Gut microbiome in the development of rheumatoid arthritis

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" Gut microbiome in the development of rheumatoid arthritis "

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par

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La Faculté de médecine, sur les préavis du Comité directeur du MD-PhD, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

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Antoine Geissbuhler
Doyen

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives à la présentation des thèses de doctorat à l'Université de Genève".

Résumé

Les recherches récentes suggèrent fortement que la santé des muqueuses et le microbiome jouent un rôle dans la genèse de la polyarthrite rhumatoïde (PR). Cependant, les données les plus convaincantes pour l'hypothèse d'une « origine muqueuse de la PR » reposent sur des expérimentations animales. Cette thèse visait à reproduire chez l'homme ces résultats expérimentaux récents, et ce en utilisant des échantillons prélevés chez des individus ayant un risque de PR. Nous avons cherché 1) à évaluer la corrélation entre un « risque de PR » accru et la présence dans les selles de bactéries associées à la PR ; 2) à évaluer la corrélation entre un « risque de PR » accru et les biomarqueurs sérologiques liés à l'intégrité intestinale ; 3) à évaluer la réactivité des anticorps sériques des patients contre les microbes associés à la PR et contre le microbiote fécal autologue.

Nous avons recueilli environ 380 échantillons de selles, pairés avec des échantillons de sérum, chez des individus à risque accru de PR au sein de la cohorte SCREEN-RA. Les individus ont ensuite été classés selon les phases pré-cliniques de PR : comme témoins, témoins à haut risque génétique, individus asymptomatiques mais avec des auto-anticorps spécifiques de la PR, et individus avec des symptômes articulaires suspects ou une PR nouvelle.

De manière inattendue, nous avons constaté que la présence de bactéries associées à la PR dans le microbiome fécal ne différait pas de manière significative entre les différentes phases pré-clinique de PR. De même, les biomarqueurs sériques de l'intégrité intestinale (I-FABP), de l'inflammation intestinal (calprotectine fécale) et de l'inflammation systémique (LBP et calprotectine) étaient similaires entre les phases pré-cliniques de PR. En outre, les Prevotellaceae et en particulier *Prevotella copri*, qui sont des bactéries pathogéniques dans les modèles murins de la PR, ne semblaient pas être ciblées de manière significative par les IgG sériques des patients atteints de PR ou des participants à haut risque de PR.

Ces données suggèrent qu'il n'y a pas, durant les stades précliniques de la PR, d'inflammation intestinale notable, du moins telle que mesurée par les biomarqueurs fécaux et sériques habituels. Par ailleurs, l'hypothèse selon laquelle une bactérie fécale particulière serait à l'origine de l'auto-immunité ou du développement de la PR semble aujourd'hui réductrice. En particulier, nous n'avons trouvé aucune preuve d'une réactivité humorale anormale contre les bactéries du genre *Prevotella* dans les phases précliniques de PR.

Abstract

The available evidence strongly suggests that mucosal health and mucosal microbiome are relevant for RA development. Currently, the most convincing evidence for the so-called “mucosal origins hypothesis” comes from mice experiments. This thesis attempted to confirm some of these recent experimental findings in an observational setting of individuals at risk for RA. We aimed 1) to assess the correlation between increased “risk of RA” and the presence of RA-associated bacteria in feces; 2) to assess the correlation between increased “risk of RA” and serological biomarkers pertaining to intestinal integrity; 3) to assess patient’s serum immunoglobulin reactivity against the RA-relevant microbes or autologous fecal microbiota.

We collected about 380 stool samples paired with serum samples, in individuals at increased risk for RA from the SCREEN-RA cohort. The individuals were then categorized in preclinical stages of RA development: controls, controls with high genetic risk, asymptomatic individuals with detectable RA autoimmunity, and individuals with suspect articular symptoms or new-onset RA.

Unexpectedly, we found that the presence of RA-associated bacteria in the fecal microbiome did not significantly differ between the various preclinical stages; nor did the fecal calprotectin levels. Furthermore, serum biomarkers of intestinal integrity (I-FABP) and systemic inflammation (LBP and calprotectin) did not significantly differ between preclinical stages. Also, Prevotellaceae and in particular *Prevotella copri*, which were bacteria of interest in mouse-models of RA, did not appear to be significantly targeted by serum IgG of RA patients or high-risk participants.

Our data suggest the absence of intestinal inflammation during the preclinical stages of RA. Moreover, the hypothesis of a specific fecal bacteria driving RA-autoimmunity or RA-development appears to be reductionist. In particular, we found no evidence for abnormal humoral reactivity against *Prevotella* species in preclinical RA stages.

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Finally, I shall mention Jean SEIGNALET, whom I did not meet but whose book and original suggestions partly inspired this thesis.

CONTENT

ABBREVIATONS

ACPA = Anti-Citrullinated Peptide Antibodies

ANOVA = Analysis of Variance

BCR = B-Cell Receptor

BMI = Body Mass Index

CCP = Cyclic-Citrullinated Peptide

CSA score = Clinically Suspect Arthralgia score

CD = Cluster of Differentiation

CV = Coefficient of Variation

DMARD = Disease-Modifying Anti-Rheumatic Drug

ELISA = Enzyme Linked Immunosorbent Assay

EULAR = European Alliance Against Rheumatism

Fab = antigen-binding Fragment

FITC = Fluorescein Isothiocyanate

Fc = crystallizable Fragment

FMT = Fecal Microbiome Transfer

HLA = Human Leucocyte Antigen

IBD = Inflammatory Bowel Disease

I-FABP = Intestinal Fatty Acid Binding Protein

Ig = Immunoglobulin

LBP = Lipopolysaccharide Binding Protein

LMR = Lactulose Mannitol Ratio

MALT = Mucosae Associated Lymphoid Tissue

MCP = Metacarpophalangeal (joint)

MHC-I = Major Histocompatibility Complex Class I

MHC-II = Major Histocompatibility Complex Class II

MTX = Methotrexate

NSAID = Non-Steroidal Anti-Inflammatory Drugs

NHS = Nurses' Health Study

PBS = Phosphate-Buffered Saline

PBA = Bovine serum albumin blocking buffer (PBS + Albumin)

PCoA = Principal Coordinate Analysis

PCR = Polymerase Chain Reaction

PERMANOVA = Permutational Analysis of Variance

QMP = Quantitative Microbiome Profiling

RA = Rheumatoid Arthritis

RA-FDRs = First Degree Relatives of Rheumatoid Arthritis Patients

RF = Rheumatoid Factor

RMP = Relative Microbiome Profiling

rRNA = Ribosomal Ribonucleic Acid

SCQM = Swiss Clinical Quality Management foundation

SCREEN-RA = Screening Strategy for Rheumatoid Arthritis

TLR = Toll-Like Receptor

TCR = T-Cell Receptor

ULN = Upper Limit of the Norm

PREFACE

Back in 2018, I met Prof. Axel FINCKH, whose previous doctoral student (Desire ALPIZAR) was working on a preliminary study of the gut microbiome in the context of rheumatoid arthritis (Alpízar et al., 2019). At that time, I was finishing my master work under the supervision of Prof. Jacques SCHRENZEL, systematically reviewing studies on fecal microbiome transfer.

Even though I have not always been an assiduous student, I have had all along my studies a great interest in how environmental factors could trigger autoimmunity, for having read thoroughly Dr Jean SEIGNALET's controversial suggestions as well as the great immunology textbook of Dr. Charles Alderson JANEWAY.

Prof. FINCKH was (surprisingly) kind enough to accept me as a doctoral student and to build this research project, despite the fact that such a translational topic falls outside of his "comfort zone" as an epidemiologist. Prof. SCHRENZEL and Prof. MERKLER joined as co-directors.

I confess our initial intention was to pragmatically tear apart causality by proceeding to fecal microbiome transfer in individuals with new-onset or imminent rheumatoid arthritis. However, our first study proposal was rejected by the Swiss National Science Foundation – in part due to ethical and safety considerations. We had to "know more" on the matter before considering a clinical trial. The project was consequently reshaped as a descriptive translational study, accommodated with a few innovative analyses. Although much "safer" to obtain, the findings derived from this type of research are also more difficult to interpret; overall, it is not obvious to demonstrate "descriptively" the role of the gut microbiome in the onset of human autoimmune arthritis.

"*Much wisdom, much grief; the more knowledge, the more sorrow*."

Ecclesiastes 1:18.

Benoît Thomas P. GILBERT

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I. BACKGROUND

RHEUMATOID ARTHRITIS

Definition of rheumatoid arthritis

RA is a chronic rheumatic disease characterized by progressive joint destruction, extra-articular manifestations and permanent disability. The diagnosis is made by a rheumatologist, who considers all the elements at his disposal. However, given the heterogeneity of RA patients, it has been necessary to create so-called "classification" criteria, which define more homogeneous patient populations for clinical studies. A new revision was carried out in 2010, with the European League Against Rheumatism (EULAR) (**Table 1**).(1,2)

Table 1 : Classification criteria for RA (EULAR/ACR 2010)

Domain	Category	Points
A	Joint involvement (0-5 points) [a]	
	1 large joint	0
	2-10 large joints	1
	1-3 small joints (large joints not counted)	2
	4-10 small joints (large joints not counted)	3
	>10 joints including at least one small joint	5
B	Serology (at least one test needed for classification; 0-3 points) [b]	
	Negative RF and negative ACPA	0
	Low positive RF or low positive ACPA	2
	High positive RF or high positive ACPA	3
C	Acute-phase reactants (at least one test needed for classification; 0-1 point) [c]	
	Normal CRP and normal ESR	0
	Abnormal CRP or abnormal ESR	1
D	Duration of symptoms [d]	
	<6 weeks	0
	≥6 weeks	1

RF = Rheumatoid Factor. ACPA = Anti-citrullinated Peptides Antibodies. CRP = C-Reactive Protein. ESR = Erythrocyte Sedimentation Rate. The points from each of domains A through D are added and the sum is considered to be the total score. A total score of ≥6 is needed to classify a patient as having definite RA.

[a] Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. Distal-interphalangeal joints, first carpo-metacarpal joints and first metatarsophalangeal joints are excluded from assessment. *Large joints* refers to shoulders, elbows, hips, knees and ankles. *Small joints* refers to metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints and wrists. [b] Negative means less than or equal to the upper limit of normal (ULN); low positive means >ULN; high positive means >3x ULN. [c] Normal and abnormal are determined by local laboratory standards. [d] Duration of symptoms as per patient's self-report.

After ACR/EULAR definition (1,2).

These 2010 criteria seek to improve the sensitivity of the classification of early RA. With this latest version, patients with at least 6 points on the proposed score, or with at least 3 joint erosions,(3) are classified as "definite" RA. This categorization does not imply that a formal diagnosis is made. The rheumatologist will confirm the diagnosis, often after a few months, and the classification criteria is used to support the clinical reasoning. Other elements are supportive of RA diagnosis, such as characteristic radiographic findings (4) or sterile and turbid synovial fluid with inclusion-bearing neutrophils.

Prevalence and incidence of rheumatoid arthritis

We reviewed the global epidemiology of RA elsewhere, I will only underline the key aspects.(5)

Prevalence

Based on the GBD-2017 study, the age-standardized prevalence of RA approximates 0.5% and tends to be higher in westernized regions such as the Americas or Europe.(5) Importantly the prevalence is markedly higher in specific genetically defined populations, in particular indigenous groups of northern and southern America.(6,7)

Incidence and disease burden

Incidence is the number of new cases by unit of time for a given number of persons. The incidence rate of RA approximates 7 to 15 new cases per 100’000 patient-years.(8–17). Age at onset is typically around 50 years-old, though older in registry studies (18) and with worldwide geographical variations.(19) RA also increases mortality, similar to patients with lymphoma or ischemic heart disease,(20) mainly due to a greater risk of infections, cardiovascular events, pulmonary involvement and lympho-proliferative malignancies.(18,21,22)

Risk-factors for rheumatoid arthritis

In epidemiology, a risk factor is a variable associated with an increased risk of disease. Thus, it is *correlational* (i.e., more frequent in diseased than in healthy individuals) but not necessarily *causal*. However, the interest of listing these "disease-associated-factors" is that they are likely to be either causal or closely related to an underlying cause – thus helping to formulate relevant hypotheses about disease's causes. Furthermore, certain traits of correlational associations can constitute solid arguments in favor of a possible causal link, for instance dose-dependency, reduced-risk after exposure cessation, etc. **Figure 1** summarizes known risk factors for RA.

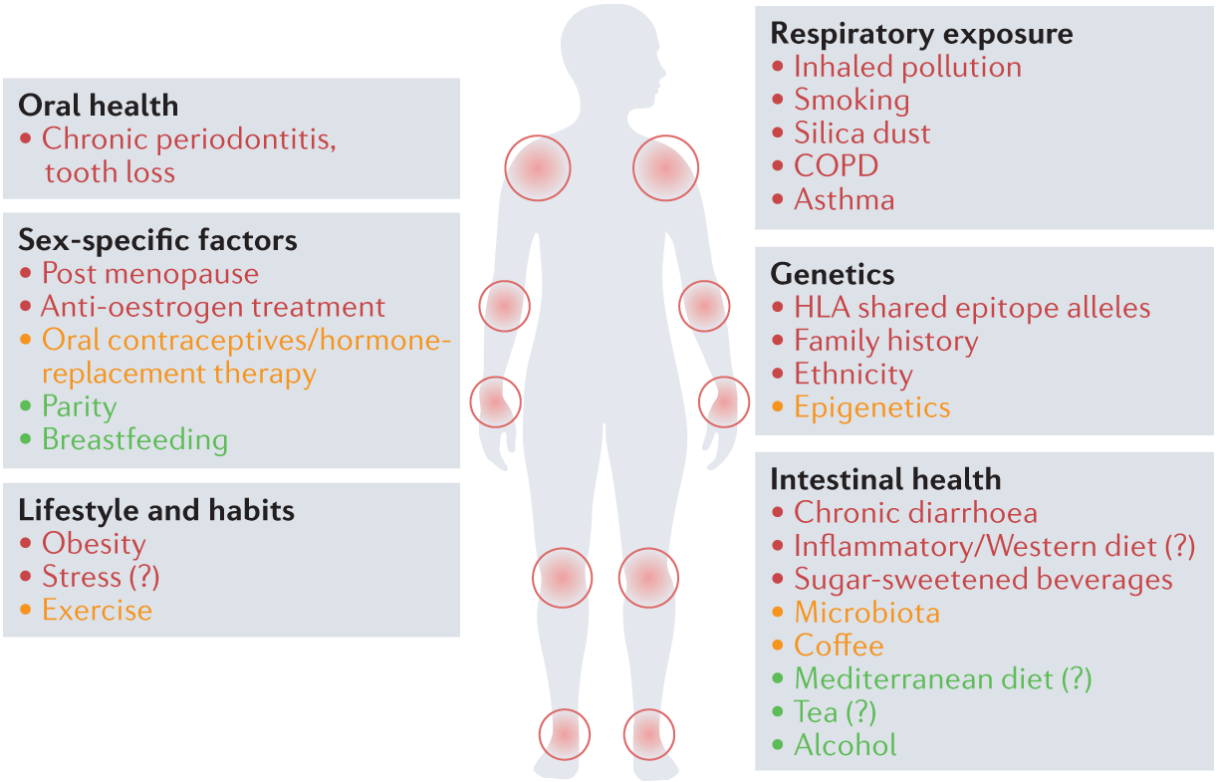


Figure 1: Risk-factors for rheumatoid arthritis. Overview of the known risk factors for rheumatoid arthritis (RA), based on epidemiological data. COPD, chronic obstructive pulmonary disease. Red = increasing risk of RA. Green = decreasing risk (protective). Orange = controversial reports and/or depending on subpopulation. Reproduced from Finckh et al. 2022, doi: 10.1038/s41584-022-00827-y (license: author reuse).

Non-modifiable risk factors

Non-modifiable risk factors have something to do with the intrinsic biological constitution of the patients.

Genetic risk factors

First-degree relatives (FDRs) of RA patients have a 3 to 5-fold increased risk of developing the same disease. This correlation is even higher in families with multiple cases of RA.(23) The most prominent susceptibility genes are a set of HLA-DRB1 alleles sharing a common sequence referred to as the 'shared epitope'. The concerned peptides are located in the hyper-variable region of the β_1 subunit of the MHC-II.(24) The point is that these alleles impact the shape of the binding groove in MHC-II molecules, which is involved in the response to extracellular immune ligands.(24) That being said, a couple of other alleles of HLA-DPB1 (i.e. β_1 chain of HLA-DP gene) and also HLA-B (notice this one is MHC-I) have also been reported to increase the risk of RA.(24)

Specific alleles in others genes also increase risk for RA, even though to a lesser extent.(24–26) The involved genes comprise for instance PTPN22,(27) IL-6 receptor,(26) RASGRP1,(28) PADI4,(29) CCR6(30) and others.(24–26) In a nutshell, RA risk-genes all point towards alterations of TCR signaling, MHC peptide presentation, reactivity of innate immune cells in response to bacterial ligands, chemotaxis, B- or T-cell differentiation and proliferation, hematopoiesis, etc.

