gut viruses (the virome) are a largely overlooked component of the human gut microbiome which is surprising as they represent its largest component[15]. A major factor in our limited understanding of the gut virome is the lack of suitable tools and analytical methods. The virome consists mainly of viruses that infect bacteria (phages), which most likely control the structure and function of bacterial populations in the gut[16,17]. Like bacterial populations, the composition of viruses in the gut varies according to environmental factors such as diet and antibiotics[15,18].

A study of the virome in inflammatory bowel disease (IBD) demonstrated changes to the intestinal virome in a disease state, with an increase in the number of different viruses present and an associated decrease in bacterial populations, suggesting a predator-prey relationship between the two[19]. The intestinal virome has not yet been characterized in patients with RA, though as phages are able to significantly alter bacterial populations it is possible they may be initiating factors for microbial dysbiosis or alter the abundance of MTX response-associated bacteria. Alternatively, the ability of viruses to cross the intestinal barrier, gain entry to the body and induce host immune responses could provide a trigger for the generation of cross-reactive antibodies in RA[19,20].

2.3 Diet and RA

Environmental factors such as smoking, diet, and infection are associated with risk of RA[21–23]. Many of these factors also influence the composition of the human gut microbiome[24]. Microbiome studies therefore need to account for environmental factors to provide a more comprehensive and insightful analysis, although this is not always done. For instance, dietary analysis has been absent from microbiome studies in RA, despite diet being a potential causal factor in disease-associated microbial dysbiosis[25]. RA patients often feel that their symptoms are associated with dietary fluctuations[26], and diet represents an easily modifiable risk factor for RA. However, little reliable evidence exists for the benefits of using dietary intervention to improve RA disease activity[27], and no studies have been carried out to assess whether diet is associated with RA treatment response or response-associated microbiota profiles. Identifying dietary components associated with microbial dysbiosis in RA could enable the generation of personalized treatment programs incorporating diet recommendations, which would be a simple and cost-effective way of enhancing treatment response in RA.

2.1 Summary

Microbial dysbiosis has been established in RA in several studies, although the cause of dysbiosis and its relevance to clinical management of RA is unknown. Additionally, the ability of microbes in the gut to initiate immune responses contributing to RA; possibly via increased intestinal permeability, is not well understood. This study will primarily investigate whether gut microbial dysbiosis; or more specifically microbial diversity (Shannon index), is associated with MTX treatment response in RA. This will build on a previous study[9] in which differences in the abundance of specific bacterial groups were identified between responders and non-responders to MTX, by defining a more specific early RA cohort on MTX monotherapy, characterizing changes to the viral as well as bacterial components of the microbiome, investigating intestinal permeability in these participants and exploring microbiome associations with diet and lifestyle factors. We will investigate longitudinal changes to the microbiota induced by MTX over 6 months and attempt to correlate a continuous microbiome parameter (α-diversity/Shannon index) with change in DAS28-C-reactive protein (DAS28-CRP).

Correlations between specific microbial abundances; including that of viruses, and treatment response will also be identified. Together, these analyses will help identify response-associated microbiota alterations prior to and during MTX treatment, which could be targeted to improve MTX treatment response. Any response-associated microbiota alterations will be tested for association with diet and lifestyle variables, to provide insight into whether these alterations could be caused by environmental factors affecting the microbiota. Finally, we will investigate whether systemic immune responses against gut microbes are present in RA, which could suggest increased intestinal permeability and a possible "gut origin" for RA.

3 Hypothesis

A single microbiome parameter (α-diversity, microbial diversity measured by Shannon index) in treatment-naïve early RA patients positively correlates with MTX response at 3 months (measured by change in DAS28- CRP) and can be used to predict treatment response in these patients.

4 Study objectives

This study has been designed primarily to provide information about changes in gut bacteria and viruses of RA patients undergoing treatment with MTX. The primary outcome in this study is DAS28-CRP (a measure of disease activity recorded at every routine clinical appointment) studied as a continuous variable. More information on the collection of disease activity variables including DAS28-CRP can be found in Section 5.7.

4.1 Primary aim

To determine alterations in gut bacteria and viruses of early RA patients and if specific changes are associated with the outcome of MTX treatment, by estimating the correlation between microbial diversity (α-diversity measured by Shannon index) and response to MTX measured by change in DAS28-CRP.

4.2 Secondary aims

1. To explore more broadly the correlations between baseline gut microbiome parameters; such as compositional and functional profiles, and MTX outcome or disease severity measured by DAS28-CRP, Simplified Disease Activity Index (SDAI), CRP, ESR or autoantibody titer.

2. To explore longitudinal alterations to the composition of gut bacteria and viruses in early RA patients throughout 6 months of MTX treatment.

3. To explore if environmental factors such as diet and lifestyle can be used to explain response-associated alterations in bacterial and/or viral populations in early RA patients.

4. To determine if RA patients generate autoimmune responses to their own intestinal microbes (indicative of increased intestinal permeability) and if this changes with treatment, by testing the humoral (antibody) and cellular (peripheral blood mononuclear cell [PBMC]) reactivity against commensal microbes.

5 Study design and methods

5.1 Timeline

A prospective cohort of 30 early RA patients will be recruited to complete this study, with data collection taking place at 3 time points: a) before treatment, b) during treatment (3 months) and c) following treatment maintenance or escalation depending on successful or unsuccessful response (6

months). An outline of the study is provided in Figure 2, study flowchart describing the involvement of participants in the study in Figure 3, and data collection time points in Figure 4.

5.2 Setting

Sample processing and data analysis will take place using facilities available at the Norwich Research Park (NRP) in Norwich, including the Quadram Institute Bioscience (QIB) and University of East Anglia (UEA) including the Bob Champion Research and Education Building (BCREB). Identification of participants will take place mainly at the Norfolk Arthritis Register (NOAR) office at UEA, and recruitment of further participants may take place at the Rheumatology Department at Norfolk and Norwich University Hospital (NNUH) or at participating General Practitioner (GP) clinics around Norfolk. Study visits will coincide with routine appointments and take place at the Rheumatology Department at NNUH.

Figure 2. Study outline

5.3 Recruitment policy

A total of 30 participants will be recruited to complete this study, although the recruitment aim will be at least 35 to account for drop out. Recruitment will run for 11 months from October 2018 through August 2019, following successful ethical approval and funding applications. This will allow for preparation and sequencing of final samples and analysis of data before September 2020, with the final study appointments finishing in April 2020.

5.3.1 Recruitment from the NOAR

Potential participants will be identified primarily via the incoming stream of GP referrals to the Early Arthritis Clinic at NNUH received by the Norfolk Arthritis Register (NOAR); an ongoing inception cohort of inflammatory arthritis patients in Norfolk, by Jacqueline Chipping (NOAR Clinical Manager and Research Nurse). NOAR was established in 1989 and investigates the development and treatment of inflammatory polyarthritis along its natural course. More than 4,000 patients are registered in NOAR, which involves longitudinal 12-monthly study visits where the Research Nurse collects information on historical and current health and joint symptoms and takes a blood sample. Further information on NOAR can be found in the NOAR protocol (Appendix 13) and at the NNUH website: http://www.nnuh.nhs.uk/departments/rheumatology/norfolk-arthritis-register/

5.3.1.1 NOAR participants meeting ACR 2010 criteria for RA

NOAR recruits approximately 18 participants per month, of which approximately 5 would potentially be suitable for this study as they meet ACR criteria for RA at baseline. Participants will be invited via the ongoing stream of referrals to NOAR, allowing around 55 participants to be invited to this study over the 11 month recruitment period. Existing NOAR members will also be invited to this study. As the NNUH appointments are part of an 18 week pathway, it will be possible to invite existing NOAR participants meeting the study criteria and recruited to NOAR within the last 4-5 months (approximately 25 potential participants), as they will not have had their initial appointment yet. This provides a minimum of 80 participants who can be invited to this study, which should be sufficient; alongside recruitment from GPs and Rheumatology Clinicians, to obtain 35 participants.

5.3.1.2 Recruitment procedure from NOAR

Figures 2 and 3 shows representations of the study timeline from the Study Team and participants point of view, respectively. A patient presenting to their GP with inflammatory arthritis and being referred to the Early Arthritis Clinic at NNUH will be notified to NOAR as part of their normal recruitment procedure, and at the same time as sending out the NOAR participant information sheet and consent form (Appendix 14 and 15); if they meet the eligibility criteria for this study, the NOAR Research Nurse will also send out an invitation letter (Appendix 2), Participant Information Sheet (PIS) (Appendix 3) containing a response slip, watermarked versions of the consent forms (Appendix 24 and 25) and prepaid envelope for the MyRA study. If a positive response slip is received, the participant will be contacted via telephone by Ellie Sayers or Jacqueline Chipping to discuss the study further and explain what will happen at their first Early Arthritis Clinic appointment. Existing NOAR members suitable for this study; who have not yet had their initial NNUH Early Arthritis Clinic appointment, will also be identified by the NOAR Research Nurse and a copy of the MyRA invitation letter (Appendix 2) and PIS (Appendix 3) will be sent to them inviting them to the study, along with watermarked versions of the consent forms (Appendix 24 and 25) so they may review these prior to their first study visit if they chose to take part.