Female sex and hormones

RA is more prevalent in women,(31–34) with a female-to-male sex ratio ranging from 4:1 in younger individuals to less than 2:1 in older populations.(35) Given this sex-bias, researchers have further focused on the relation between sex hormones and RA.(36)

Epidemiology seems to support the link. For instance, RA diagnosis is often associated with menopausal age, suggesting that estrogenic function decline (menopause, anti-estrogenic therapies) may be a risk factor for the development of RA, while high estrogen exposure is reported as protective.(35)

Modifiable and environmental risk factors for RA

Based on twin studies, it is possible to state that RA has an overall 30-60% "heritability",(37) the latter being a statistical concept reflecting "the proportion of observed variance explained by genetic components".(37) This heritability predominates for ACPA- or RF-positive RA,(38) with ~30-40% owing only to the shared epitope.(39,40) In terms of raw numbers : within 207 monozygotic twin pairs, only 27 (~13%) were concordant; which means, in 87% of RA cases, the genetically identical sibling was not affected.(37,41)

In other words, about half of the attributable risk for RA is the result of environmental factors – consequently, a significant proportion of RA cases could be prevented if we knew which lifestyle to recommend.(42)

Inhaled factors and lung disease

Tabaco smoke, including passive smoking in childhood,(43) is the strongest risk-factor, representing up to 25% of the population attributable risk for seropositive RA.(25,44,45) It must be pointed out that this risk is particularly increased in shared-epitope homozygous individuals who are heavy smokers, underscoring a well-documented gene-environment interaction (Odds-ratio of 52.6).(46–54) The effect of smoking is dose-dependent and decreases slowly after smoking cessation.(44,55) Studies further linked air pollution or various inhaled particulates with increased risk for RA(54,56,57,57–64) as well as chronic airways diseases, such as asthma.(65–67)

Diet and lifestyle

We have reviewed elsewhere the nutritional factors associated with RA.(68) Overall, a healthy diet may be protective, however the precise definition of "healthy" remains debated. Most available evidence is derived from a few public cohorts, but conclusions can be contradictory depending on how the data is analyzed.

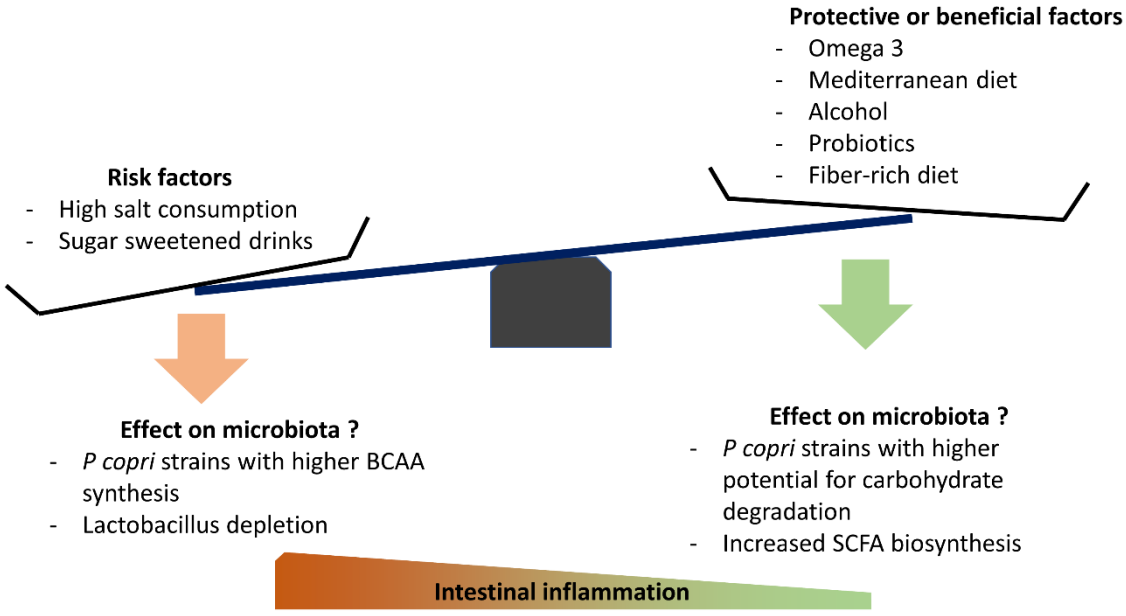


Figure 2: Dietary factors involved in the risk of RA, and their potential effects on the microbiota. "Beneficial factors" refer to interventions that have shown modest but positive effects in established RA. BCAA = Branched Chain Amino Acids. Based on literature review from Alpizar et al. 2021, https://doi.org/10.3390/nu13010096 , license: author reuse, under CC BY license.

Oral Health

Periodontitis (or "*periodontal disease*") is a biofilm-driven inflammatory disease caused by bacteria that adhere onto subgingival teeth surfaces and that lead to the destruction of tooth-supporting tissues.(69)

A higher prevalence of periodontitis is often reported in RA patients compared to healthy controls. Odds-ratios range from 1.82 to 8.05 after adjusting for confounding factors such as plaque accumulation and gingival inflammation.(70–76) Conversely, an increased prevalence of RA has been found in patients with periodontitis, compared with periodontally healthy subjects (odds-ratio ranging from 1.16 to 4.28).(73,77,78)

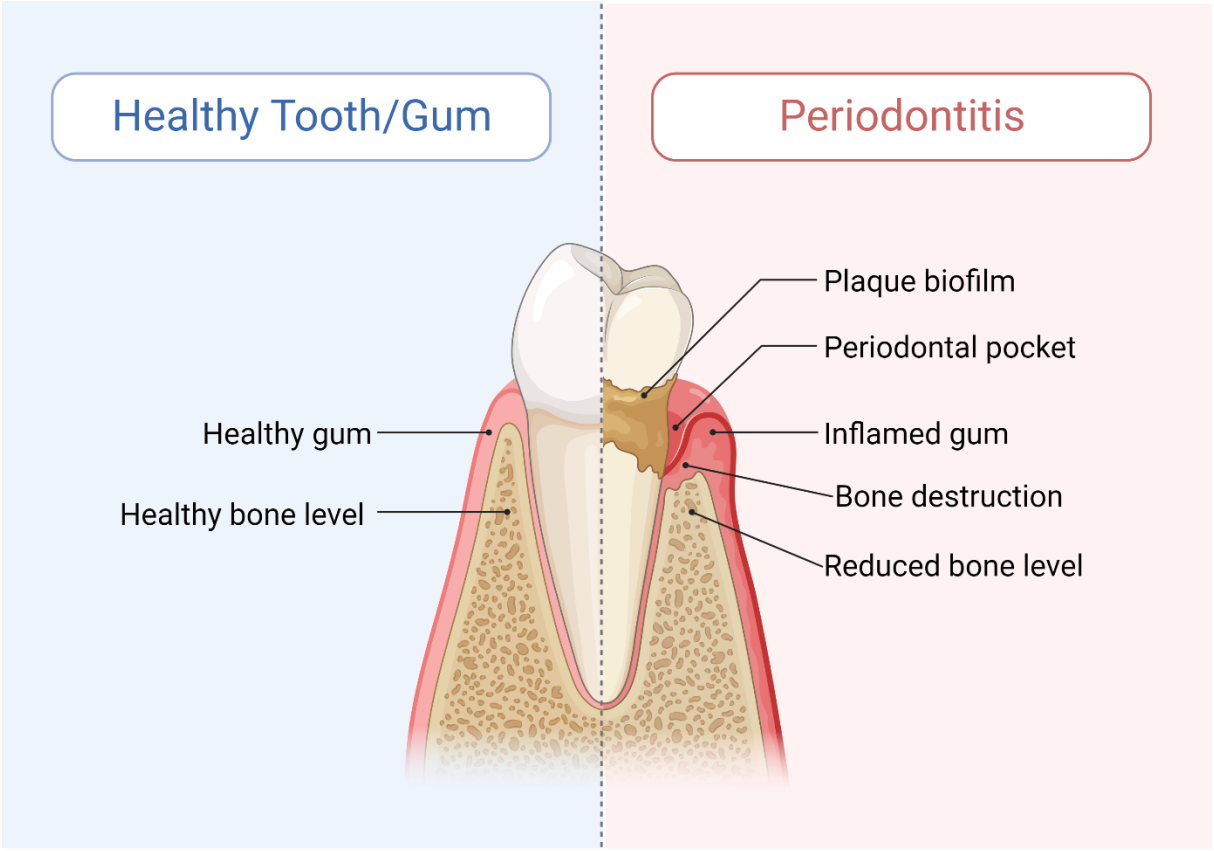


Figure 3: Gum health, normal versus periodontitis. Periodontitis results from biofilm formation, gingival inflammation, and adjacent bone destruction. Created with www.Biorender.com .

The pathogenesis of rheumatoid arthritis

RA results from a multi-step process, whereby environmental factors in genetically susceptible individuals induce a pathological autoimmune activation of the immune system, followed by an asymptomatic/pauci-symptomatic or pre-clinical phase that eventually leads to the clinical onset of the disease.(79) Thus, individuals may pass through a series of 'at risk' stages as defined by the European Alliance Against Rheumatism (EULAR) (80):

- 1 - Having genetic risk factors.
- 2 - Being exposed to environmental risk factors.
- 3 - Having developed systemic autoimmunity.
- 4 - Developing arthralgia without synovitis, i.e. articular pain without clinically obvious swelling.
- 5 - Early arthritis, i.e. clinically obvious swelling and pain but not yet diagnosable as RA.
- 6 - Classified RA, i.e. fulfilling the EULAR-ACR/2010 classification criteria.

Together, these phases are termed "pre-clinical phases of RA" (**Figure 4**). However, until properly diagnosed as having RA, it is still remains very unclear if an individual categorized in such a "pre-clinical phase" will indeed develop RA or not. Also, the progression of an individual from one pre-clinical phase to the next is not necessarily linear across those categories. Consequently, the term "pre-RA" must not be used, unless the researchers are certain of a subsequent RA diagnosis, for instance, in case of a cohort study – we could analyze serum samples taken 1 year before formal RA diagnosis; this way we will with certainty analyze the actual "pre-RA" samples.

We have already mentioned the genetic and environmental risk factors (pre-clinical stages n°1 and 2). Pre-clinical stages n°3 to n°6 are discussed below.

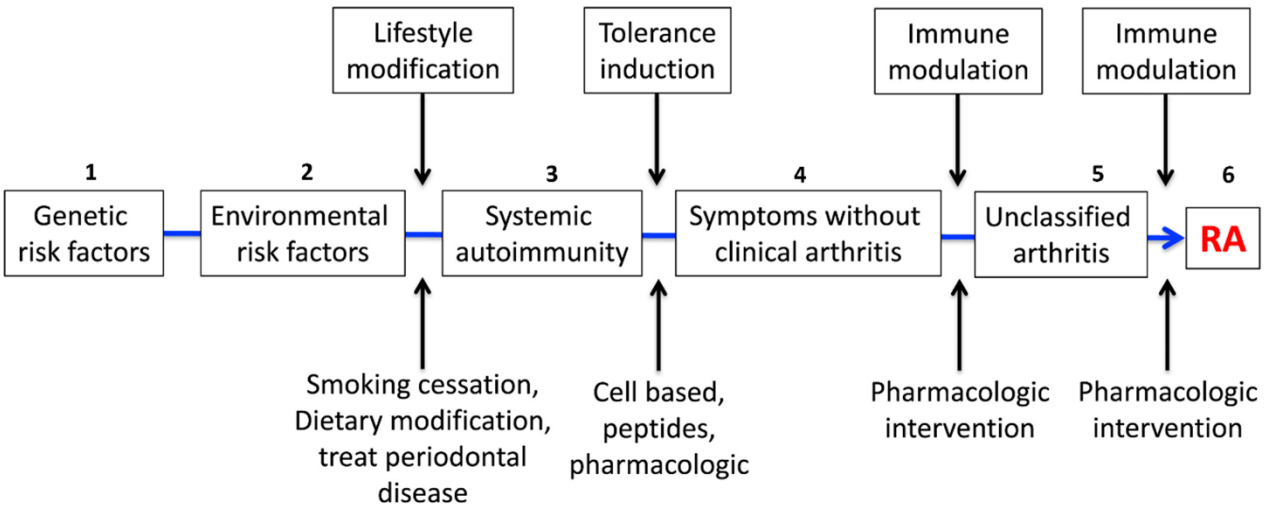


Figure 4: Overview of strategies for RA prevention. Points of intervention are highlighted (arrows). Numbers 1 to 6 correspond to the preclinical RA stages evoked hereabove. Reproduced from Cope, A. P. , Emerging therapies for pre-RA, 2017, https://doi.org/10.1016/j.berh.2017.08.005 , under license n°5601330162944 .

Asymptomatic systemic autoimmunity associated with rheumatoid arthritis

The first manifestation of RA is usually latent autoimmunity corresponding to hereabove stage n°3. In the context of EULAR recommendations, such “autoimmunity” is restricted to the following definition: the presence of certain auto-antibodies in the bloodstream (hence “systemic”). In the context of established RA, a synonym is “seropositivity”.

Researchers found that these auto-antibodies were often present in serum for several years before the first articular symptoms,(81–89) sometimes up to 10 years prior the diagnosis (**Figure 5**).(83) To date, it is not obvious which auto-antibodies come first, the literature is often conflicting on this subject.(81,84) Even though only Rheumatoid Factor (RF) and Anti-Citrullinated Peptides Antibodies (ACPA) are part of the classification criteria for RA (**Table 1**), many other auto-antibodies have been associated with RA and its preclinical phases.(90,91) Their common point is that all of them are either directed against intracellular content, which normally is not accessible to immune cells unless improper apoptosis or cell lysis occurs, or against post-translationally modified proteins, most probably resulting from chronic inflammation and neutrophilic activation. Auto-antibodies relevant to RA are reviewed in appendix “AUTO-ANTIBODIES IN RHEUMATOID ARTHRITIS”, page 182.

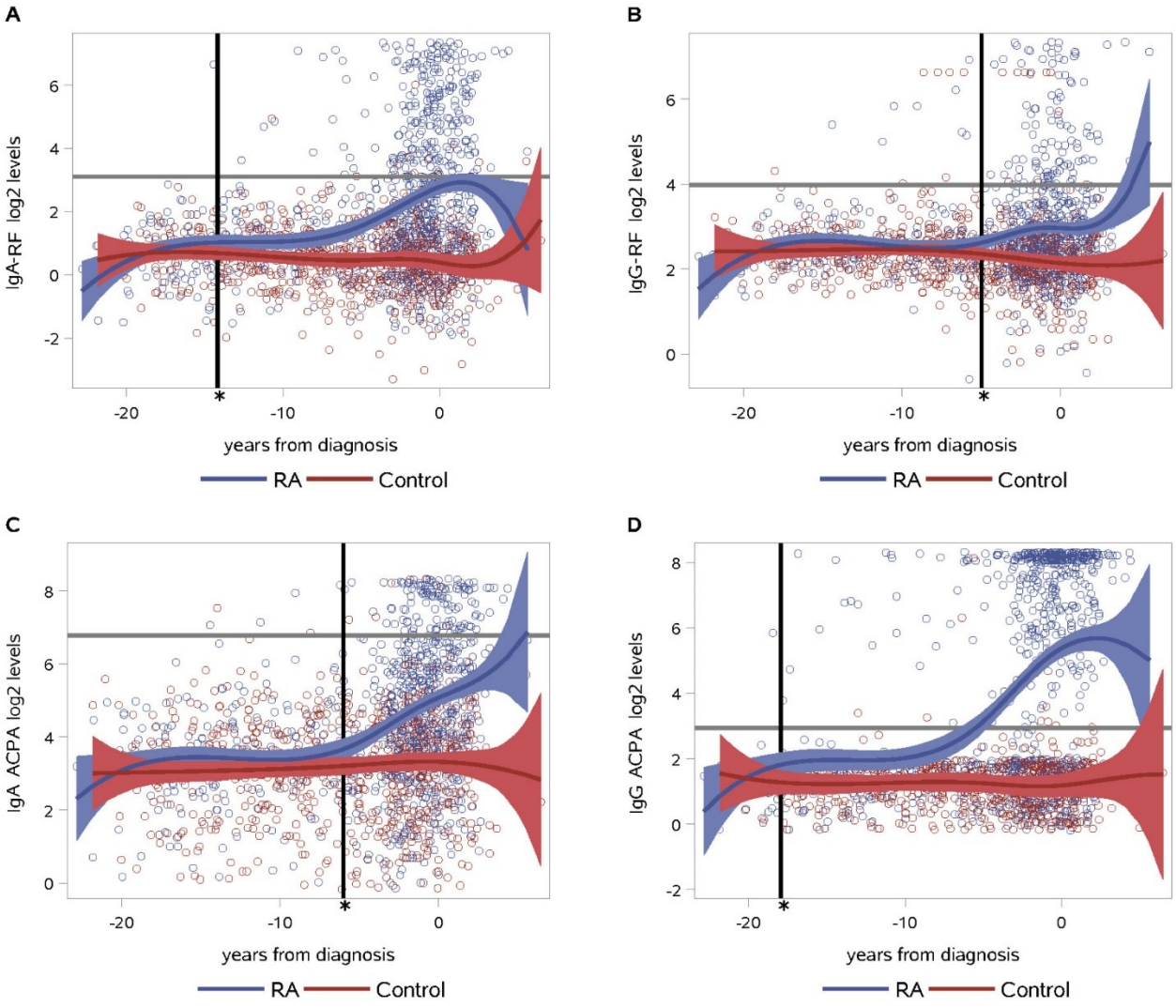


Figure 5: Auto-antibody elevation timing prior to RA onset. IgA-RF, IgG-RF, IgA ACPA, and IgG ACPA levels in RA cases and controls, from a US-military personnel biobank. **A** and **B**, Mixed model estimates of levels of IgA-RF (**A**) and IgG-RF (**B**). **C** and **D**, Mixed model estimates of levels of IgA ACPA (**C**) and IgG ACPA (**D**). Horizontal lines and shading show the mean ± SD; circles represent individual subjects. The gray horizontal lines represent the cutoff for positivity determined in 156 controls. Vertical lines indicate the time point at which values diverged between cases and controls. * = $p < 0.05$. Figure reproduced from Kelmenson et al., Arthritis Rheumatol, 72: 251-261, https://doi.org/10.1002/art.41091 , under license n° 5601350834235.

The symptomatic preclinical phases of the disease

Symptomatic preclinical phases refer to the pre-clinical stages n°4 and n°5 proposed by EULAR (80) :

- 4 - Developing arthralgia without synovitis (i.e. articular pain without clinically obvious swelling)

- 5 - Early arthritis (i.e. clinically obvious swelling and pain, but not yet diagnosable as RA)

The difference between phases n°4 and n°5 is that in phase n°4 the joints look normal, and the definition mostly relies on patient-reported symptoms. These symptomatic "preclinical-RA" patients can be identified using specific questionnaires and/or physical examination.(92) In particular, the European League Against Rheumatism (EULAR) has proposed clinical characteristics of arthralgia at risk for RA,(93) namely "clinically suspect arthralgia" (CSA), which increase the risk of developing RA.(94)

Of course, these symptomatic phases can occur in combination with other hereabove mentioned preclinical phases, such as genetic and environmental risk factors and detectable auto-antibodies – the progression of real-life individuals is not necessarily linear across the pre-clinical stages from the EULAR terminology.