5.3.2 Recruitment from the participant's GP or NNUH Rheumatology Clinician

In addition to NOAR, MyRA participants will be recruited directly via the patient's own GP or NNUH Rheumatology Clinician. GPs and clinicians will be provided with study information to give to

potential participants entering their clinics who might fit the eligibility criteria. Participating GPs and clinicians will be provided with this Study Protocol so they may answer brief questions about the study, but potential participants will still be asked to return the response slip to QIB and will receive a telephone call from Ellie Sayers or Jacqueline Chipping prior to visiting NNUH for their initial Early Arthritis Clinic appointment. If a potential participant is recruited from their initial Early Arthritis Clinic appointment at NNUH and responds positively using the response slip, they will receive the telephone call prior to their Nurse Practitioner appointment at NNUH.

5.4 Eligibility criteria

5.4.1 Inclusion criteria

- 18-65 years of age
- RA diagnosis based on ACR 2010 classification criteria with symptoms starting within the last 2 years
- Referred by GP to the Early Arthritis Clinic at NNUH
- Commencing methotrexate monotherapy for the first time

5.4.2 Exclusion criteria

- *Initially* commencing combination therapy (prior to first stool sample) rather than methotrexate monotherapy i.e. MTX combined with another DMARD or prednisolone
- Commencement of MTX therapy prior to first stool sample or cessation of MTX therapy at any point during the study
- History of psoriasis
- Those currently suffering from, or have ever suffered from, any diagnosed gastrointestinal disease, gastrointestinal disorders including regular diarrhoea and constipation (excluding hiatus hernia unless symptomatic) and/or have undergone gastrointestinal surgery.
- Those regularly (3+ times/week) taking self-prescribed over the counter medications for digestive/gastrointestinal conditions
- Use of laxatives within 7 days prior to sampling unless these have been used on a regular basis (3+ times/week) for more than one month prior to the study and will continue to be used throughout the study period
- The use of over-the-counter medications or food/drinks containing pre and/or probiotics within 7 days prior to sampling, unless these have been used on a regular basis (3+ times/week) for more than one month prior to the study and will continue to be used throughout the study period
- Significant alteration of the participant's normal diet at any point during the study (e.g. adoption of the 5:2 fasting diet)
- Regular (3+ times/week) or recent (within 3 months) use of colonic irrigation or other bowel cleansing techniques
- Recently returned to the UK following a period abroad, and who have suffered gastric symptoms during the period abroad or on return to the UK. These will be assessed on an individual basis
- Currently taking or finished a course of antibiotics within the last 3 months
- Currently pregnant or lactating
- Living with or related to any member of the Study Team
- Those who have limited or no understanding of spoken and written English

5.5 Study procedures

5.5.1 First appointment: Early Arthritis Clinic

5.5.1.1 Informed consent

At the participant's first Early Arthritis Clinic appointment, any member of the Study Team appropriately trained in taking Informed Consent will be present to ensure the participant knows what the study involves and has read and understood the PIS before asking for written Informed Consent, both for participation in the study and for long-term storage of samples at the Norwich Research Park (NRP) Biorepository (Appendix 4 and 5). The consent to store samples long-term in the NRP Biorepository is completely optional. Alternatively, the Rheumatology Clinician in charge of the participant's care may take written Informed Consent on behalf of the study team. The clinicians will have copies of this protocol and will have completed mandatory General Clinical Practice (GCP) training which involves guidelines for taking Informed Consent. Consent will not be sought from potential participants who heard about the study for the first time on the same day.

If the participant is identified by their Rheumatology Clinician at their initial Early Arthritis Clinic appointment and responds positively to the invitation letter, they will not provide informed consent at this stage. Therefore, informed consent for these participants will be taken at their Nurse Practitioner (NP) appointment by an appropriately trained member of the study team before taking the first study samples. Watermarked versions of the consent forms (Appendix 24 and 25) will be posted to the participant prior to this appointment for them to review and they will have had an initial telephone call with a member of the study team.

After consent, a letter to the participant's GP will be generated to inform them of their patient's involvement with MyRA (Appendix 6). The participant will have agreed to this and provided their GP details as part of the consent process.

5.5.1.2 Providing initial study materials

The participant will then be provided with a stool collection kit containing a home stool collection device (Fecotainer), a clear plastic zip-lock bag to place the Fecotainer in and instructions for taking the stool sample (Appendix 7). A 7-day food diary (Appendix 8) will be provided to be completed the week prior to the participant's Nurse Practitioner (NP) appointment (approximately 3 weeks after their first Early Arthritis Clinic appointment), where they will commence methotrexate (MTX) treatment as part of their normal clinical care. Participants will be asked to bring a fresh stool sample to the NP appointment (collected within 2 hours of the appointment), or if they are unable to do this they may wish to organize collection of the stool sample with Ellie Sayers or Jacqueline Chipping either the day before their NP appointment, or the day after provided they have not yet begun MTX treatment at the time.

5.5.1.3 Collection of samples and data if the participant is started on methotrexate at their first Early Arthritis Clinic appointment

Should the Rheumatology Clinician begin their patient on MTX at this first appointment; without arranging an NP appointment, stool and blood samples may be collected at this time point (if the clinician agrees it is suitable to do this prior to commencing the first dose of MTX). Collection of samples and data would be as per Section 5.5.2.1, 5.5.2.2 and 5.5.2.3, except the participant would be asked to fill in the 7-day food diary from this point forward and bring it to their next Routine Treatment Review where the study team will be present. In this case, the participant would then only be seen at their 3 and 6-month Routine Treatment Review appointments (Section 5.5.3) and not at an NP appointment. No samples or data will be collected before the participant has provided written informed consent to take part in this study.

5.5.2 Second appointment: Nurse Practitioner

As part of their normal care pathway, the participant will commence MTX treatment after this appointment. Should the participant or their clinician decide for any reason not to commence MTX treatment, they would be excluded from the study from this point. If continuing the study, participants will be provided with another stool collection kit at the end of this appointment (Fecotainer, instructions for use [Appendix 7], clear plastic zip-lock bag). No further 7-day food diaries will be provided.

5.5.2.1 Collection of stool sample

At the participant's NP appointment their stool sample will be taken from them by a member of the study team, if they were able to produce this sample within the 2 hours prior to this appointment. If this was not possible, collection of a fresh stool sample from the participant's location may be organized between the participant and Ellie Sayers or Jacqueline Chipping for the following day, provided the sample is produced prior to the participant taking their first dose of methotrexate. If the participant was consented at their NP appointment, they will be asked to produce a stool sample before taking their first dose of methotrexate. The participant will be asked to bring another fresh stool sample (taken within 2 hours) to their next appointment in 3 months' time, or to arrange collection of this sample at a time more suitable to them, using the stool collection kit provided.

5.5.2.2 Collection of blood sample

The on-duty Healthcare Assistant (HCA) or Phlebotomist at the NNUH; who will be taking approximately 4ml blood as part of the participant's routine clinical care (to test for inflammatory biomarkers), will be asked to obtain an additional 40 mL blood sample during the same procedure to minimize inconvenience to the participant. This additional 40 mL blood sample will be collected solely for the purpose of this study, and in the form of 2 x 10 mL whole blood (20 mL in Vacutainer EDTA tubes) and 2 x 10 mL serum (20 mL in Vacutainer SST tubes).

5.5.2.3 Collection of 7-day food diary, baseline health and lifestyle questionnaire and clinical data

The participant's completed 7-day food diary will be collected, and any member of the Study Team will go through a baseline health and lifestyle questionnaire (Appendix 9) with the participant at this stage. No further 7-day food diaries will be provided to the participant for the purpose of this study. A methotrexate treatment record will be provided to the participant (Appendix 23) where they will record the dates and dosages of methotrexate taken. Participants will need to keep this document until they have completed the study, so it will be retrieved at the final study visit (6 month routine treatment review).

After the appointment, a member of the study team with appropriate access to NNUH hospital systems will obtain clinical data including DAS28-CRP (recorded by the clinician) using the Case Report Form (Appendix 11).