Fluctuations and seronegative rheumatoid arthritis

These "pre-clinical" stages can be quite perplexing. Not many studies have repeatedly sampled individuals in pre-clinical stages, thus it is only recently that we realized the auto-antibodies can regress and disappear from peripheral circulation before re-occurring later.(95–98)

It is important to note that the classification criteria for RA can be fulfilled even without having circulating ACPA or RF – consequently, if no other diagnosis is found, rheumatologists may diagnose so-called "seronegative RA". However, this expression may be challenging for two reasons:

- Strictly speaking, "seronegative RA" means "RA without neither serological ACPA, nor RF". But the latter does not necessarily imply a *total absence of auto-antibodies*. For instance, anti-CarP, anti-PAD4, anti-acetylated peptide antibodies and others can often be found in so-called "seronegative" RA.(99–102)

- On one hand, "seronegative RA" seems to have a slightly better articular prognosis,(103) on the other hand, it is debated if a seronegative RA could not simply be, at least in some cases, a misclassified diagnosis, such as spondyloarthitis.(104)

While it is not our intention to change the current terminology, let us remember that in some cases, individuals with "seronegative RA" or "seronegative pre-clinical phases" may still present other unidentified auto-antibodies.

Value of biomarkers and clinical scores in predicting RA onset

Subjects with arthralgia and RA-specific auto-antibodies have approximately a 30% risk of developing RA within one year.(105) Similarly, 1/3 of individuals with undifferentiated (i.e., non-classifiable) arthritis will develop RA in the following year.(106) The parameters that associate with the onset of RA one year prior the diagnosis include: female sex, older age, location of affected joints, intensity of morning stiffness, number of swollen joints, number of painful joints, and ACPA, RF and CRP serological status.(107) Nonetheless, the prognostic value of a biomarker or a clinical score of RA onset risk depend greatly on the population that is considered. We have reviewed this question elsewhere.(108)

Progression and later complications

Only a fraction of individuals in "pre-clinical" stages of RA eventually develop the disease. I describe below the natural, i.e. if untreated, course of the disease.(109)

Clinical presentation

RA usually starts as a symmetric arthritis involving the small joints of the hands and feet with no bone damage, most frequently affecting the metacarpophalangeal, proximal interphalangeal and wrist joints. The distal interphalangeal, the sacroiliac and the lumbar spine joints are rarely affected the latter is more typical for "seronegative" spondyloarthropathies.

Synovitis is particularly apparent in the morning, accompanied with stiffness of the joints during a prolonged period (this is a subjective sign). When tendons are involved, we may speak of tenosynovitis. Fever, fatigue, weight loss is also present together with increased blood inflammatory parameters.

Pannus formation and articular destruction

Cell types affected in joints are mostly synovial cells, and cartilage,(110) the latter chondrocytes being progressively destroyed and replaced by fibrotic tissue (**Table 3**).

Synovium is normally the soft tissue lining of di-arthrodial joints, tendon sheaths and bursae, it is responsible for lubricating cartilage allowing periarticular movement and chondrocytes feeding.(111)

The synovium is composed of an intima (20-40 µm thick) and underlying tissue (subintima, up to 5mm thick). In RA, pro-inflammatory monocytes infiltrate the intima attracting neutrophils and other leucocytes.(111,112) Surprisingly, infiltrated B-cells may even differentiate on site into plasma cells and locally secrete antibodies, in particular, ACPA.(113) In some cases lymphoid follicles are even created.(114) Synovium inflammation leads to neovascularization, activation of fibroblasts, until reaching a tumor-like proliferation activity: it is the *pannus*.(115) The expansion of the pannus is the main cause of adjacent bones, tendons and cartilage destruction.

Extra articular manifestations

Chronic RA can extend to many organs – such manifestations include,(110,116) subcutaneous rheumatoid nodules, immune pulmonary involvement, ocular manifestations, vasculitis, systemic manifestations (amyloidosis), lymphoproliferative disorders, and cardiovascular co-morbidities which are the most common cause of death for RA patients.(117)

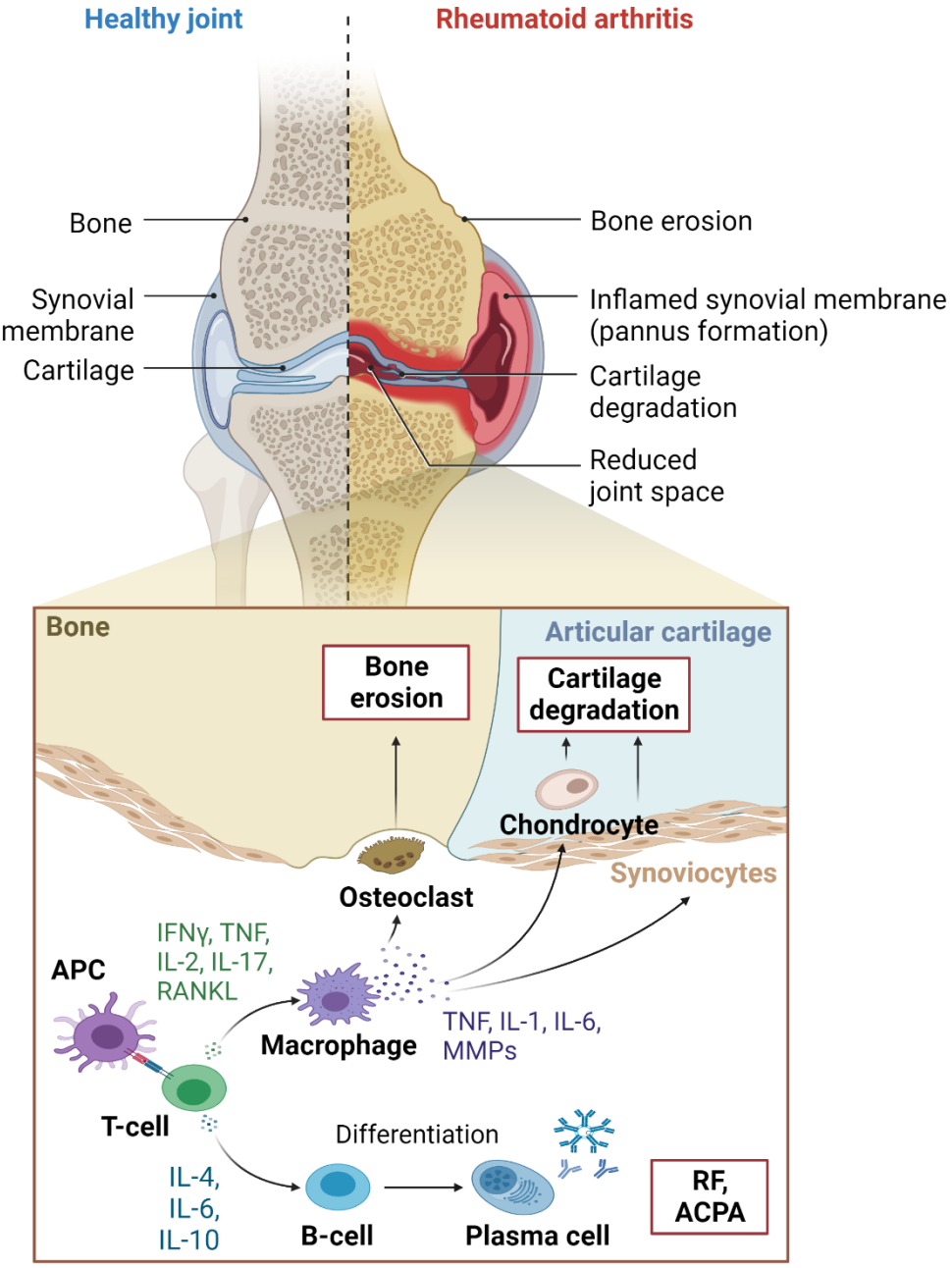


Figure 6: Joint inflammation in rheumatoid arthritis. Joint inflammation in RA leads to pannus formation, as well as bone and articular destruction by dysregulation of chondrocytes and osteoclasts. APC = Antigen Presenting Cell. RF = Rheumatoid Factor. ACPA = Anti-Citrullinated Peptide Antibodies. MMPs = Matrix Metalloproteinases. Created with www.biorender.com .

Treatments for rheumatoid arthritis

Conventional strategy

Medications used to treat RA are termed “Disease Modifying Anti-Rheumatic Drugs” (DMARDs), for they have demonstrated their capacity to improve disease-related parameters, in particular, limiting structural joint damage.(118) Available treatments include:

- conventional synthetic DMARDs (csDMARDs) could be seen as the first generation of DMARDs. They are synthetic molecules from various origins, and their mechanism of action is not always clear. Methotrexate is the most popular and considered the current gold standard.(119) Other compounds in this category include : sulfasalazine, hydroxychloroquine, leflunomide, azathioprine.

- biological DMARDs (bDMARDs) are purposely designed to target a specific cytokine or receptor relevant to RA. They are termed “biological” since being antibodies or likewise recombinant proteins. They are also often used in combination with a csDMARD. The most popular molecules in this class are antibodies targeting TNF-α.(120) Other bDMARDs include : anti-IL6, anti-IL-17, anti-IL-23. Also, rituximab is a recombinant antibody inducing destruction of mature B-cells by targeting CD20.(121)

- targeted synthetic DMARDs (tsDMARD). They are synthetic molecules developed to specifically inhibit intracellular enzymes relevant to inflammation and immunity. Several inhibitors of the Janus-kinases, which are involved in transducing intra-cellular signals in response to cytokines, interferon and growth factor, are available on the market.(122)

- Glucocorticoids, mainly prednisone, are considered when initiating or changing DMARDs or managing acute relapses in different dose regimens and routes of administration. Currently, complete discontinuation of glucocorticoids is recommended as soon as possible and no longer for permanent use.

These DMARD therapies have been extensively studied in clinical trials, and I also had the chance to compare their effectiveness in the Swiss RA registry.(123) RA treatments work “relatively” well, which means about 80% of the patients feel better, and ~30% are still in remission after 12-months. However, these molecules are not able to “cure” the disease, which often relapses sooner or later. Furthermore, 10-15% of patients never reach clinical remission, i.e. absence of symptoms on therapy.(124,125)

Consequently, there is still room for improvement in RA management. An interesting development is the current field of research focusing on “preventive interventions”.

Preventive interventions

RA diagnosis is made when the underlying autoimmune process has already turned uncontrolled and has become destructive. Hence, the idea of preventive interventions or treating the disease as early as possible to stop the immune process before the full establishment of the autoimmune vicious circle. Research interest is thus increasingly turning to the "pre-clinical" phases preceding the development of RA, as they represent opportunities for such preventive interventions.(80,126)

At least, in an animal model of RA (here the zymosan-induced arthritis in SKG-mice) early administration of methotrexate can suppress the development of arthritis.(127) Trials have likewise tested hydroxychloroquine, that appeared to reduce rates of progression from palindromic rheumatism (a form of preclinical RA) to persistent inflammatory arthritis.(128–130) At least six other different randomized controlled trials test presently the preventive approaches in RA.(131,132) The PRAIRI study (for "Prevention of clinically manifest RA by B-cell-directed therapy in the earliest phase of the disease") assigns individuals at high risk of RA to a single infusion of rituximab versus placebo. This experiment demonstrated a significant (55%) reduction in the development of RA at one year.(133) Other randomized trials currently compare the preventive effect of methotrexate (TREAT EARLIER),(134) abatacept (APIPPRA),(135) hydroxychloroquine (STOP-RA),(136) and statins (STAPRA),(137) with placebo. Nonetheless, the evidence available so far only demonstrated a delay in disease onset – not a long-term prevention *per se*.

Even though drug interventions may be relevant from a medical point of view, potential users of preventive interventions would seem to prefer non-medicinal interventions,(138) such as dietary changes, stress reduction or physical exercise.(127,131) Therefore, exploring the efficacy of modifying risk factors, such as lifestyle behaviors, in particular, tobacco smoking, body weight reduction, oral health and nutritional habits, is also explored.(139) Further studies even attempt to modify the microbiome of at risk individuals by researching the impact on RA development of periodontal intervention (140) or administering probiotics.(141–143)

THE MUCOSAL ORIGIN HYPOTHESIS

History – *Streptococci*-induced arthritis

At the end of the 19th century several physicians convinced themselves of a microbiological origin of rheumatoid arthritis, seeing it as an infectious arthritis without even suspecting that they might have detected contaminants in the synovial fluid.(144,145) Since then, the theory of a direct infectious origin was regularly brought to light and disproved.

For instance, in 1942, noticing the efficacy of sulfanilamide in the "*so called septic forms of arthritis*", Nanna Svartz created salazopyrin in an attempt to combine both anti-infectious properties of sulfanilamide and anti-pyretic action of aspirin. She reported that "*if the disease is still in an active stage, the treatment often yields good results in cases of this type too*".(146) The infectious theory for RA matured since then, and it took three more decades for Svartz to draw a very relevant parallel between post-streptococcal reactive arthritis and rheumatoid arthritis, hence proposing in 1972 a microbial origin of RA from poorly pasteurized milk. Her mice experiments consisted in injecting, among others, intra-peritoneally mixtures of dead or alive *Streptococcus agalactiae* cocci, which provoked *"in rats an RF-like macroglobulin* [3-4 weeks after first injection] *and slight arthritis, as was found on many occasions with agalactiae from RA patients*".(147)

Of course injecting mice with live streptococci is a source of diffuse septic arthritis,(148) and this might have happened in some experiments of Svartz. But how the heat-killed *Streptococcus agalactiae* could still induce a delayed arthritis is very interesting.(147) This model was refined a couple of years later, and it became clear that a peptidoglycan-polysaccharide, part of the bacterial cell wall, was responsible for the arthritis following intraperitoneal injection.(149,150) Indeed, the peptidoglycan-polysaccharides can resist degradation and deposit in synovial tissue where they trigger local immune reaction.(149,150) The latter also works with other oral streptococcal strains sampled in RA patients, even though the standard for inducing sustained arthritis in susceptible mice is now to inject them with bacterial or yeast cell-wall glucan compounds such as curdlan or zymosan.(151) These experiments illustrate a first connection between microbes and sterile arthritis, but our knowledge on the matter has rapidly evolved since then.

The formal mucosal origin hypothesis

The "mucosal origin hypothesis" of RA postulates that the autoimmune processes leading to the development of RA are triggered in the mucosa-associated lymphoid tissues, located in the lungs, the oral cavity, genitals and the gut, before systemic spread of auto-reactive cells.(152,153) Articular manifestations would already be a "late" stage of the process.

Holers et al. are often cited,(153) but it is unclear who has the paternity of this framework. Jean SEIGNALET had already put together most of the concepts in the early 90',(154) as follows:

- RA is multifactorial, meaning that even though genetic risk factors are involved, the disease is mostly triggered by environmental factors (see hereabove "Risk-factors for rheumatoid arthritis", page 12).

- Apart sex and hormones, all non-modifiable risk factors are genes that have a direct connection with MHC-II alleles or subsequent signaling in the response to extracellular ligands (see hereabove "Genetic risk factors", page 13).

- Other environmental risk-factors usually involve a certain degree of chronical inflammation at the mucosal level: smoking, air pollution, periodontitis, pro-inflammatory diet, chronic bronchitis, chronic diarrhea, genital infections, etc. (see "Modifiable and environmental risk factors for RA", page 13).

- These mucosae are the site of a complex interplay between host immune cells and a huge load of extracellular ligands from commensal microbes and poorly digested food antigens, which can be tolerated most of the time, but also targeted in case of excessive or damageable proliferation.

- We also know that commensal or pathogenic microbes can express surface epitopes with structure homology to the self, which renders them even more delicate to eliminate by the host's immune system, since they are difficult to distinguish from the "self". Acute situations can even result in self-damaging cross-reactivity or distant inflammation triggered by bacterial glycan compounds deposition in host tissue, as an example: rat post-streptococcal arthritis, or which models human rheumatic fever.

- **Hence:** what would happen in susceptible individuals if, due to a chronic localized inflammation, the submucosal space was continuously infiltrated by pro-inflammatory bacteria and microbe's debris mixed up with various exogenous antigens, some of which may have structural similarities with the self? Would that, together with triggering factors such as infection, stress, etc. be able to provoke autoimmunity?

Mechanistical scenario

It is unclear though how exactly autoimmunity is triggered. Several mechanisms are proposed, and maybe all of them happen simultaneously, to a certain extent (**Figure 7** page 28). The "microbe-driven" mechanistic hypothesis generally involves the following.

Generation of neo-autoantigens

To explain how citrullinated peptide generation could connect to mucosae and generation of ACPA, we can consider that:

- Some microbes, for instance *Porphyromonas gingivalis,* are known to possess citrullinating PAD enzymes. It is therefore speculated that they could citrullinate host or bacterial peptides making them more immunogenic and subsequently break the immune tolerance for self-structures.(155,156)

- Neutrophils also possess PAD4 enzyme that is necessary to citrullinate histones during neutrophil extracellular traps formation (NETosis).(157) Persistent mucosal inflammation and local recruitment of neutrophils could lead to exocytosis of active PAD4 and self-citrullinated-peptides, which will mix with various bacterial debris and be sampled by activated mucosal dendritic cells to mount immune responses.