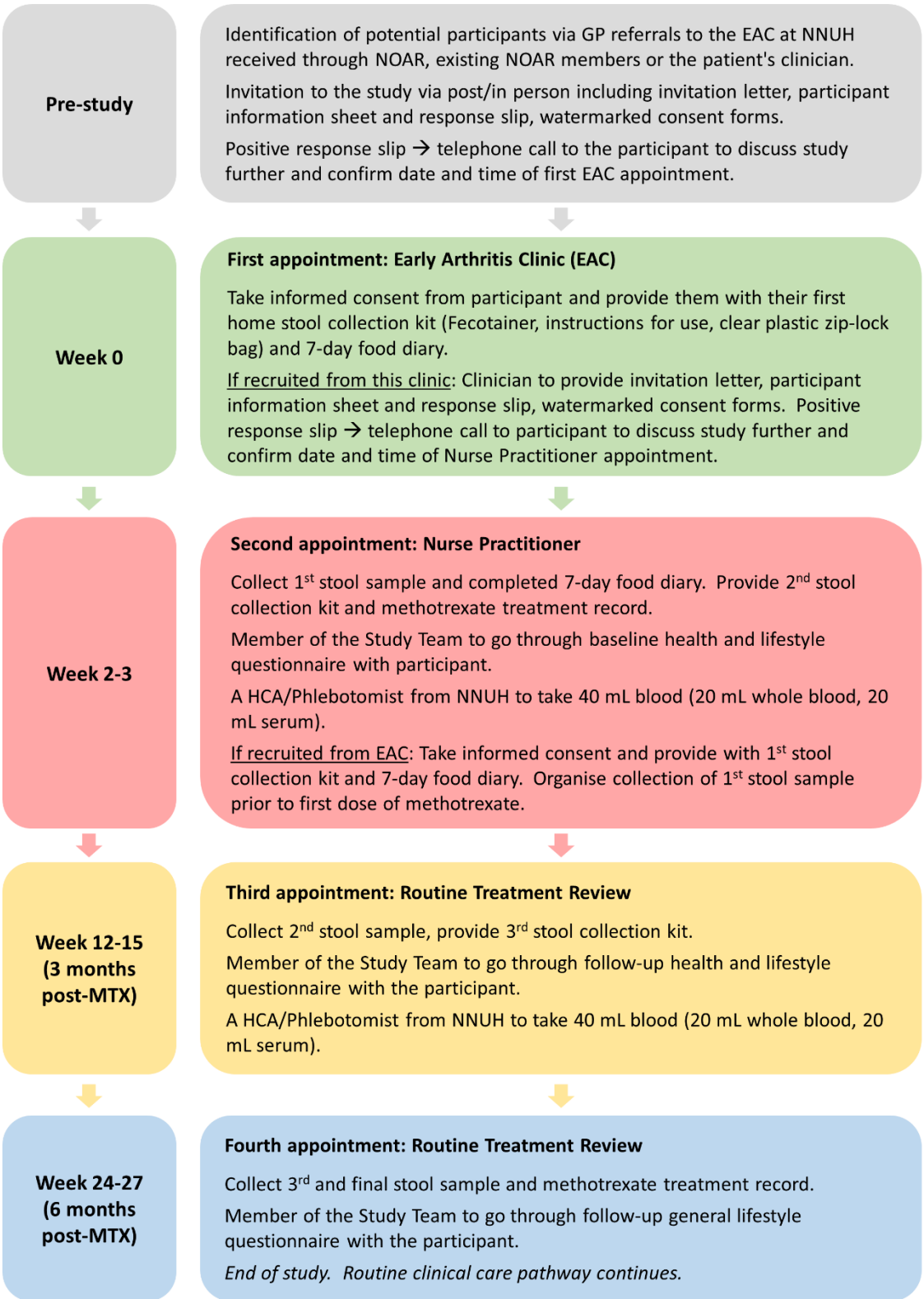
5.5.3 Third and fourth appointments: Routine Treatment Review

The following two data collection points will coincide with routine treatment review appointments organized as part of the participant's normal care pathway (at approximately 3 and 6 months from the NP appointment or time of commencing MTX/post-MTX).

The 3-month appointment will reflect the NP appointment in that another 40 mL blood sample will be taken for the purpose of this study by the on-duty HCA/Phlebotomist; as per Section 5.5.2.2, and the participant's stool sample will be collected from them as per Section 5.5.2.1. Clinical data will be recorded by a member of the study team with appropriate access to NNUH hospital systems using the Case Report Form (Appendix 11). Any member of the Study Team will go through another health

and lifestyle questionnaire with the participant, although this will be a shortened partial version of the questionnaire aimed at identifying changes (if any) from the NP appointment (Appendix 10). The participant will be provided with another stool collection kit for the final stool sample to be

Figure 3: Flowchart of involvement of participants in the study



brought to their next treatment review (at 6 months post-MTX, approximately 3 months after their 3-month treatment review).

At the 6-month treatment review, the participant's final stool sample will be collected as previously, any member of the Study Team will go through a final follow-up health and lifestyle questionnaire (Appendix 10) with the participant and their methotrexate treatment record (Appendix 23) will be collected. Clinical data will be recorded by a member of the study team with appropriate access to NNUH hospital systems using the Case Report Form (Appendix 11). No further blood samples will be taken for the purpose of this study at this 6-month treatment review. Following this final appointment, no further information will be required from the participant and their involvement with this study will end. Their routine care will continue as normal.

5.6 Withdrawal procedure

If at any point during the study the participant wishes to withdraw, they may do so without giving a reason and their clinical care and participation in future studies at QIB, UEA or NNUH will not be affected. The participant will be sent a withdrawal letter (Appendix 12) explaining this and thanking them for their participation so far. Any samples or data collected up to the point of withdrawal will be kept and used in the study.

The withdrawal letter (Appendix 12) will also be sent to participants if they need to be withdrawn from the study by a member of the study team due to meeting exclusion criteria; for instance, due to cessation of MTX therapy or starting MTX prior to collection of the first stool sample.

5.7 Data collection and storage

Data will be collected in the form of a health and lifestyle questionnaire (Appendix 9 and 10) and 7-day food diary (Appendix 8), in addition to clinical metadata collected by any member of the Study Team with the necessary access to hospital systems (Ellie Sayers, Jacqueline Chipping, Alexander MacGregor). Clinical data including DAS28-CRP, CRP, erythrocyte sedimentation rate (ESR), anti-cyclic citrullinated peptide (anti-CCP), rheumatoid factor, height/weight and Bristol stool score will be collected by means of a study-specific Case Report Form (Appendix 11).