- Alternatively, self-peptide citrullination by NETosis could also result from bacterial antigen dissemination in the blood stream, for instance, lipopolysaccharides can activate platelets and subsequently, circulating neutrophils, inducing NETosis.(158)

Loss of tolerance by molecular mimicry

Molecular mimicry is a situation in which a foreign antigen possesses structure or sequence similarities with self-antigens.(159) It happens for instance in the case of acute rheumatic fever,(160,161) and surface glycans are good candidate molecules for such scenario (also for instance in multiple sclerosis).(162) Glycan-related molecular-mimicry is also discussed in the context of intestinal bowel diseases,(163) and it seems that intestinal microbiota has an important role in shaping this glycan-specific antibodies repertoire.(164)

Molecular mimicry might also be involved in lupus and other connective tissue diseases, given that certain bacteria express proteins with sequence similarities to human ribonucleoprotein Ro60 (termed “orthologs”), a common auto-antigen in Lupus.(165) Germ free mice colonized with these ortholog-containing microbes develop anti-Ro60 antibodies, while not being exposed to the human antigen, and suffer the deposition of glomerular immune complexes.(165) Similar experiments were conducted on mouse models of antiphospholipid syndrome, where *Roseburia intestinalis* exhibits such “molecular-mimicry-epitopes” (“mimotopes”).(166) However, these mouse models are extremely susceptible to autoimmunity, and it is not clear to what extent such evidence is transposable to humans.

Similarly, *Chlamydia trachomatis* and *Chlamydia pneumoniae* known to cause reactive arthritis(167) are occasionally targeted by lymphocytes from RA patient joints.(168,169) Remarkably, *C. trachomatis*

produces antigenic microbial heat-shock proteins, such as Hsp60, a stress molecule. The latter has sequence homology with its human counterparts (170) suggesting immunogenic Hsp60 protein produced by persistent *Chlamydiae* could induce synovial inflammation and autoimmunity in chronic diseases such as RA.(171) Prevotellaceae bacteria have also been hypothesized to be involved in such a cross-reaction (see "Loss of tolerance: *Prevotellaceae?*" on page 38).

Bystander activation of auto-reactive cells

It is known that a minority of T-cells have a self-reactive TCR but are maintained quiescent or in an anergic state.(172) Part of these cells are physiological and will become regulatory T-cells, however, in some cases they acquire a pro-inflammatory effector phenotype and get involved in allergy or autoimmunity.(173) Bystander activation of T- (or B-) cells designates an activation that is independent from TCR or BCR signaling. It can be mediated by cytokines, super-antigens or other co-receptor pathways, especially in the context of inflammation, mucosal breach etc. Hence, bystander activation is proposed to explain how quiescent autoreactive T-cells could be activated and cause autoimmunity,(174) since super-antigens are common in the human gut microbiome.(175)

Bystander activation is also applied to autoreactive B-cells. Indeed, humans also host many autoreactive B-cells, which normally produce mild-affinity polyreactive natural antibodies, in a thymus-independent manner.(176) However, exposition to high loads of pro-inflammatory cytokines, or TLR-ligands, might dysregulate such cells and promote a stronger-affinity response, until creating a pathological autoimmunity.(176)

Antigen dissemination

Not to be confused with "epitope spreading", antigen dissemination designates the translocation of exogenous molecules or even whole bacteria through a permeabilized mucosa to a distant site. For instance, it is common to find bacterial DNA in synovial fluid,(177) or lymphoid tissues in mouse models,(178) or in the liver of patients with lupus and autoimmune hepatitis.(179) Bacterial cell wall compounds can also translocate to synovium to trigger arthritis as it happens in case of post-streptococcal reactive arthritis,(149,150) but the plausibility of whole bacteria dissemination is still debated.(180) Antigen dissemination is then hypothesized to induce:

- local innate inflammation and cell lysis with aberrant self-proteins exposition or citrullination.

- NETosis in the blood compartment and immune complexes deposition. It has been known for a long time that RA patients have an intriguing joint deposition of immune complexes.(181)

- Antigen accumulation in the joints, IFN-λ expression and aberrant MHC-II antigen-presentation by inflamed synovial cells.

Dual antigen receptor T-cells

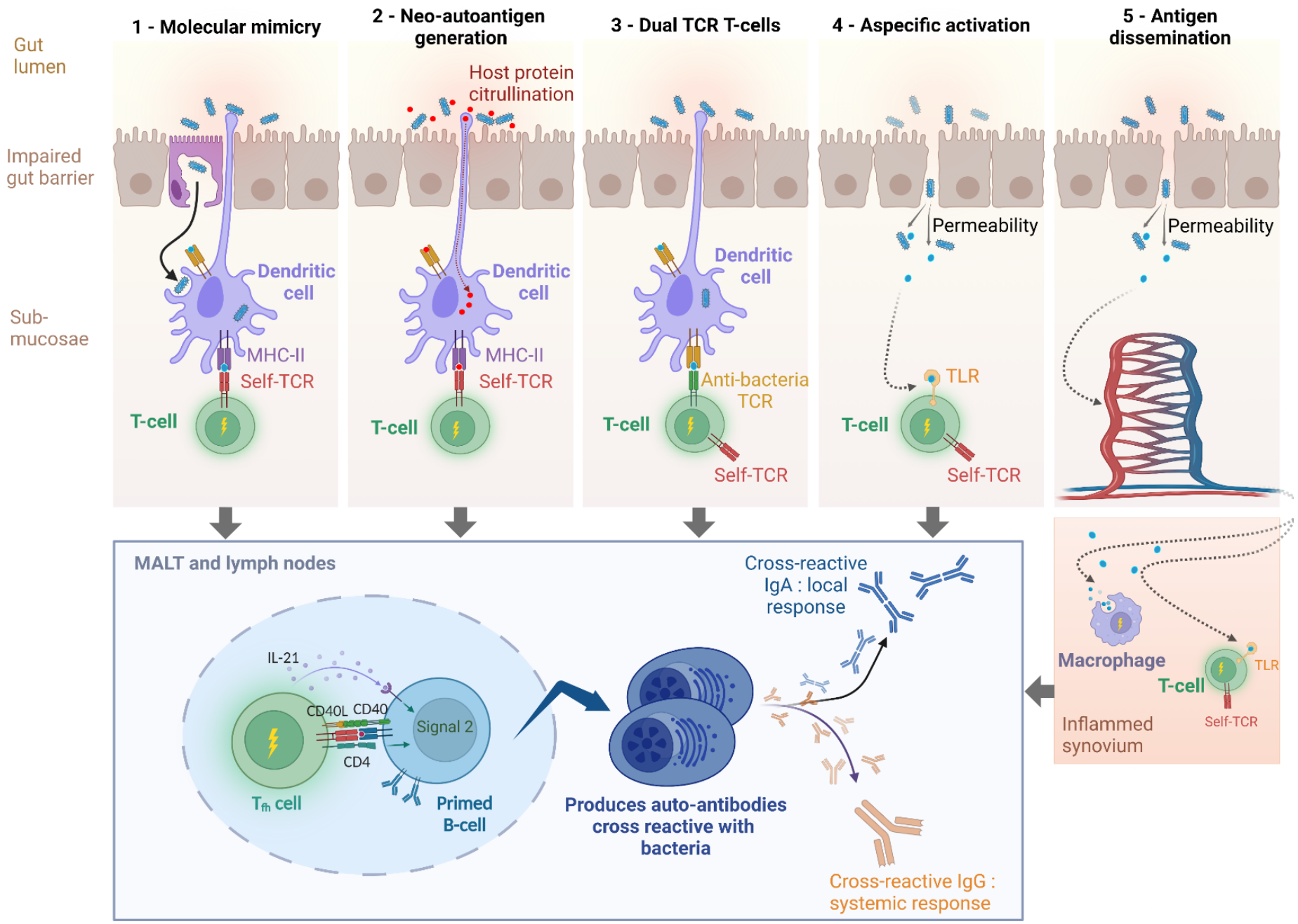
The majority of T-cells express a TCR composed of only one α chain and one β chain, even though two alleles of each exist in the genome (one per chromosome n°14). This is because of allelic exclusion, i.e. the silencing of one of the alleles, so only the second is mutated and involved in thymic selection during T-cell development.(182) However, it has been noticed in the 80's that about 10% of T-cells actually express dual surface TCR α chains, for which the allelic exclusion is less stringent that β chain.(183) Also, about 1% of T-cells express dual surface TCR β chains.(183) As a result, such T-cells have two different TCRs and can recognize two different antigens!

Dual TCR T-cells are poorly studied, but they are probably involved, for instance, in response to auto-antigens or alloreactivity in graft-versus-host disease.(184,185) It is theoretically possible that such dual TCR cells have one TCR recognizing exogenous antigens (for instance bacterial) and a second TCR mistakenly undeleted, with reactivity to an auto-antigen. Hence, T-cell activation by the exogenous ligand would result in possible autoimmune reaction against the self-peptide by either:

- involving auto-antibodies, if the self-peptide if ever presented by antigen-presenting-cells with the appropriate cytokine microenvironment.

- involving the T_H17 axis and recruitment of innate immune cells, where the self-peptide is expressed. The latter has been shown to happen in mouse models of RA-related autoimmune lung disease, where dual TCR T_H17 reactive against segmented filamentous bacteria also infiltrate lung tissue.(186) Still, such mouse models aberrantly express a very skewed TCR repertoire, about 2/3 of dual TCR T-cells, and it is questionable if such phenomenon is truly representative of what could happen in genetically normal mice, or humans.(183)

Figure 7: Proposed mechanisms to explain the influence of gut microbiota on autoimmunity. In purple are represented antigen-presenting cells. Light blue represents bacteria or derived peptides. Green cells are T lymphocytes that will activate local B-cells (dark blue). The latter will result in the production of cross-reactive Ig. The five possible mechanisms are presented. TCR = T-Cell Receptor. TLR = Toll-Like Receptor. MALT = Mucosa-Associated Lymphoid Tissue. Created with www.biorender.com .


1 - Molecular mimicry
2 - Neo-autoantigen generation
3 - Dual TCR T-cells
4 - Aspecific activation
5 - Antigen dissemination
Gut lumen
Impaired gut barrier
Sub-mucosae
Host protein citrullination
Dendritic cell
MHC-II
Self-TCR
T-cell
Anti-bacteria TCR
Permeability
TLR
MALT and lymph nodes
IL-21
CD40L
CD40
CD4
Signal 2
Tfh cell
Primed B-cell
Produces auto-antibodies cross reactive with bacteria
Cross-reactive IgA : local response
Cross-reactive IgG : systemic response
Macrophage
Inflammed synovium

Key steps and microbiome

Some findings in the pre-clinical stages of RA elegantly fit in line with this mucosal hypothesis, for instance, transcriptional analysis revealed that IgA production is significantly enriched in RA and its pre-clinical stages,(187) which is also suggested by the elevated IgA-plasmablast levels in individuals with asymptomatic seropositivity for RF or ACPA.(188) (it should be remembered that IgA is mainly produced to be secreted on mucosal surfaces). Similarly, serum IgA ACPA predominates in seropositive individuals who will develop RA,(189) or in at-risk individuals.(190,191) Finding IgA auto-antibodies in the sputum of RA patients or at-risk individuals is also suggestive of an initial mucosal trigger, especially since some individuals were ACPA positive in the sputum, but not in the serum ! (192–194) This means the APCA-producing B-cells were located in the MALT compartment and produced auto-antibodies locally.

The “secretory component” is a fragment of the polymeric immunoglobulin receptor, which remains bounded to dimeric IgA after their secretion. It is unclear how secretory IgA can sometimes circulate back in the serum after having been secreted.(195) Rheumatoid factor linked to a secretory component is detectable in RA patients’ serum.(196) Secretory ACPA have also been detected in a subgroup of early RA patients (about 17%).(197) Actually, the prevalence of secretory-anti-CarP, -ACPA and -RF is increased in RA patients, compared with healthy controls, further suggesting a mucosal origin of these auto-antibodies.(198)

It is, therefore, appealing to speculate that after the loss of tolerance to self-antigens, auto-reactive B-cells spread in central lymphoid organs and produce higher affinity IgG or IgA in the bloodstream.(153) Such trafficking has recently been evidenced in mouse models.(199) Last but not least, microbial antigens and their metabolic products have a major impact on the immune system,(200) and are extensively discussed by many recent reviews linking them to RA.(200–203)

Hence the three key steps in the theory:

- Chronic local inflammation and increased mucosal permeability – maybe due to, or driven by, the mucosal microbiome. In this regard, inter-epithelial cell tight junctions, the mucus layer, and secreted antimicrobial peptides should not be forgotten as important constituents of a healthy mucosa (**Figure 8**, page 33).(153)

- Loss of tolerance for self-antigens, due to interference of a foreign antigen or modified proteins, of which microbiome would be a major source.

- The systemic spread of auto-reactive B- or T-cells and shift from a local reaction to a sustained and higher-affinity self-damageable immune response characterized by the presence of IgA or IgG auto-antibodies in the serum.

Microbiota – microbiome definition

A subtle distinction exists between "*microbiome*" and "*microbiota*", even though the terms are used interchangeably in most cases.

> Microbiome: the suffixes *-biome* is derived from ecosystem biology and designates a given ecosystem as a whole. *Micro-* further specifies that we are talking about the microscopic world. Hence, *microbiome* has a broad sense: at the mucosal level, it encompasses bacteria, but also viruses, archaea and fungi including their genomes and metabolites.(204,205)
>
> Microbiota: the suffix *-biota* is rather claimed to refer to the organisms living within a *-biome*. Hence microbiota has a slightly more restrictive meaning.(204,205)

Even though the title of my thesis contains "microbiome", which at the mucosal level is, indeed, our study object, for various technical reasons, in most cases we only studied the bacterial taxonomy referring to it as the *microbiota* and ignored the metabolites, the whole genomes and the ~1% fungi and viruses. So did most of the previous studies. Hence, I am using "microbiota", unless I refer to the "whole" ecosystem or unless an additional investigation was made to explicitly characterize whole genomes, other biomarkers or functional profiles.

Sometimes, the term "dysbiosis" is used to designate the process leading to a supposedly pathological (i.e. "dysbiotic") microbiome. Within a "dysbiotic" microbiome, the "good" microbes would have been outnumbered by the "bad" ones leading to detrimental shift in function, metabolite production, host tissue invasion, etc. However, looking closer, there is no clear-cut definition of what a dysbiotic microbiome is, simply because it is still extremely unclear, which are the "good bugs" and which are the "bad" ones, especially since this distinction seems to depend on the context! (206)

Most studies have sampled microbiome both in a group of patients and in a group of controls. Then, hypothesizing a causal relationship between the disease of interest and the patient's microbiome (whereas this was the research question...), researchers have labeled as "dysbiotic" the patient's microbiome, if they found any characteristic differing from the control group. That is to me the real current meaning of "dysbiosis": i.e. the "dysbiotic" microbiomes, if analyzed with clustering techniques within the setting of a given case-control study, will be classified distinctly from those of healthy controls. But these difference in microbiome do not really establish causality, as patients and controls differ in many other parameters such as treatment, diet, lifestyles, etc. Since "dysbiosis" is largely undefined, I will avoid this terminology in my thesis and rather describe the compositional alterations of interest.

GUT MUCOSA IN RHEUMATOID ARTHRITIS

Inflammation: diet and microbiota?

Diet (see "Diet and lifestyle", page 14) and chronic diarrhea are risk factors for RA (207) but it is unclear what exactly would cause a chronic gut inflammation in the context of RA. Most available evidence is derived from other contexts or mouse models and suggests that the gut microbiome is driving the inflammation. I think diet shall not be disregarded either. The key element is that intestinal inflammation could lead to a permeabilized epithelium infiltrated by different exogenous antigens. The latter introduces the concept of intestinal permeability.

Intestinal permeability

Terminologically, we should refer to the following definitions: (208)

> Intestinal barrier: functional entity separating the gut lumen from the inner host, consisting of mechanical elements (glycocalyx, mucus, epithelial layer), humoral elements (defensins, IgA), immunological elements (lymphocytes, innate immune cells), muscular and neurological elements.(209) In physiological conditions, bacteria do not access the epithelium, due to a compact inner mucus layer of ~200µm thickness (even though what we know on this matter is mostly derived from mice) (**Figure 8**).(210)
>
> Intestinal permeability: functional feature of the intestinal barrier at given sites, measurable by analyzing flux rates across the intestinal wall as a whole or across wall components of defined molecules that are largely inert during the process and that can be adequately measured in these settings.

Today, standard measures of gut mucosal barrier permeability imply the ingestion of passively absorbed probes, most commonly lactulose and mannitol, ideally labeled with ^{13}C, which can be subsequently measured in the urine at the timepoint of interest - a higher urinary lactulose/mannitol ratio (LMR) is for instance believed to indicate a higher small-bowel permeability.(211)

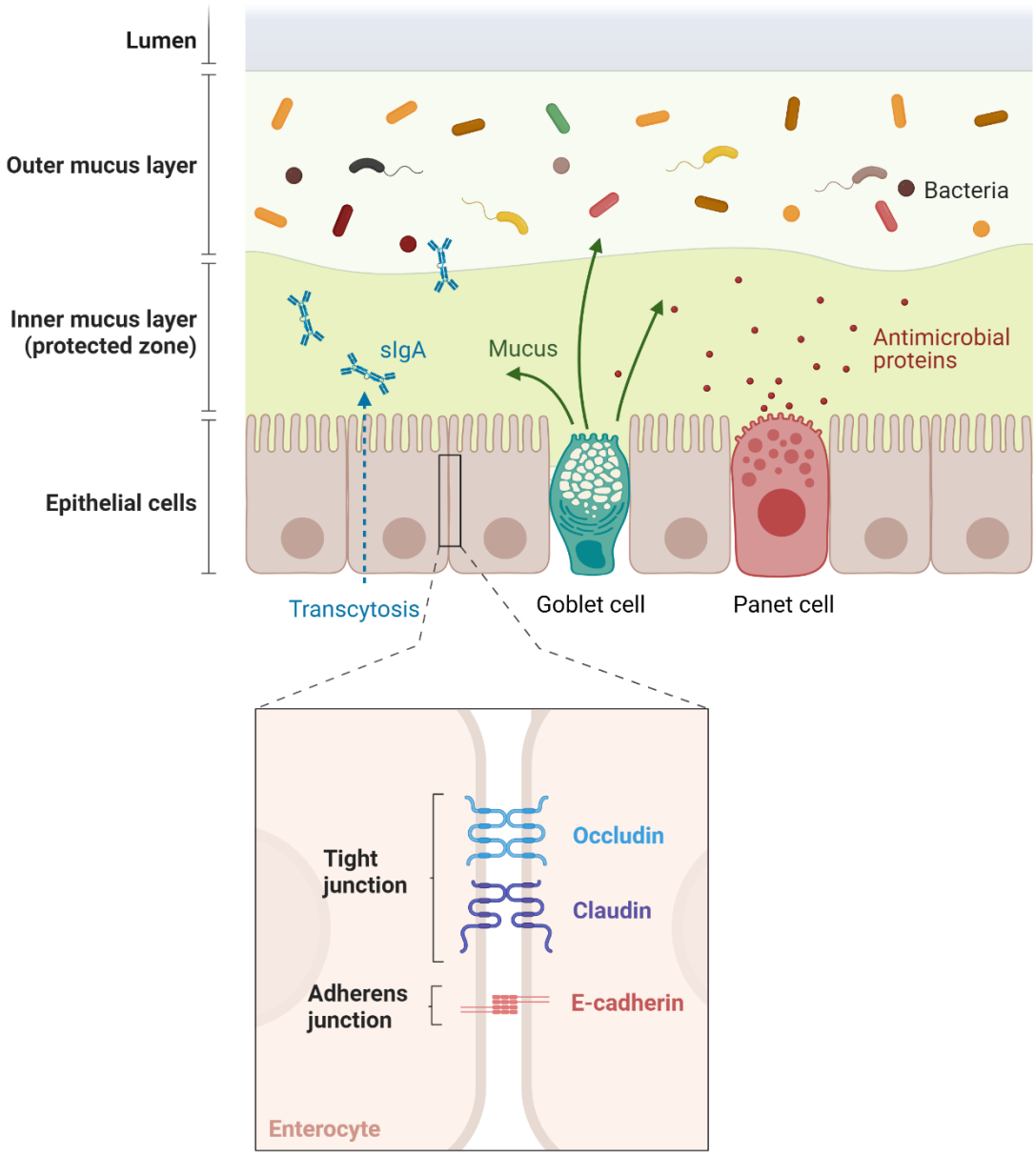


Figure 8: Structure of intestinal mucosal barrier. Two layers of mucus protect epithelial cells, the inner one remining sterile in physiological conditions. Tight junctions ensure permeability of the intestinal barrier towards luminal content. Created with www.biorender.com .

However, functional tests of gut permeability are logistically complicated, time-consuming, and can be compromised by concomitant intestinal diseases, or NSAID intake.(208,211,212). To simplify gut mucosal assessment, several biomarkers, such as Lipopolysaccharide-Binding Protein (LBP), Intestinal Fatty Acid Binding Protein (I-FABP) and zonulin, have been proposed, but their reliability is debated.(213) I will comment these biomarkers briefly.

Lipopolysaccharide-Binding Protein (LBP) is a protein mostly secreted by the liver.(214) LBP can opsonize gram negative bacteria to facilitate phagocytosis.(215) It also binds circulating LPS, before the resulting complex can link CD14,(216) a monocyte receptor, which is also expressed by hepatocytes, to induce antibacterial response.(217) Furthermore, CD14 exists in a soluble form (sCD14), which works with LPB-LPS complexes to neutralize LPS.(214) Because of a relatively long half-life, serum LBP levels remain elevated for several days after bacteremia. Given the technical limitations of direct serum-LPS assessment,(218) elevated serum LBP are sometimes proposed to be a reflect of chronic LPS translocation, from the intestinal lumen to the systemic circulation.(219–223) In clinical studies, LBP has demonstrated useful diagnostic properties in discriminating patients with infections from those without and correlates well with clinical response to antibiotics.(224) Elevated LBP levels have also been shown in patients with inflammatory bowel diseases and were associated with higher inflammatory bowel disease activity.(224) LBP has also been studied as a marker of inflammation and disease activity in RA patients.(225) However, as discussed in more detail below (Section “COMMON DISCUSSION”, page 109), LBP is linked primarily to the host’s antibacterial response and not directly associated with intestinal permeability.

Intestinal Fatty Acid Binding Protein (I-FABP), also known as Fatty Acid Binding Protein 2 (FABP-2), is a tissue specific intracellular protein only expressed in enterocytes.(226) It is released in peripheral circulation after epithelial cell injury and thus is used as a marker of intestinal damage, for instance during small bowel ischemia,(227) or in obesity.(228) However, I did not find much evidence of its correlation with altered functional permeability testing (see “COMMON DISCUSSION”, page 109).

Zonulin is the eukaryotic analogue of the *Vibrio cholerae*-derived *zonula occludens toxin*. This protein is involved in controlling the intestinal permeability by promoting reversible disassembly of epithelial inter-cellular tight junctions (tight junctions are also named *zonula occludens*).(229) It is secreted luminally by mammalian’s small intestine enteric cells when exposed to bacteria, even nonpathogenic strains.(230) The latter could be interpreted as a basic immunological reaction to “flush out” these too-close bacteria. Serum zonulin is thus associated with increased intestinal permeability.(231) Interestingly, in type 1 diabetes, - an

autoimmune disease -, serum zonulin levels are increased, even before the onset of the disease.(232) In coeliac disease or Crohn's disease, other immune-mediated conditions, zonulin is upregulated during flares of the disease.(233,234) Serum zonulin was also associated with increased intestinal permeability in the context of RA, and in ACPA positive asymptomatic individuals.(235) However, commercially available zonulin ELISA kits have demonstrated low reliability and specificity, and apparently do not target zonulin itself (also known as pre-Haptoglobin2),(236) and cross-react with other proteins such as complement C3 or albumin.(237) It is consequently still unclear what the commercially available zonulin tests actually detect *in vivo*.(238)

In vitro assessment is also possible, using cells lines or biopsies with immunohistochemistry protocols to assess expression of tight junction proteins (such as claudin, occludin, and zonulin).(239) Finally, onsite measurements have been tried, for instance using endoscopic confocal microscopy which tracks leaks of fluorescein injected intravenously,(240) or by measuring *in vivo* epithelial impedance.(241)

Overall, the literature about gut permeability is confusing. Many authors describe "increased gut permeability" while only measuring proxy biomarkers of this construct. While "proxy permeability" has been linked to various diseases, such as celiac disease (242) or depression (243) it remains unclear if gut permeability is a cause or a consequence of these diseases.

Causes of increased gut permeability

Some bacterial strains can invade or damage mucus layer and induce epithelial stress, lesions and inflammation. However, the causality might be circular since an inflamed epithelium is also be more susceptible to uncontrolled bacterial growth.(244) Based on *in vitro* and mice studies, a few strains have been characterized as protective of detrimental for intestinal permeability.(244) Known pathogen such as *Clostridium difficile*,(245) shiga-toxin producing *E. coli*,(246) or *Salmonella* species (247) can disrupt tight junctions, while certain strains of *Lactobacillus rhamnosus* are known to restore barrier function.(248)

In the clinical context, bacterial invasion of the mucosa occurs for instance in spondyloarthitis (SpA) associated with inflammatory bowel disease (IBD).(249) Biopsies taken in SpA-IBD patients showed adherent and invading Gram-negative (but not Gram-positive) bacteria within the lamina propria of the ileum, contrary to "regular" IBD patients and healthy controls.(249) Histochemical staining also revealed downregulation of claudin-1 and claudin-4 (tight-junction proteins), while serum levels of I-FABP, LPS and sCD14, were elevated compared to "normal" IBD patients.(249) Damaged mucosal barrier due to the presence of adherent and invasive bacteria was also shown in ileal biopsies of 20

ankylosing spondylitis patients.(250) Serum levels of LPS, LBP and I-FABP were also allegedly increased compared to healthy subjects.(250)

Similarly, in mouse models of lupus-like autoimmunity, certain bacterial strains seem to be able to translocate from the intestine to the mesenteric veins, lymph nodes, the liver and even the spleen.(179) The involved *Enterococcus gallinarum* down-regulated ileal molecules related to barrier function, the mucus layer, antimicrobial defense, and up-regulated those related to inflammation, also promoting immune complex deposition, and anti-dsDNA / anti-RND IgG production, which are similar to the auto-antibodies found in lupus and autoimmune hepatitis. These effects were abrogated by antibiotic treatment.(179)

Based on mice studies, Spadoni et al. focused on the gut endothelial cells and proposed to add the concept of gut-vascular barrier, which they shown to be disrupted by *Salmonella*. (251) Analyzing biopsies from celiac patients with increased ALAT blood levels revealed an over-expression of plasmalemma vesicle-associated protein-1 (PV1), a tissue marker of endothelial cell permeability,(252) while the epithelial intestinal cells did not appear damaged thus suggesting that regulation of luminal compounds in the blood stream is even more sophisticated than we think and does not only depend on enterocytes.(251) Adapting from Camilleri et al., factors reportedly modulating intestinal permeability are reviewed in appendix **Table 10** on page 189.(209,253)

Gut permeability in rheumatoid arthritis

Solid evidence about gut permeability in RA patients is largely lacking. The first studies in the 80s used orally administered probes but were all biased by NSAID usage.(212,254,255) NSAID are nowadays known to induce small bowel lesions, referred to as NSAID-induced enteropathy, including mucosal breaks.(256,257)

Later studies relied on uncertain biomarker measurements, sometimes completed by gut biopsies. For instance, in 2022 Ayyappan et al. (258) compared serum from RA patients to healthy age-sex matched controls and assessed various antimicrobial response factors such as CD14 (sCD14), lipopolysaccharide-binding protein (LBP), lysozyme, CXCL16, and LPS. They found significant elevation of sCD14 (so did previous reports (259,260)), LBP, lysozyme (as previously (261)), CXCL16, which was interpreted as suggesting a systemic exposure to microbial products. However, one could argue that these finding might as well result from stereotypical inflammation.

Similarly, Audo et al., comparing 59 RA patients with 33 healthy controls, showed elevated serum LBP and sCD14, but these biomarkers are expected to increase in the context of inflammation anyway.(262) Audo et al. also assessed colonic biopsies from 20 RA patients and 20 controls, showing elevated epithelial zonulin expression, but no difference in occludin and claudin-2 content.(262) Ruling out

NSAID-treated patients, Matei et al. (239) have shown elevated LBS, LBP and I-FABP in RA patients compared to healthy controls.

Lately, Tajik et al. using serum zonulin as a marker, showed allegedly increased intestinal permeability in early and established RA patients, compared to healthy controls.(235) Interestingly, they confirmed this finding on ileus mucosal biopsies from 10 healthy controls, 10 new-onset untreated RA patients, and five chronic RA patients, showing lower expression of tight-junction proteins and increased levels of immune cells in the lamina propria compared to healthy controls. This was also associated with increased lactulose/mannitol urinary excretion ratio.(235)

Other small clues could point to abnormal permeability in the context of RA. It is known for instance that bacterial DNA, as well as bacterial cell-wall components such as peptidoglycan–polysaccharide complexes or muramic acid, can be found in the joints of RA patients.(263,264) But it is unclear if these findings are specific for RA, since a small degree of bacterial translocation seems to occur in physiological condition.(265–268)

The gut microbiome and rheumatoid arthritis

Mouse models provide most of our knowledge about gut microbiome and RA. Relevant mice-derived evidence is discussed in appendix “Mice data overview“, page 190. I focus below on human data.

Research on the fecal flora of RA patients is not new and early investigations using culture-based identification methodology have revealed an enrichment of certain species, particularly *Clostridium perfringens* which was hypothesized at that time (1968) to contribute to RA pathogenesis via secretion of alpha-toxins.(269,270) Computerized chromatography also measured in 1994 a difference in feces composition between early RA subjects and other patients, excluding gastrointestinal diseases, attributed to anaerobic bacteria.(271)

Later on, genomic probing and sequencing approaches allowed broader characterization. Most of these modern analyses focus on a few variable regions (V1 to V6) of the gene coding for the 16S subunit of bacterial ribosomal RNA (hence “16S-sequencing”), which is specific for bacteria and widely conserved. Though convenient and powerful, this method relies on assumptions that are debated today. For instance, it is not obvious which thresholds of sequence similitude to use for taxa definition; while certain species can have several copies of the 16S gene.(272)

Between 2013 and 2019, 3 studies using 16S sequencing demonstrated an increased relative abundance of Prevotellaceae, presumably mainly *Prevotella copri*, among pre-clinical RA and/or early RA patients compared to healthy controls.(273–275) Also, *Prevotella*-enriched microbiota, when transferred from patient to mouse models, aggravated colitis or arthritis.(273,275)

Nevertheless, later studies (mostly Asian) using 16S-based sequencing did not identify any difference in the relative abundance of *Prevotellaceae* between RA subjects and controls, contradicting previous results.(276–283) The variability in the populations of interest further confuses how to interpret these discrepancies. Other bacteria of interest were proposed. For instance, *Collinsella* genus was enriched in RA patients, and authors further demonstrated that compared to *E. coli*, *Collinsella aerofaciens* had the ability to increase gut permeability, promote pro-inflammatory conditions, and increase incidence of collagen-induced arthritis in mice.(276)

Hence, *P. copri* might not be the only bacteria involved in the RA pathogenesis. Other species could promote inflammation and alter gut permeability. We underline that *Collinsella aerofaciens* has previously been associated to inflammatory bowel disease, and exacerbated murine colitis compared to other control bacteria.(284) Others have searched for microbes inversely associated with RA activity ('protective bacteria'), and a recent publication suggested that *Parabacteroides distasonis* could be a probiotic able to alleviates inflammatory arthritis (at least it worked in the mouse model).(283)

Microbiome can also be assessed by so-called "shotgun" sequencing, which aims at providing whole-genome data.(285) Available shogun sequencing studies confirmed a significant difference of the composition of the gut microbiome of RA patients compared to controls, but confusingly the involved taxa were not always those reported by 16S-based studies.(286–289)

In a nutshell, several microbes were associated with RA, and have demonstrated the ability to worsen arthritis in mouse models. Future research should investigate what these bacteria have in common, such as harmful metabolites, human-resembling antigens, mucus-invading capacity, etc. Available microbiome studies in RA are listed in appendix "Gut microbiome studies in rheumatoid arthritis", page 196.

Loss of tolerance: *Prevotellaceae?*

A few words on the evidence linking the gut microbiome to the loss of immune self-tolerance in humans.

The *Prevotella copri* hypothesis

Given the fact that Prevotellaceae from RA patients worsen colitis and arthritis in mouse models,(273,275) researchers have suggested that *P. copri* or other Prevotellaceae might have epitopes conferring cross-reactivity to arthritis-related autoantigens.(275) In oncology mouse models, *P. copri* exacerbates carboplatin-associated intestinal colitis,(290) and it was also associated to ankylosing spondylitis.(291)

Interestingly, in 2017 Pianta et al. isolated an HLA-DR-presented peptide on mono-nuclear cells from the peripheral blood of an established RA patient. The peptide originated from *Prevotella copri* (protein

Pc-p27).(292) Antibody response to this protein or to the whole *P. copri* was then tested with serum samples from 127 patients with new-onset RA or chronic RA, 28 patients with connective tissue diseases, 28 patients with spondyloarthropathy, 70 patients with Lyme arthritis, and 50 healthy controls. (292) Only the group of RA patients had a specific cellular and/or humoral response to Pc-p27, a protein of *P. copri*.

Wang et al. later demonstrated that some HLA-DR presented self-peptides, isolated from mono-nuclear cells extracted from blood and synovial tissue of chronic RA patients, were originating from self-proteins N-acetylglucosamine-6-sulfatase (GNS) and filamin A (FLNA).(293) In this context, Pianta et al. predicted these self-derived-peptides to bind strongly to the HLA-DR shared epitope alleles (idem for the previous *P. copri*-derived peptide), that they had 67% and 80% epitope homology with some *Prevotella* proteins (and Parabacteroides but no homology found with *P. gingivalis*).(294) When IgG and IgA responses were considered together, 56 out of the 101 patients with RA (55%) had antibody responses against GNS and/or FLNA, and this strongly correlated with *P. copri* antibody responses, suggesting a possible cross-reaction.(294)

Apart from the molecular mimicry mechanism, *Prevotella* species have been hypothesized to drive inflammation by other means.(295) For instance, *Prevotella* species take part in the process of biofilm formation, which in the oral context connects to periodontitis,(296) in particular for *P. nigrescens* and *P. intermedia* promoting inflammation and T_H17 immune responses.(297–299) Also, in the oncologic context *Prevotella copri* is among the bacteria increasing response to anti-PD1 therapy, hence, it is "immunogenic" and favors immune responses.(300) The latter "immunogenicity" could be a consequence of an immune-stimulant glycolipid that *P. copri* produces (alpha-galactosylceramid – though a hundred times less than Bacteroides fragilis (301)), which can activate non-conventional T-cells, though there is no certainty that this applies to every strain.(302,303)

Challenging findings

Pianta et al. and Wang et al. thus suggested that *Prevotella copri* could be involved in a cross-reaction with the GNS and FLNA self-proteins. Later findings also challenged these views.(293,294)

First, it is not clear how anti-Prevotellaceae immunity is related to gut microbiota or even oral microbiota, since Pianta et al. did not analyze concomitant stool samples; thus, we do not know if patients with anti-*Prevotella* Ig had noticeable amounts of these bacteria in their microbiome.

Second, the *Prevotella* genus was discovered in the 90s, and comprises several gram-negative anaerobic bacteria with saccharolytic abilities.(295,304) Moreover, when microbiomes are clustered different "enterotypes" can be identified including one dominated by *Prevotella* species.(305) The latter, though, still represents a debatable issue. Thus, it is unclear if the authors reporting "relative

expansions of *Prevotella* species" found more "normal" *Prevotella* enterotypes, or if they found increased abundance of *Prevotella* species.