All potentially patient-identifiable and other physical data (e.g. forms) will be stored in a locked file cabinet at QIB, with access restricted to study scientists. Patients will be provided with a unique patient identifier consisting of an unrelated sequence of numbers which does not contain any personal information (MyRA_year_month of consent_participant number [MyRA_18_10_01), and samples will be anonymized at the point of receipt using a suffix to this identifier (_time point and sample type [stool or blood] e.g. S1 or B1 _date sample taken [MyRA_18_10_01_S1_251018]). Linked information will be kept in separate files from the personal information pertaining to each participant, and if the participant is also taking part in the NOAR study this information will include their unique NOAR identifier. The location of each sample in QIB (or later the NRP Biorepository) will be recorded. Any data electronically inputted or generated in relation to forms and samples will be stored in a secure (password-protected or encrypted) database on QIB computers.

5.7.1 Collection of disease activity variables

5.7.1.1 DAS28-CRP

The DAS28 is a composite score made up of counts of the number of swollen (SJC) and tender (TJC) joints out of a total 28 measured, erythrocyte sedimentation rate (DAS28-ESR) or C-reactive protein (DAS28-CRP), and a health assessment questionnaire (patient global assessment of disease activity [PtGA], measured via a visual analogue scale [VAS]) filled in by the patient. While DAS28-ESR and

DAS28-CRP are often used interchangeably in clinical practice, DAS28-CRP will be used for this study; as ESR is not always measured in clinical laboratories.

DAS28-CRP is used by clinicians to monitor patient progress and inform treatment strategies, and will be recorded by the clinician at each routine appointment. DAS28-CRP in the participant's electronic or paper notes will be recorded by a member of the study team with appropriate access to hospital systems via the Case Report Form (Appendix 11). Should the DAS28-CRP not be recorded during a routine clinical appointment, each component of the DAS28-CRP is listed individually to enable calculation by the study team using the below equation[28]:

$$0.56 \times \sqrt{TJC} + 0.28 \times \sqrt{SJC} + 0.36 \times \ln(CRP + 1) + 0.014 \times PtGA + 0.96$$

5.7.1.2 Other measures of disease activity

Although DAS28-CRP is the standard measure used in medicine to measure RA disease activity, other measures may be used by some clinicians. Therefore another disease activity variable which we will record in this study via the Case Report Form (Appendix 11) is the Simplified Disease Activity Index (SDAI), which uses the same variables as DAS28-CRP but with the addition of provider global assessment of disease activity (PrGA). PrGA is a similar measure to PtGA and is also measured on a VAS, but is an indicator of the clinician's opinion on their patient's disease activity. The SDAI is calculated as a cumulative sum of SJC, TJC, PrGA, PtGA and CRP.

Figure 4. Overview of data collection time points

Activity	Pre-study	Week 0 (Early Arthritis Clinic)	Week 2-3 (Nurse Practitioner)	Week 12-15 (3 month Routine Treatment Review)	Week 24-27 (6 month Routine Treatment Review)
Identify suitable participant and send PIS and response slip	✓				
Telephone discussion to discuss study and confirm date and time of next appointment (on receipt of positive response)	✓	✓*			
Informed consent		✓	✓*		
Provide Fecotainer to participant		✓	✓	✓	
Provide 7-day food diary to participant		✓	✓*		
Provide methotrexate treatment record to participant			✓		
Stool sample and health questionnaire collected			✓	✓	✓

7-day food diary collected			✓	✓*	
Methotrexate treatment record collected					✓
Clinical data collected via case report form			✓	✓	✓
40 mL blood sample (20 mL serum, 20 mL whole blood)			✓	✓	

*If participant is identified at the Early Arthritis Clinic, these activities will be carried out at this later stage.

5.8 Diet and lifestyle assessment

7-day food diaries have been provided by the European Prospective Investigation of Cancer (EPIC) study, are validated and approved for re-use in this study. Ellie Sayers will be responsible for the data entry and analysis of these food diaries, for which expertise will be drawn from diet and nutrition researchers at UEA. Dietary analysis software such as Tinuviel (WISP) will be used for determination of micro/macronutrient intakes from food diaries, as it has been in previous studies[29–31]. The specific diet components to be analyzed will be based on current known dietary risk factors for RA, including red meat, fatty fish (omega 3), and vitamin C intake[32–34]. Other components to be investigated will be determined in an analysis of epidemiological data from the EPIC/NOAR link (participants in EPIC who are also registered with NOAR, for which there is historical 7-day food diary information available). Additional dietary components identified in association with methotrexate response from the EPIC/NOAR link data will be drivers for the analysis of food diaries in this study.

The health and lifestyle questionnaires are taken from the Microbiome of the Ageing Gut and its Effect on Cognition (MOTION) study, and have been approved for re-use in this study. The IRAS number for the MOTION study is 241617, and the ethical application is still in progress. The health and lifestyle questionnaire was reviewed by Patient Participation Groups (PPG) from four Norfolk GP sites. Twelve members from the four PPG groups returned comments on the questionnaire. Comments were noted and amendments made. The questionnaire consists of several questions related to factors which can affect microbiome composition, such as the use of over-the-counter or prescription drugs, living arrangements and recent travel. Recording these factors at baseline and changes at each subsequent time point will help account for confounding variables during the analysis of microbiome data.