Confusingly, *Prevotella* species are also reported to have beneficial effects in other circumstances.(306) For instance, *Prevotella histicola* reduced arthritis severity and intestinal permeability (by increasing expression of tight junction proteins) in a mouse model, compared to colonization with *Prevotella melanogenica*.(307) Furthermore, regarding glucose metabolism, Kovatcheva et al. have shown that healthy subjects exhibiting improved glucose metabolism after a very rich in fibers barley kernel-based bread supplementation had expanded their abundance of *Prevotella* species, specially *P. copri*,(308) which has also been linked with beneficial cardiometabolic markers.(309) On the other hand, Pedersen et al. identified *P. copri* to be one on the main species driving insulin resistance in type II diabetic patients.(310) This apparent contradiction might be explained by the high variability of *P. copri* at the strain level.(311) Using shotgun sequencing, Scher et al. revealed that new onset RA patients do not exactly host the same strains of *P. copri* as control patients.(273)

At the strain level, *P. copri* is composed of four distinct clades, with different carbohydrate metabolism repertoires.(312) Actually, *P. copri* is commonly reported in healthy individuals.(313) The authors could not associate one particular clade to diseased state.(312) Recently, Nii et al., compared *P. copri* strains isolated from RA patients versus healthy controls.(314) They did not find much differences, except for a ~100kbp conjugative transposon that usually has horizontally transferred virulence factors etc. and was specific to the *P. copri* strains of interest.(314) In DBA/1J mice that were colonized with *P. copri* after antibiotic treatment in specific pathogen free conditions, the collagen-induced arthritis was more severe when the mice were colonized by RA patients-derived *P. copri* with the conjugative transposon region.(314) The latter proved the strain-level relevance when considering *P. copri*.

II. THESIS WORK

RATIONALE AND OBJECTIVES

The predictability of rheumatoid arthritis

This thesis assumes that RA does not start in the joints. Hence, it should theoretically be possible to detect the early processes leading to RA, before irreversible joint damage occurs, and to administer a preventive intervention. However, it is still problematic to reliably identify at-risk individuals who would most likely benefit from such interventions. Because therapeutic interventions with antirheumatic therapies carry a substantial risk of toxicity, we shall avoid to mistakenly treat individuals who were not susceptible to developing RA.

The gold-standard way to identify reliable biomarkers able to predict RA would be to:

- Enroll, in a cohort study, the individuals at risk for RA who have not yet developed the disease.
- Sample them regularly (blood, microbiome, etc.) as frequently as possible.
- Continue sampling and following-up until RA diagnosis.
- Then, once enough individuals in the cohort have developed RA, group them together, find matched controls, and check the potential biomarkers in the samples taken at minus 6 month, minus 12 months, etc., before the RA diagnosis.
- A good “predictive” biomarker would be at least highly specific for the incident-RA group, at a timepoint with clinical relevance (for instance one year prior to RA diagnosis).
- Finally, test the biomarker in a prospective validation cohort.

Even though this approach is close to the design of the cohort that I have worked with, that it is NOT the approach that has been taken in this thesis; for the simple reason that the “incident-RA” group that we could have access to was still not large enough, and most of the patients lacked a past stool sample.

Consequently, we had to content ourselves with the second-best option which is:

- Constitute groups of individuals at different stages of disease development based on their present phenotype.
- Assess in these groups the biomarkers of interest, at the present timepoint, so to see if the biomarkers correlate with later “stage” of the disease or “pre-disease”.
- Eventually, test the biomarker relevance in a validation cohort or with longer-term follow-up.

The challenge with such a design is that it is not obvious to assess which "*stage* of disease development" an individual is in ("staging"); since we do not know with certainty what will happen to the patient in the future. We know for instance that in the general population, having RA-autoimmunity without symptoms has a predictive positive value for inflammatory arthritis onset of ~9%,(315) which increases to ~30–50% in the next 3 years with concomitant genetic risk factors.(316) Or, in case of suspicious inflammatory arthralgia, the predictive positive value for future RA is ~30%,(94) and climbs up to ~60% in case of concomitant autoimmunity associated with RA,(317) etc. Therefore, we could design risk-groups using international recommendations for categorizing the pre-clinical stages of RA.(93) Such staging is our best guess based on the existing literature and patient's data.

Objectives

Our objectives were to:

1) Assess a potential correlation between increasing "risk of RA", and the presence of RA-associated bacteria in feces.
2) Assess the correlation between increased "risk of RA", and the serological concentrations of biomarkers pertaining to intestinal inflammation and gut permeability.
3) Provide exploratory assessment of patient's immunoglobin reactivity against RA-relevant microbes or against the autologous fecal microbiota.

We proposed a work-package for each of these objectives.

COMMON METHODS

Here I describe methodological aspects that apply to all three work-packages.

Study design: the SCREEN-RA cohort

The SCREEN-RA cohort (Evaluation of a SCREENing strategy for Rheumatoid Arthritis) was created in 2009 by Prof. Axel FINCKH, with the support of the Swiss National Science Foundation. It aims to discover or validate novel biomarkers predictive for RA development. The SCREEN-RA cohort recruits adult individuals who are first-degree relatives of RA patients (RA-FDRs).(318) The enrolled participants do not have the disease at inclusion but they are known for having a familial susceptibility to it. The SCREEN-RA cohort has been approved by the relevant ethic Committees (project PB_2016-00889), and my thesis was conducted under the scope of this ethical approval. All participants signed an informed consent before enrolment, in accordance with the Declaration of Helsinki.

As of May 2023, the cohort enrolled 1550 individuals. I took advantage of this setting to perform a cross-sectional study, which means we sampled the individuals in such a way as to obtain a representative "screenshot" of the cohort. Then, we grouped the participants according to their "pre-clinical RA stage" and performed the comparisons of interest. Consequently, the same cohort will be the source of the individuals for the control group. We reasoned that this was the best way to exclude confounding by genetic background, which would have occurred if we had recruited healthy controls from the general population.

I have extensively described the cohort in a separated publication ("1 – BMJ Open - Cohort Profile: SCREEN-RA: design, methods and perspectives of a Swiss cohort study of first-degree relatives of patients with rheumatoid arthritis", page 132),(96), which can be briefly summarized as following:

Recruitment methods include e-mails to patients, articles in magazines, promotion via patient associations, and, since 2018, campaigns on social networks. Enrollment has been conducted through 10 collaborative centers: Geneva (Hôpitaux Universitaires de Genève), Lausanne (Centre Hospitalier Universitaire Vaudois), Fribourg (Hôpital Fribourgeois), Neuchâtel (Réseau Hospitalier Neuchâtelois), Bâle (Universitätsspital Basel), Zurich (Universitätsspital Zurich), Berne (Inselspital – Hôpital universitaire de Berne), Aarau (Kantonsspital Aarau) and Saint-Gall (Kantonsspital St.Gallen).

Upon enrollment, each participant is given a clinical examination by a specialized nurse or a rheumatologist to assess potential tender and swollen joints and rule out the presence of RA or other autoimmune conditions.(319) In addition to biological sampling, this examination is repeated yearly for the "high-risk" participants.

All participants receive an annual follow-up questionnaire assessing articular symptoms, presence of immune diseases, current medications, and environmental factors such as smoking, nutritional or exercising habits. Questionnaires are available in three languages (French, English and German) and were established in collaboration with other ongoing studies of at-risk populations, such as the American SERA cohort (320), to allow replication studies in future.

Exclusion criteria are an established diagnosis of RA, or the presence of active co-morbid inflammatory arthritides (i.e., patients with psoriatic arthritis, spondylarthritis, or known microcrystalline arthritis) to avoid outcome misclassification. After enrollment, all participants are followed using yearly questionnaires to detect new symptoms or signs of the disease.

Data are collected through a secured online interface, stored using REDCap (Research Electronic Data Capture) software and hosted on institutional servers with secure backup. The database is password protected and changes are tracked in logfiles. Previously, paper questionnaires were used, presently most of them are sent out to the participants by e-mail. Reports of physical examinations and results of serological analysis are entered into REDcap. Each patient is identified by a numerical code of two to four digits, which is also used to label the biological samples.

Sampling procedure

All biological samples are processed following standard operative procedures and stored at -80°C, in a dedicated biobank at HUG/UNIGE. Samples from the collaborative centers are regularly shipped on dry ice to Geneva.

Blood and serum

For all SCREEN-RA participants, full blood samples are collected at the initial enrollment time in EDTA collection tubes for genetic testing (HLA) and additional aliquots for a genomic DNA library; and in Tempus collection tubes for RNA extraction (not used in this thesis). Serum samples are collected for the assessment of autoantibodies (ACPA, RF, and anti-Ra33 in a subset of participants). The serum aliquots are stored at -80°C in a serum library. Assessment of the serological status is performed using various commercially as well as non-commercially available kits:

- CCPlus Immunoscan® (anti-CCP2) IgG ELISA (Svar Life Science, Malmö, Sweden)
- QUANTA Lite® CCP3.1 IgG/IgA ELISA (INOVA Diagnostics)
- QUANTA Lite® CCP3 IgG ELISA (INOVA Diagnostics)
- QUANTA Flash® CCP3 IgG CIA (INOVA Diagnostics)
- QUANTA Lite RF IgM ELISA (INOVA Diagnostics)

- QUANTA Lite RF IgA ELISA (INOVA Diagnostics)

- Elia RF IgM (Phadia AB)

- Elia RF IgA (Phadia AB)

- ELIA anti-Ra33 (IgA, IgG or IgM isotype), research use only (Phadia AB)

In case of testing with different kits, only the highest titers of autoantibodies obtained are used in the study. This variability of assays is due to (1) availability changes or updates from the suppliers or laboratory partners, and (2) the fact that several generations of anti-cyclic citrullinated peptides (CCP) assays exist (the synthetic citrullinated probes used as target may vary depending on the generation of the kit; CCP2, 3, 3.1, etc.). Clinically, these anti-CCP assays are interpreted equivalently, as we do in SCREEN-RA, nonetheless, we have noted in our pre-clinical population some discrepancies. The latter most probably must have manifested themselves due to early immune reaction restricted to a few epitopes, when a participant is positive, for instance, in the anti-CCP2 assay but not in the anti-CCP3.1; which happens less often in the established RA population for which these kits are primarily designed.

Stool samples

Between September 2019 and October 2021, we invited the SCREEN-RA participants to provide a stool sample paired with a serum sample. They received stool collection-devices allowing the creation of several aliquots and proceeded to stool sampling at home. These devices were obtained from Prof. Jeroen RAES (Leuven, Belgium), with whom we further collaborated for the faecal microbiome profiling. The participants were instructed to immediately freeze the collected stool samples at -20°C and without further delay bring the frozen samples to the study centres to be stored at −80°C without any additive, as previously described.(321) During a study visit, a blood sample was also taken, clotted and centrifuged to store several serum aliquots at -80°C according to SCREEN-RA usual standard operating procedures.(96)

Other samples

Additional samples are taken as part of the bigger study. In a subset of the cohort, gingival crevicular fluid is collected at one site in each dentition quadrant using membrane strips. The salivary microbiome is sampled by collecting unstimulated saliva in a sterile plastic tube. Finally, the subgingival microbiome is sampled using sterile paper points inserted into the bottom of the gingival pockets, at four different oral sites. They were not analyzed in my thesis but will be used in future complementary projects.

Exposure of interest (case and control definition)

To assign the participants to one of the four following groups we used (a) the results of serum auto-antibodies assessment, (b) the physical examinations and (c) the follow-up questionnaire:

1) Control group, i.e., healthy asymptomatic RA-FDRs, without clinically significant autoantibody titers (ACPA < the upper limit of the norm (ULN), RF < 3x the ULN, anti-Ra33 < 3x the ULN);

2) High genetic risk group, i.e., healthy asymptomatic RA-FDRs with two copies of the shared epitope (SE), which doubles the risk of RA compared to having one single copy.(322)

3) Autoimmunity group, i.e., RA-FDRs without articular symptoms, but with clinically significant autoimmunity (ACPA titers above the ULN, or RF or anti-Ra33 at least 3x the ULN), which strongly increases the risk of developing RA among FDRs.(83,323,324)

4) Symptomatic group, i.e., RA-FDRs a with clinically suspect arthralgia (CSA) score equal to or greater than 4 based on the EULAR questionnaire. When one of the CSA items was missing or in case of concomitant autoimmunity, a CSA score greater than 3 was used to define clinically suspect symptoms for RA.(93) Due to the fact that the number of incident RA cases were insufficient to constitute an independent group, the RA-FDRs who developed incident RA and a small number of untreated new onset RA recruited as positive controls, were also included in this group. We only recruited untreated patients given the possible effect of DMARD on microbiome.(325)

Clinically suspect arthralgia is defined as follows:

Table 2: EULAR defined characteristics describing arthralgia at risk for RA

History taking:	
Joint symptoms of recent onset (duration <1 year)	1
Symptoms located in MCP joints	1
Duration of morning stiffness ≥60 min	1
Most severe symptoms present in the early morning	1
Presence of a first-degree relative with RA	1
Physical examination:	
Difficulty with making a fist	1
Positive squeeze test of MCP joints	1
MCP = Metacarpophalangeal. RA = rheumatoid arthritis. Maximum score = 7. In SCREEN-RA, the history-taking items are administered using the online follow-up questionnaire.	

There are a few subtilities:

- Stool and serum samples were not always collected exactly on the same day and some follow-up questionnaires might also have been completed a few days later. In such cases, the classification is defined at (1) the time of serum sampling for serum-derived variables with exception of anti-Ra33 titers that are derived from a previous sample, and (2) in case of stool sampling, in the 30 days before and after the date of the stool sampling for variables included in the CSA score, and the maximum score was retained).
- As the data that we could obtain from previous tests were not sufficient for the assignment of the participants to one of the four groups, our strategy was to invite as many participants as possible during the sampling campaign and to make stool sampling and the visit for a physical examination as prerequisites for the participation in the study.
- To be able to take advantage of the collected samples we assigned each participant to a particular pre-clinical stage, even in case of mild-phenotype or partially missing information. Our preference was to place a participant in a highest possible risk group when the risk factor was known with enough certainty, in other words, we gave a priority to specificity over sensitivity.

The **Figure 9** below shows the group assignment algorithm.

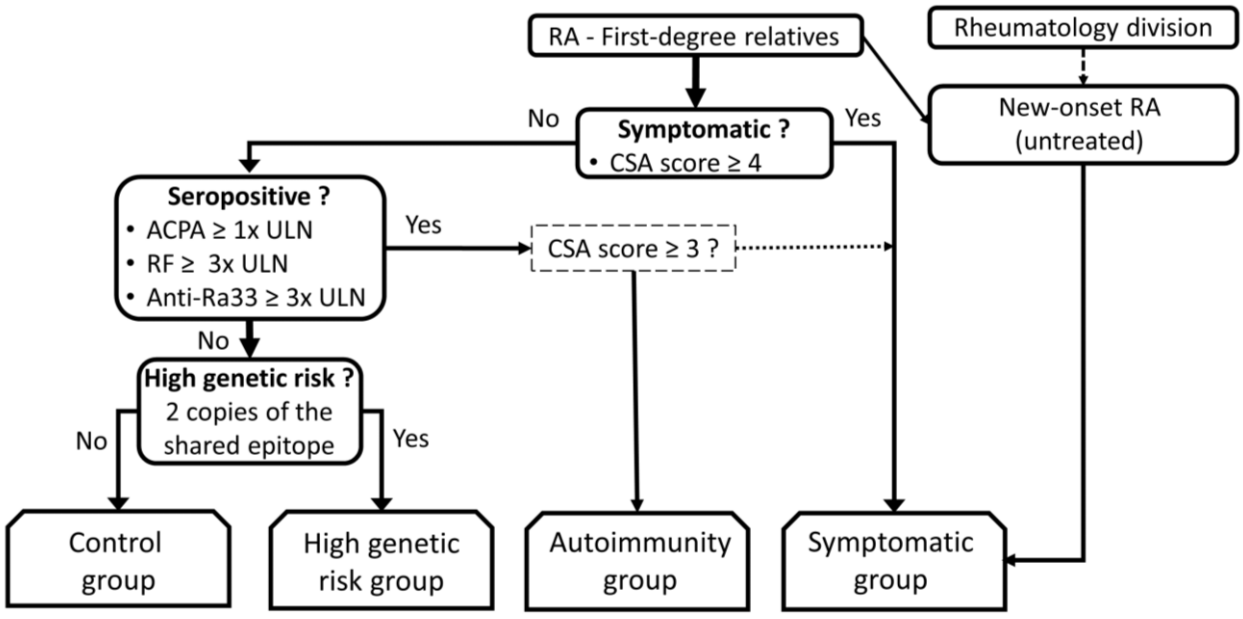


Figure 9: Screen-RA group assignment algorithm. RA = Rheumatoid Arthritis. CSA = Clinically Suspect Arthralgia, defined using EULAR score. ACPA = Anti Citrullinated Peptide Antibodies. RF = Rheumatoid Factor. ULN = Upper Limit of the Norm. New-onset RA recruited from the rheumatology division are not necessarily RA-First-degree relatives.

Figure 10 below shows the group repartition of the SCREEN-RA participants as of June 2023. The proportions of the different subpopulations are similar to those of our 2019-2021 sampling campaign.

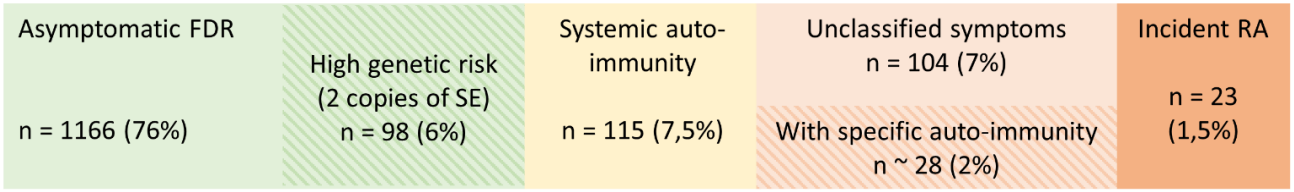


Figure 10 : Repartition of SCREEN-RA participants by preclinical stage. FDR = First Degree Relative of RA patient. SE = Shared Epitope. RA = Rheumatoid Arthritis.

WORK-PACKAGE 1 – STOOL PROFILING

Rationale

There are several coinvestigators within the SCREEN-RA consortium who are currently involved in the national and international research exploring the role of the oral microbiota and periodontitis in the development of RA.(326–330) For this reason, our project is focused only on the intestinal microbiome: still and all, as we work in close collaboration within the SCREEN-RA consortium, an integration of the analysis of both research areas will be possible in the future.