5.9 Sample processing and analysis

5.9.1 Blood samples

5.9.1.1 Collection and storage

Blood samples will be collected at the second and third appointments by the on-duty HCA or Phlebotomist at the NNUH Rheumatology Department who will be taking blood samples as part of the participant's routine clinical care. A total of 80 mL blood will be taken for the purpose of this study at the second and third appointment (pre- and post-MTX), in the form of 2 x 10 mL Vacutainer SSTs and 2 x 10 mL Vacutainer EDTA tubes. Serum samples will be aliquoted and frozen in a -20°C freezer, whereas whole blood samples will be processed to isolate PBMCs which will be stored in cryovials in liquid nitrogen until further analysis can be carried out. Both samples will be stored at QIB (within a maximum of 1 hour after receipt) for the duration of the study and will be transferred to the NRP Biorepository at the end of the study for long-term storage.

5.9.1.2 Immunological analyses

Serum and whole blood samples will be used in immunoassays currently being developed at QIB. The serum assay will involve enzyme-linked immunosorbent assays (ELISA) in which systemic antibodies against components of the participant's own microbiota (derived from their stool sample) are detected. This analysis will identify if the participant has systemic immunity against their own gut microbiota, which could be an indicator of a "leaky gut". PBMCs will be isolated from whole blood samples and frozen for future use in cellular assays for immune reactivity to commensal microbes; for instance, using proliferation assays and cytokine production assays. Together, these assays will provide information on systemic immune reactivity towards components of commensal microbes, giving an indicator of intestinal permeability and the ability of these microbes to elicit immune responses in the host. Changes to the participant's immune reactivity against their own gut microbes may occur with treatment (for instance if there was increased intestinal permeability prior to treatment which resolved with MTX), which will also be assessed in this study.

5.9.2 Stool samples

5.9.2.1 Collection and storage

Stool samples will be collected by the participant and received by any member of the Study Team at the second, third and fourth appointments. These samples will either be delivered to the NNUH by the participant at their routine appointment or collected by a member of the study team from their home a day either side of this appointment. Collection by the study team from the participant's location will have been organized between the participant and study team to arrange a suitable time. Each stool sample will be separated into aliquots and frozen at -80°C in a QIB freezer until further analysis can be carried out. This will be done as soon as possible after collection of the fresh sample (within a maximum 1 hour after receiving the sample), to avoid unwanted changes to the microbial communities present in the sample. Any remaining original sample will be discarded, and at the end of the study stool samples left over will be transferred to the NRP Biorepository for long-term storage.

5.9.2.2 Shotgun metagenomic sequencing

Total microbial DNA will be extracted from stool samples, quantified and sent to Novogene (Hong Kong) for shotgun metagenomic sequencing on the Illumina platform (insert size, 300bp; paired-end read length, 150bp; depth, 10GB data per sample). The resulting sequencing data received from Novogene will be used to identify the α-diversity (Shannon Index) and bacterial composition and functional profiles in the stool samples. The raw sequences will also be used to identify viral sequences, using a pipeline currently being developed at QIB. Correlations will be drawn between α-diversity (Shannon Index) and change in DAS28-CRP to answer the primary research aim. Various statistical analyses (see Statistical Methods) will be carried out to determine the diversity (e.g. Shannon index, Bray-Curtis dissimilarity) and differential abundance of microbial genera between responders and non-responders to MTX; which will inform on the reliability of microbiome parameters as predictors of MTX response. This analysis will be carried out by Ellie Sayers with the support of QIB Bioinformaticians.

6 Dissemination of findings

Data generated from this study will be anonymized and published as part of a postgraduate thesis and research journal articles. The data may also be presented in anonymized form at conferences. At the end of the study, a brief, lay summary of the findings of the study will be posted to participants on QIB headed paper, meeting the HRA transparency requirements under the new

General Data Protection Regulation (GDPR) legislation. Anonymized datasets will be kept indefinitely and available to other researchers to comply with journal requirements when required.

7 Statistical methods

7.1 Sample size calculations

Sample size calculations were carried out by Dr. George Savva, a QIB statistician. Previous studies suggest that change in DAS28 score in response to treatment has a standard deviation of 1 to 1.3 points[35,36]. The minimum clinically relevant difference in DAS28 has been reported in two different studies as 1-1.2 points[36,37].