To assess the intestinal microbiome we sampled and analyzed fecal material. The only reason for sampling fecal material is that it is easily accessible, and has been previously used by other researchers. Nonetheless, in our discussion we question if feces microbiota really reflects the intra-intestinal ecosystem.

The previous studies, discussed in the section "GUT MUCOSA IN RHEUMATOID ARTHRITIS" page 32, did not come to a clear conclusion if the "expanded relative abundance" of *Prevotella copri* in the fecal flora of preclinical-RA or new-onset RA patients implied an absolute increase of *Prevotella* bacterial cell-counts per gram of stool; or if it rather resulted from a relative decrease in other taxa. Maeda et al. have used qPCR to try to assess this and showed increased *P. copri* loads in their RA subjects,(275) but this needs to be confirmed.

Furthermore, other confounding factors such as stool transit, age, sex, or nutritional habits, which may differ in individuals developing an autoimmune disease, could be taken into account. In order to strengthen the evidence for a causative role of the gut microbiota and *Prevotella* species in RA development, we thus decided to replicate a microbiota profiling on a larger and stratified sample of patients; the creation of the SCREEN-RA cohort would also ensure access to a significant amount of metadata and allow a long-term follow-up of sampled participants.

We built a collaborative relationship with Pr. RAES from the laboratory of molecular bacteriology of the REGA institute, KU Leuven, to use an improved methodology taking into account the bacterial loads of each analyzed sample.(321)

We hypothesized that more individuals would have increased abundance of *P. copri* or other Prevotellaceae when considering high-risk pre-clinical phases of RA, taking (if possible) potential confounding factors into account. Also, we considered that increased counts of RA-associated bacteria could be linked to a subclinical intestinal inflammation. We, therefore, also aimed at measuring fecal calprotectin.

Calprotectin is a zinc and calcium binding protein expressed by activated macrophages, granulocytes and monocytes. It has a bactericidal effect and promotes inflammatory response.(331) Since calprotectin is translocated into the extracellular fluids, or the intestinal lumen, by activated neutrophils, it is known as a fecal biomarker for intestinal inflammation, particularly in the context of inflammatory bowel diseases.(332) It is also reliable for detecting mild mucosal inflammation in other contexts and was, for instance, elevated in antiphospholipid patients, whose immune system is cross-reacting with bacterial intestinal epitopes.(166) Fecal calprotectin has also been studied in rheumatic diseases, notably ankylosing spondylitis, where it appears to be elevated.(333–335) However, it is uncertain whether this results from NSAID induced enteropathy or associated mucosal inflammation.(336)

Contribution statement

Benoît GILBERT was involved in the study design, online SCREEN-RA interface design (Redcap), data-management of SCREEN-RA cohort (together with Denis MONGIN) and selection of eligible participants. He personally contacted a few dozens of participants and assessed them at study visits, including clinical examination, serum sampling and fecal sample storage. Benoît GILBERT also performed the data analysis in R language, with help and supervision from Raul TADEO, which also involved sequencing data processing, statistical work and developing custom plotting functions.

Olivia STUDER, together with Eric TRUNK and study nurses of collaborative centers, contacted most of the participants and organized the study visits and biological samplings.

The RAES lab (Leuven, Belgium) took care of stool-sample processing (16S-sequencing, fecal calprotectin assessment, fecal cell counts and moisture measurements).

Céline LAMACCHIA was involved in the study design, manuscript writing, oversaw serum sample storage and SCREEN-RA biobank management, and collaboration with HUG central lab for ACPA, anti-Ra33 and RF serologies as part of routine SCREEN-RA workflow.

Axel FINCKH (principal investigator), Jeroen RAES, and Delphine COURVOISIER, contributed to the study design and funding, supervision, and manuscript preparation.

Methods

Population

Apart from the main grouping, we also selected as a secondary exposure of interest 20 most pronounced phenotypes from each group, matching the control individuals for sex and age. This subgrouping was pre-planned and used for a parallel project on anti-glycan Ig (not published yet).

Operationally, this selection was made by ranking participants based on grouping criteria, more specifically:

- In the symptomatic group, by decreasing order of having RA, ACPA, RF, anti-Ra33 and highest CSA score.
- In the autoimmunity group, by decreasing order of ACPA, RF, anti-Ra33.
- In the high genetic risk group, by being negative for ACPA, RF and anti-Ra33, and having a CSA score of 1 (minimum in this cohort).
- For the control group, by being negative for ACPA, RF, anti-Ra33, and having a CSA score of 1.

Match() function from R *Matching* package was used to further select the control individuals (in excess), matching to the symptomatic subgroup on sex and age.

Stool and serum sampling

Between September 2019 and October 2021, SCREEN-RA participants have been invited to provide a stool sample paired with a serum sample. All the participants were provided with stool collection-devices that allow creation of several aliquots and proceeded to stool sampling at home. They temporarily froze the fresh stool sample at −20°C, and rapidly brought it to the study centres to be stored at −80°C without any additive, as previously described.(321) During the study visit, a blood sample was also taken, clotted and centrifuged to store several serum aliquots at -80°C according to SCREEN-RA standard operating procedures.(96)

Microbiota profiling

Sample processing

“16S” amplification methodology selectively amplifies (by PCR reactions) a region of the gene coding for the 16S subunit of bacterial ribosomal RNA. Because of the amplification and sequencing process a precise track of the initial non-amplified DNA content cannot be realized. Therefore, the end result reflects the proportion of each bacterial taxa in the sample but does not provide information about the “absolute” number of bacterial cells per gram of original stool sample, which is why we additionally performed fecal cell-counts by cytometry.

DNA was extracted from a thawed stool aliquot using Qiagen MagAttract PowerMicrobiome DNA/RNA bead-beating on a robotized platform. DNA samples were then randomized on 96 wells plates; and, for bacterial and archaeal characterization, the extracted DNA (dilution 1:10) was further amplified in triplicate using 16S rRNA primers 515F (5’- GTGYCAGCMGCCGCGGTAA-3’) and 806R (5’-GGACTACNVGGGTWTCTAAT-3’) targeting the V4 region and modified to contain a barcode sequence

between each primer and the Illumina adaptor sequences to produce dual-barcoded libraries. The sequencing was performed on a MiSeq platform (2x250 paired end reads, Illumina).

Microbial loads of stool samples were measured as described previously by diluting a stool aliquot in physiological solution, filtering and staining the DNA with SYBR Green and acquiring cell-counts on a C6 Accuri flow cytometer.(337) Moisture content was determined as the percentage of mass loss after lyophilization from 0.2 g frozen aliquots of non-homogenized fecal material (−80 °C) as previously described.(337)

Data analysis

Fastq files obtained from the MiSeq platform were filtered and trimmed using the DADA2 pipeline (v1.16.0) in R (v4.0.3).(338,339) Reads were truncated after 230 (forward) and 150 (reverse) nucleotides. Denoising, merging and chimera removal were performed with default parameters. This generated a set of Amplicon Sequence Variants (ASV), which were subsequently matched to the Silva 16S database (138v) using the DADA2 built-in assigner.(340)

The output of the DADA2 pipeline was visualized in R with packages *phyloseq* (v1.32.0) and *ggplot2* (v3.4.2).(338,341,342) Sample richness was assessed using Shannon Index. For Principal Coordinate Analysis (PCoA), ASV counts were corrected using the fecal cell-count data, and sample ordination was done using a Bray-Curtis dissimilarity matrix (at the ASV level) before PCoA plotting. PERMOVA was performed on the Bray-Curtis dissimilarity matrix using function *adonis2()* from R package *vegan*.

The quantitative microbiome profiling (QMP) matrix was built as described previously.(321) In brief, samples were downsized to even sampling depth, defined as the ratio between sampling size (16S rRNA gene copy number-corrected sequencing depth) and microbial load with the average total cell count per gram of frozen fecal material. Given that certain bacteria can have several copies of the 16S rRNA gene, the genome copies numbers were imputed using RasperGade16S,(343) a new tool that utilizes a heterogeneous pulsed evolution model for predicting 16S rRNA genome copies and that also provides confidence estimates for the predictions. A minimum rarefied read counts of <150 was used for QMP analyses. Rarefied ASV counts were thus converted into numbers of cells per gram.

For enterotyping, the observed genus richness was calculated on the genus matrix downsized to 10,000 reads using *phyloseq*,(341) as already reported in previous studies. (337) Enterotyping (or community typing) based on the Dirichlet-multinomial mixtures approach was performed in R as described previously.(337,344,345) This approach used a combined genus-level abundance RMP matrix including SCREEN-RA samples compiled with 1045 samples originating from the Flemish Gut Flora Project.(346) The optimal number of Dirichlet components based on the Bayesian information criterion was four.

The four clusters were named *Bacteroides1* (Bact1), *Bacteroides2* (Bact2), *Prevotella* (Prev) and *Ruminococcaceae* (Rum) as described previously.(321).

Microbial community composition and differential analysis were conducted using non-parametric tests, such as Wilcoxon rank sum and Kruskal-Wallis. To assess other taxa-specific differences between the groups, low abundance ASV were removed, i.e., ASV not present at least 10 times in 5% of the samples. Then ASV in this filtered dataset were aggregated at the relevant taxonomical level (Family or Genus level), and sequence counts were compared between groups using R package *Aldex2* accounting for multiple testing and data compositionality (*Aldex2* performs a centered-log-ratio transformation on the count data and applies Benjamini-Hochberg correction on p-values). Other p-values were also corrected for multiple testing using the Benjamini–Hochberg method, reported as *p-adj*, when multiple tests were performed on the lists of variables.

Fecal calprotectin

Fecal calprotectin concentrations were determined using the fCAL ELISA Kit (Bühlmann) on the frozen fecal material as described previously.(337)

Since the fecal calprotectin values did not follow a normal distribution, they were compared between the groups using Wilcoxon signed rank tests, comparing each group with the control group as reference and applying Benjamini–Hochberg correction in the subgroup comparison.

Results

Population description

We retrieved 387 stool samples, of which 371 were included in this study (**Figure 11**).

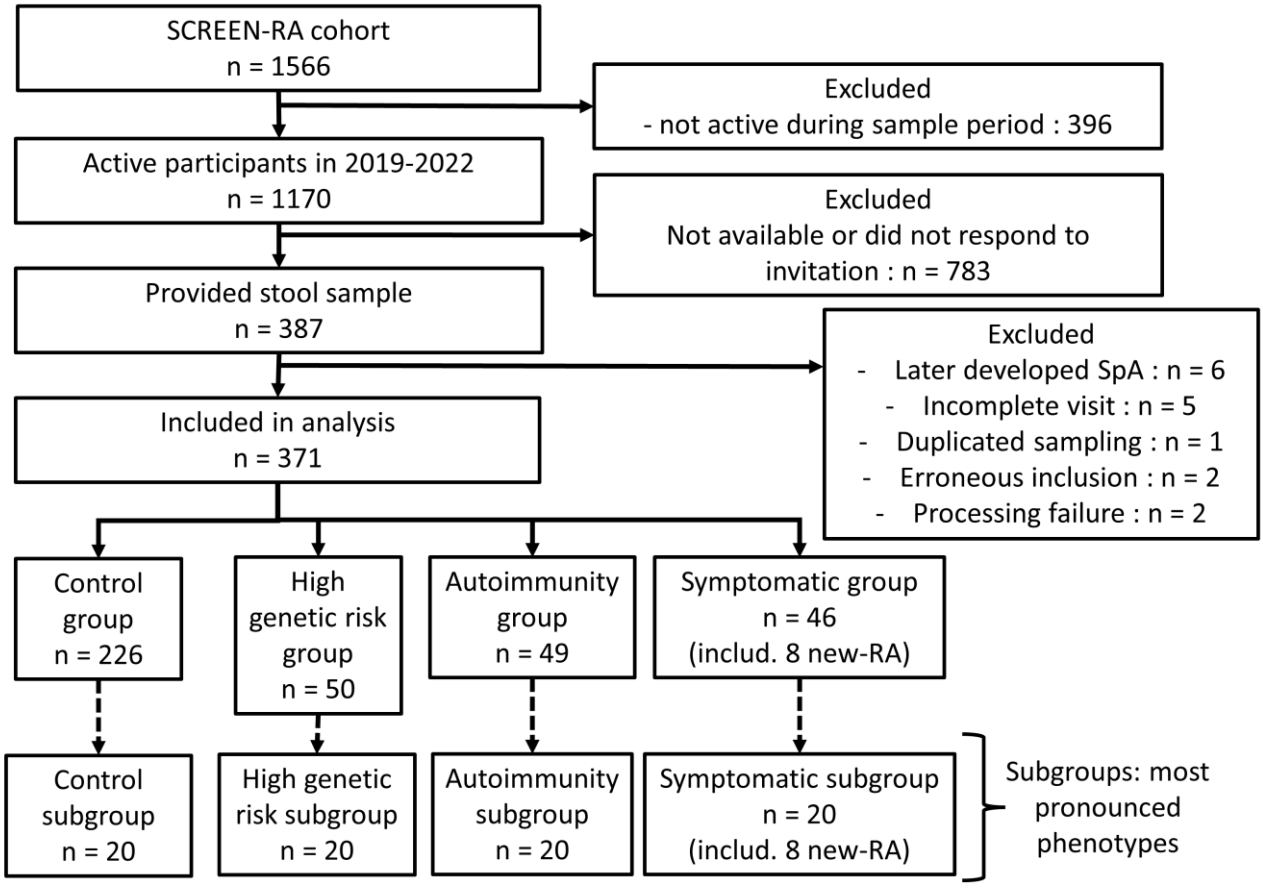


Figure 11: Study recruitment flow-chart. SpA = Spondyloarthitis. RA = Rheumatoid Arthritis. New-RA = includes both RA-FDRs from SCREEN-RA who developed a new RA at the time of sampling, and new-onset RA from Geneva Rheumatology Division, who are not necessarily RA-FDRs.

Baseline characteristics per group are presented in **Table 3**, and the most pronounced phenotype subgroups are shown in **Table 4**.

Table 3: Baseline characteristics of study population, SCREEN-RA

Variable	Control n = 226		High Genetic risk n = 50		Autoimmunity n = 49		Symptomatic n = 46		p value
	n % of total in group Otherwise: Mean (SD)								
		Miss.		Miss.		Miss.		Miss.	
Female	78 %		82 %		73 %		87 %		0.377
Age	52 (14)		53 (12)		55 (16)		54 (12)		0.523
BMI	24 (4)		25 (3)		25 (4)		25 (5)		0.7
Share epitope copies									
0	53 %		0 %		47 %		50 %		**<0.001**
1	47 %		0 %		39 %		39 %	2 %	
2	0 %		100 %		14 %		9 %		
RA autoimmunity	0 %		0 %		100 %		26 %		**<0.001**
ACPA									
Negative	100 %		100 %		67 %		83 %		**<0.001**
Low	0 %		0 %		14 %		4 %		
High	0 %		0 %		18 %		13 %		
RF									
Negative	90 %		94 %		27 %		65 %		**<0.001**
Low	10 %		6 %		2 %		17 %		
High	0 %		0 %		71 %		17 %		
Anti-Ra33									
Negative	41 %		72 %		65 %		54 %		<0.150
Low	9 %	50 %	20 %	8 %	20 %	10 %	13 %	33 %	
High	0 %		0 %		4 %		0 %		
Clinically Suspect Arthralgia (CSA)									
No	96 %	4 %	100 %		100 %		9 %	2 %	**<0.001**
Yes	0 %		0 %		0 %		89 %		
CSA score (detail)									
1	64 %		80 %		82 %		4 %		
2	23 %		16 %		18 %		4 %		
3	10 %	4 %	4 %		0 %		9 %	2 %	**<0.001**
4	0 %		0 %		0 %		61 %		
5	0 %		0 %		0 %		11 %		
6	0 %		0 %		0 %		9 %		
Antibiotics (past 2 months)	6 %		2 %		6 %		6 %		0.710
Probiotics (past month)	10 %		8 %		8 %		9 %		0.965
Surgery (past 2 months)	2 %		6 %		6 %		6 %		0.162
Travel outside Europe (past month)	2 %		2 %		2 %		0 %		0.827

SD = standard Deviation. BMI = Body Mass Index. RA = Rheumatoid Arthritis. ACPA = Anti-citrullinated Peptide Antibodies. RF = Rheumatoid Factors. CSA = Clinically Suspect Arthralgia. Miss. = Missing data.
It should be noted that 4 patients with new-onset RA, included in the symptomatic group due to their diagnosis, however, did not meet the threshold for CSA because of either missing data in the questionnaires or did not present obvious symptoms at the study visit (symptoms can fluctuate and regress between flares).