Hence, to detect a clinically relevant difference in DAS28 we will aim to recruit enough participants to estimate an effect size of one standard deviation in the outcome per two standard deviations in Shannon index, corresponding to a correlation coefficient of 0.5, typically considered a large effect. This will require 30 subjects to complete the study (true power=82.5%, size=0.05), hence our target sample size for recruitment is 35, allowing for 5 participants to drop-out of the study. Using the change score from baseline to the mean of two follow-up responses as the primary outcome will slightly improve the power of the study but it is difficult to know by how much without prior information on the longitudinal variation of DAS28. Power calculations were conducted using the *pwr* package in R.

Multivariate analyses linking detail of the structure and composition of the microbiome to outcomes are purely exploratory and will be reported as such.

7.2 Statistical methods for the analysis of study data

7.2.1 Correlating microbiome parameters with treatment outcome

Spearmans correlation coefficient will be used to identify statistically significant associations between (continuous variables) Shannon index at baseline and change in DAS28-CRP from baseline to 3 months post-MTX. In addition, Shannon index and other microbiome characteristics such as taxa and gene abundance will be analysed for covariation with other clinical metadata (CRP, anti-CCP, RF), also using Spearmans correlation coefficient. The predictive capability of the microbiome for MTX response will be assessed by generation of a cross-validated predictive model, using a machine-learning R statistical package such as *randomForest*.

7.2.2 Identifying microbiome alterations induced by methotrexate

Longitudinal changes to the microbiome during MTX treatment will be studied using a regression model. Alterations to the microbiome throughout methotrexate treatment will also be visualized using principle coordinates analysis (PCoA) and β-diversity (Bray-Curtis dissimilarity; between-sample diversity). This analysis will be done using an R package such as *vegan*.

7.2.3 Other statistical analyses

Multivariable models will be developed to explore whether diet and lifestyle factors can help account for any alterations seen in the microbiome associated with treatment response. Blood work will mainly consist of ELISA, for which parametric tests will be used to determine the signal strength of fluorescence from each sample, corresponding to the titer of antibodies against enteric microbes. All analyses will be conducted in R, GraphPad Prism or similar statistical programs.

8 Resource requirements

8.1 Staffing requirements

Processing and analysis of samples and data will be carried out primarily by Ellie Sayers, a PhD Student at UEA for whom a maintenance stipend is provided by a Faculty of Medicine and Health Science Studentship, and for which this study will form the main part of a PhD. In addition, Jacqueline Chipping or another NOAR Research Nurse may be required to take blood samples, assist with consenting participants and collecting stool samples. Novogene (Hong Kong) services will be used for shotgun metagenomic sequencing.

8.2 Material requirements

A successful funding application to the local Norfolk charity Action Arthritis (Registered Charity Number 292569) has provided £18,629.02 for lab consumables and study materials (Appendix 16). The 7-day food diaries are already available for use at the BCREB. A short general health questionnaire has been generated by Simon Carding for use in a larger project (the MOTION study, IRAS no. 241617, ethical application still in progress) to encompass factors affecting the intestinal microbiome, such as exercise, medications and lifestyle.

9 Confidentiality

All members of the Study Team will be fully compliant with the requirements of the EU General Data Protection Regulation (GDPR) 2016/679 and the UK Data Protection Act (DPA) 2018 with regards to the collection, storage, processing and disclosure of personal information and will uphold the GDPR and DPA core principles to maintain confidentiality. Personal information will be anonymized in a linked form on receipt, by using a unique identifier consisting of an unrelated sequence of characters and numbers, and stored securely in a locked file cabinet at QIB with keys available only to members of the study team. Linked data will be also only be accessible by the study team and will stored in an encrypted or password-protected database on UEA or QIB computers. The data will be stored for 15 years in case it is required for further research, and the data custodian will be Ellie Sayers.

10 Ethical considerations

As this study involves the recruitment and participation of human subjects from the National Health Service (NHS), appropriate Research Ethics Committee (REC) approval will be obtained by applying to the Health Research Authority (HRA) via the Integrated Research Application System (IRAS).

10.1 Informed consent

Written informed consent will be obtained from participants entering into the study by an appropriately trained member of the Study Team or Rheumatology Clinician, and members of the Study Team will continuously ensure the participants are happy to continue with the study and provide samples, in accordance with National Institute for Health Research Good Clinical Practice (GCP) guidelines.

10.2 Risks associated with study procedures

The risks associated with venipuncture such as discomfort and bruising will be minimized as only an appropriately trained HCA/Phlebotomist at NNUH will carry out this procedure, unless they are unable to do so for any reason in which case an appropriately trained member of the Study Team (e.g. the NOAR Research Nurse) will carry out the procedure.

10.3 Reporting of Serious Adverse Events

As this study does not involve any intervention or procedure which will not be part of the participant's routine clinical care, we will not officially record any Serious Adverse Events (SAEs).

10.4 Future research

Samples and data obtained from this study may be used in future research projects, where those projects have obtained the appropriate ethical approval to do so. No samples or data from this study will be used by other projects without the appropriate ethical approval.

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