Table 4: Baseline characteristics of most pronounced subgroups, SCREEN-RA

Variable	Control n = 20		High Genetic risk n = 20		Autoimmunity n = 20		Symptomatic n = 20		p value
	n % of total in group Otherwise: Mean (SD)								
		Miss.		Miss.		Miss.		Miss.	
Female	95 %		90 %		85 %		95 %		0.632
Age	53 (14)		58 (10)		61 (13)		54 (15)		0.149
BMI	25 (4)		27 (3)		26 (5)		24 (4)		0.260
Share epitope copies									
0	50 %		0 %		45 %		50 %	5 %	**<0.001**
1	50 %		0 %		40 %		40 %		
2	0 %		100 %		15 %		5 %		
RA autoimmunity	0 %		0 %		100 %		60 %		**<0.001**
ACPA									
Negative	100 %		100 %		20 %		60 %		**<0.001**
Low	0 %		0 %		35 %		10 %		
High	0 %		0 %		45 %		30 %		
RF									
Negative	100 %		100 %		60 %		25 %		**<0.001**
Low	0 %		0 %		5 %		35 %		
High	0 %		0 %		35 %		40 %		
Anti-Ra33									
Negative	50 %	50 %	95 %	5 %	45 %	25 %	45 %	45 %	**0.036**
Low	0 %		0 %		25 %		10 %		
High	0 %		0 %		5 %		0 %		
Clinically Suspect Arthralgia (CSA)									
No	100 %		100 %		100 %		20 %	5 %	**<0.001**
Yes	0 %		0 %		0 %		75 %		
CSA score (detail)									
1	100 %		100 %		80 %		10 %		
2	0 %		0 %		20 %		10 %		
3	0 %		0 %		0 %		5 %	5 %	**<0.001**
4	0 %		0 %		0 %		55 %		
5	0 %		0 %		0 %		10 %		
6	0 %		0 %		0 %		5 %		
Antibiotics (past 2 months)	5 %		5 %		10 %		5 %		0.901
Probiotics (past month)	5 %		15 %		5 %		10 %		0.591
Surgery (past 2 months)	0 %		10 %		5 %		5 %		0.528
Travel outside Europe (past month)	5 %		0 %		5 %		0 %		0.583

SD = standard Deviation. BMI = Body Mass Index. RA = Rheumatoid Arthritis. ACPA = Anti-citrullinated Peptide Antibodies. RF = Rheumatoid Factors. CSA = Clinically Suspect Arthralgia. Miss. = Missing data.
It should be noted that 4 patients with new-onset RA, included in the symptomatic group due to their diagnosis, however, did not meet the threshold for CSA because of either missing data in the questionnaires or did not present obvious symptoms at the study visit (symptoms can fluctuate and regress between flares).

Microbiota

Shannon index, which reflects the number of different bacterial taxa identified in each stool sample (alpha-diversity), did not differ between the groups (ANOVA p = 0.39). To overview the data each fecal microbiome can be assigned to an enterotype based on the dominant bacterial taxa.(347) Assigning samples in their respective enterotypes did not reveal significant differences between the groups (Fisher's exact test p = 0.64; **Figure 12**A).

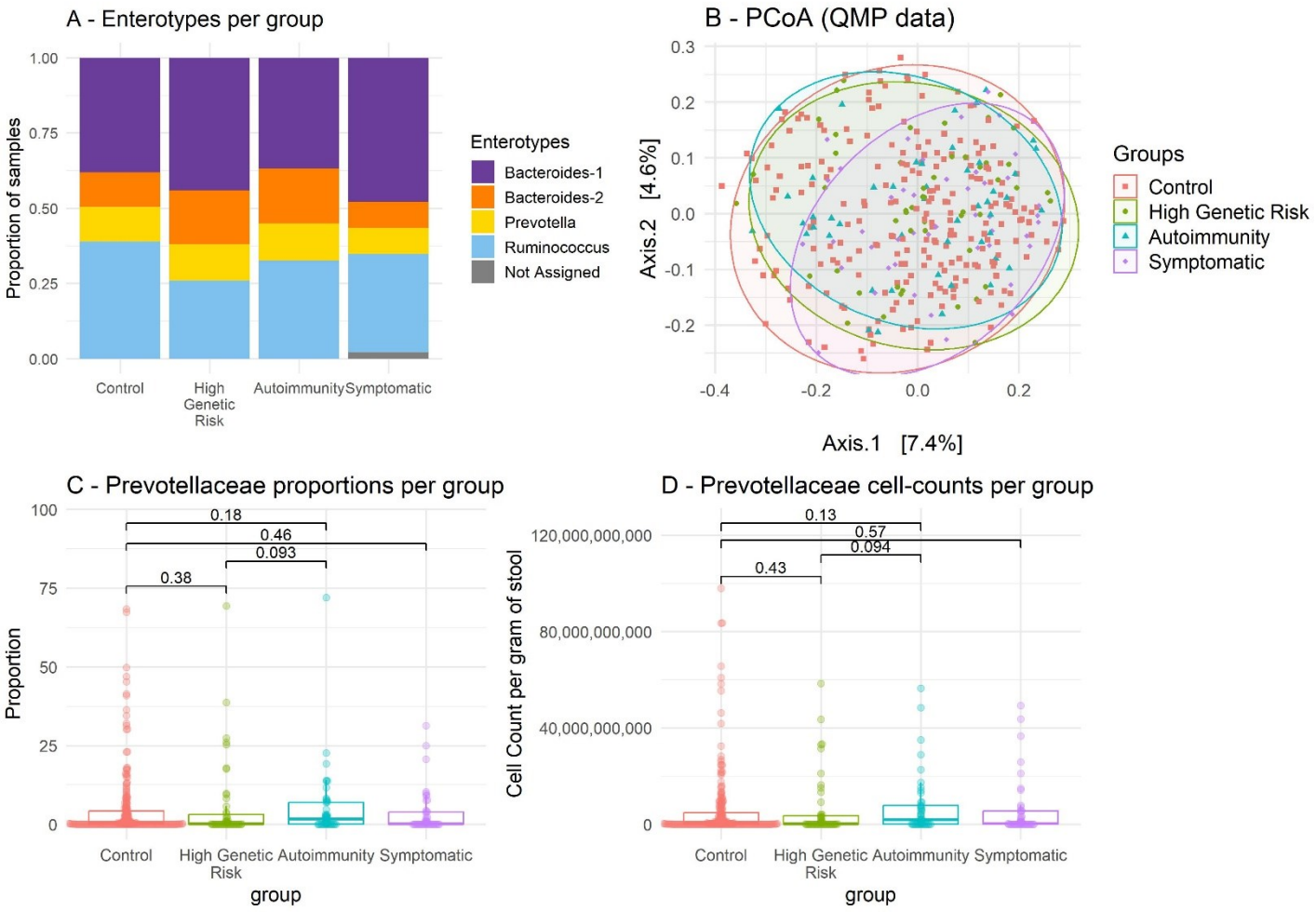


Figure 12: Gut microbiota profiling by group. A – Enterotype distribution by group; Fisher p = 0.64. Sample n°411 was not assigned due to low read-count. **B** - Principal Coordinate Analysis performed at the sequence variant level after QMP correction; distance between points reflects their dissimilarity, based on Bray-Curtis's index. PERMANOVA R2 = 0.00798; p = 0.56. **C** – Proportions of Prevotellacae bacteria per group, boxplots; p-value are Wilcoxon tests. **D** – Prevotellacae estimated cell-counts per group, boxplots; p-value are Wilcoxon tests. RMP = Relative Microbiota Profiling (provides proportions). QMP = Quantitative Microbiota Profiling (provides estimated cell counts).

To assess sample composition at the most granular level, it is possible to compare samples to each other using Bray-Curtis distance.(348) This index, ranging from 0 to 1, reflects the ecological difference between two samples in terms of counts of detected taxa; in our case, we use as input the QMP counts per gram of stool. Comparing sample composition to each other using Bray-Curtis index subsequently allows performing a Principal Coordinate Analysis (PCoA). On a PCoA plot the distance between two points increases when their compositional difference increases, as assessed by Bray-Curtis index. We found no group-wise clustering doing a PCoA on the QMP data at the 16S sequence variant level (PERMANOVA, R2 = 0.00798, p = 0.56; **Figure 12**B). Using the RMP data (bacterial proportions) yielded the same results (PERMANOVA, R2 = 0.0073, p = 0.83). Overall, stool profiling was similar between groups in both cases - when assessed as estimated cell counts or as percentages (see Family level, **Figure 15**, page 61).

More specifically, contrary to our previous report,(349) we found no group-differences in Prevotellaceae abundance (**Figure 16**, page 62, **Figure 12**C and D ; Kruskal-Wallis p = 0.29). Results were similar using either the RMP or QMP data (**Figure 16**, page 62).

To explore differential abundance of other bacterial taxa we used *Aledx2* tool. It performs centered log-ratio transformations on crude 16S-count data and applies Benjamini-Hochberg correction on Kruskal-Wallis p-values to account for multiple testing. *Aldex2* found no significant differences between groups regarding other bacterial families or genera present in the dataset (**Figure 18**, page 64). Also, contradicting the previous findings,(350) we found no association between shared epitope presence and Prevotellaceae, or *Prevotella* genera when grouping on shared-epitope genotype, (see in "LIMITATIONS", page 122).

Microbiota in most pronounced phenotype subgroups

In the sensitivity analysis, by selecting the 20 most pronounced phenotypes in each group we modestly reproduced published results regarding increased Prevotellaceae abundance in autoimmunity and symptomatic groups (**Figure 17** page 63). QMP revealed higher cell counts (**Figure 13**D), which reflects the higher proportions (**Figure 13**C). Even though we visually noticed an increase in the number of Prevotella-enterotypes, it did not reach significance (**Figure 13**A, Fisher's p = 0.55), neither using QMP cell counts on the PCoA (**Figure 13**B, PERMANOVA p = 0.14); which are more conservative analyses taking the other taxa into account.

As an alternative to Benjamini–Hochberg method, we re-assessed p-value of these subgroup findings by performing a permutation test (10'000 repetitions). Only 5.548 % of the permutation samples had a median difference of quantitative abundance (QMP) much greater than observed in the pronounced

phenotype subgroups (if comparing control versus autoimmunity), corresponding to a one-sided p-value of 0.054 (0.038 if using RMP data) (**Figure 20**, page 66).

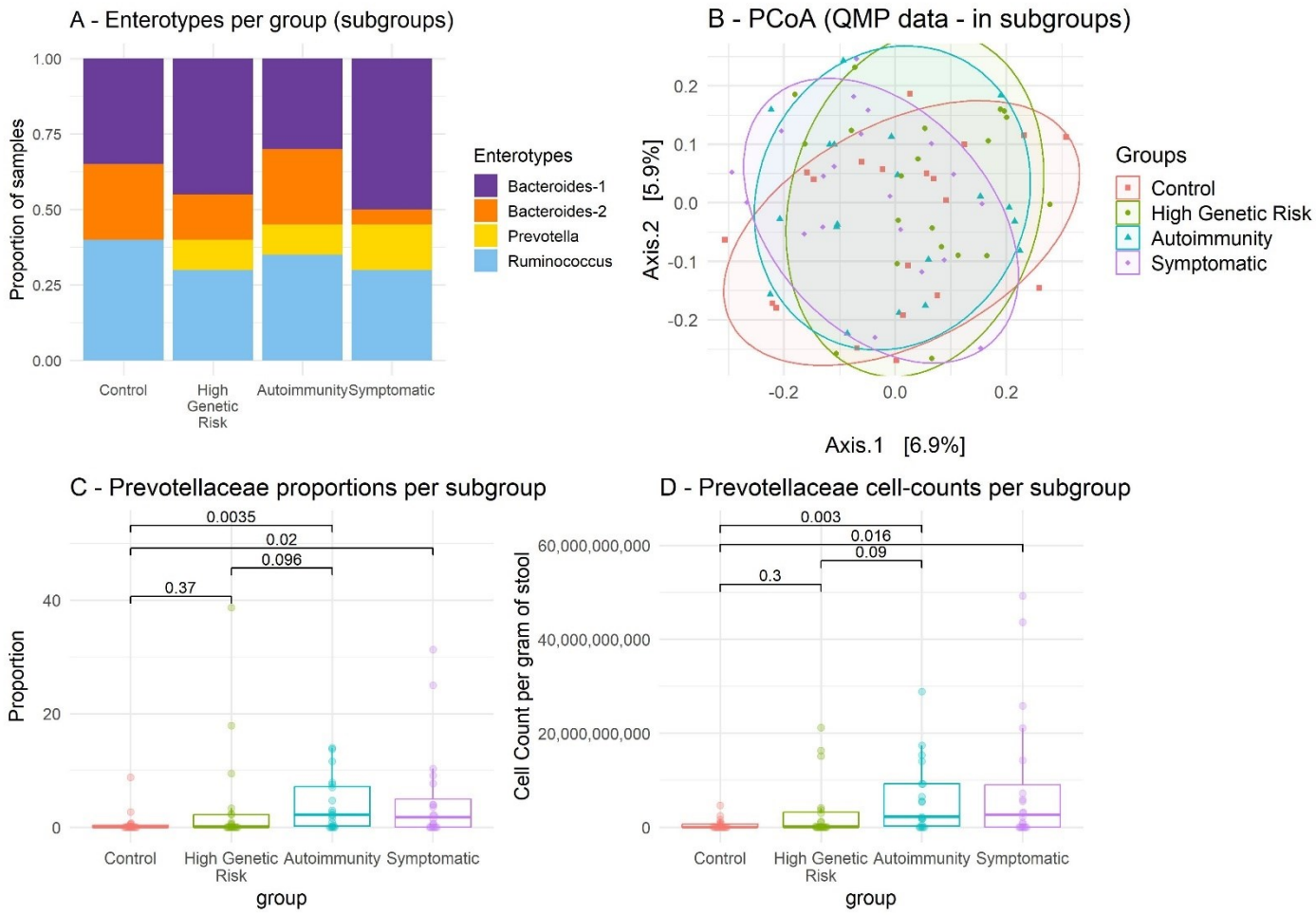


Figure 13: Gut microbiota profiling by subgroup. A – Enterotype distribution by subgroup; Fisher's p = 0.5473. **B** - Principal Coordinate Analysis performed at the sequence variant level with QMP data; distance between points reflects their dissimilarity, based on Bray-Curtis's index. PERMANOVA R2 = 0.042, p = 0.14. **C** – Proportions of Prevotellacae bacteria per group, boxplot; p-value are Wilcoxon tests (unadjusted). Adjusted p-values (accounting for panels C and D) are, from bottom to top: 0.37, 0.13, 0.04, 0.014. **D** – Prevotellacae estimated cell-counts per group, boxplots; p-value are Wilcoxon tests (unadjusted). Adjusted p-values (accounting for panels C and D together) are, from bottom to top: 0.34, 0.13, 0.04, 0.014. RMP = Relative Microbiota Profiling (provides proportions). QMP = Quantitative Microbiota Profiling (provides estimated cell counts).

Fecal calprotectin

We found no overall difference in fecal calprotectin, a biomarker of mucosal inflammation, between groups (Kruskal-Wallis p = 0.3; **Figure 14**A). When restricting the analysis to the most pronounced subgroups, a trend was noticeable with a modest increase in the autoimmunity group compared to the control group, which disappeared after correction for multiple testing (p = 0.076; p-adj = 0.23; **Figure 14**B). Also, *Prevotella* genera were not among the bacteria associated with mildly elevated (>100 ug/g) calprotectin in this dataset as assessed using *Aldex2*, associated microbes were *Streptococcus* and an unclassified *Clostridia UCG-014* (data not shown).

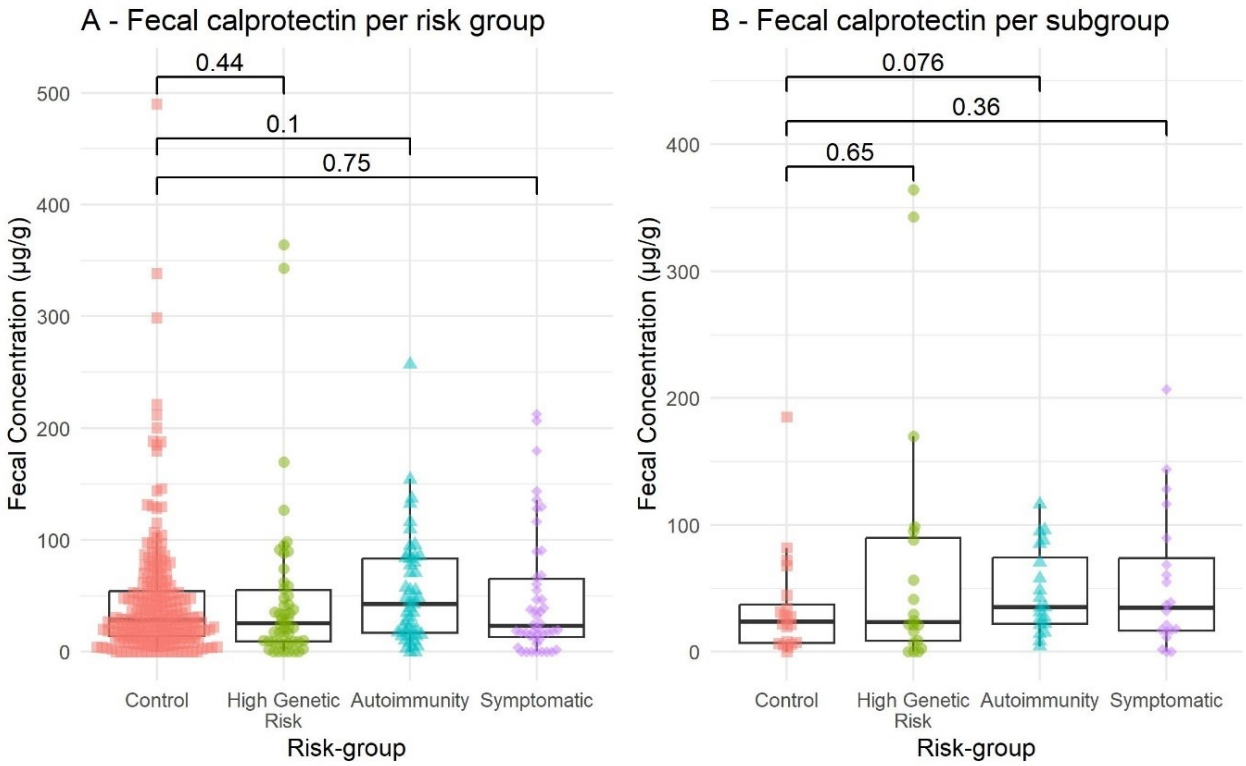


Figure 14: Fecal calprotectin by risk group. Measured with ELISA in fresh frozen stool. **A** – Fecal calprotectin in all the included stool samples. P-values are Wilcoxon tests. **B** – Fecal calprotectin only in the most pronounced phenotypes subgroups. Displayed p-values are Wilcoxon test, non-adjusted. P-adj values for subgroups are respectively (from bottom to top): 0.65, 0.55, 0.23 .

Extended figures

Few additional figures are noteworthy to be mentioned and are commented below.

Figure 15 displays the composition of each stool sample as a colored vertical bar representing different bacterial Families present. Only the most frequent are shown. It visually stresses the high complexity of fecal samples and the overall similarity in this regard within and between each of our study groups.

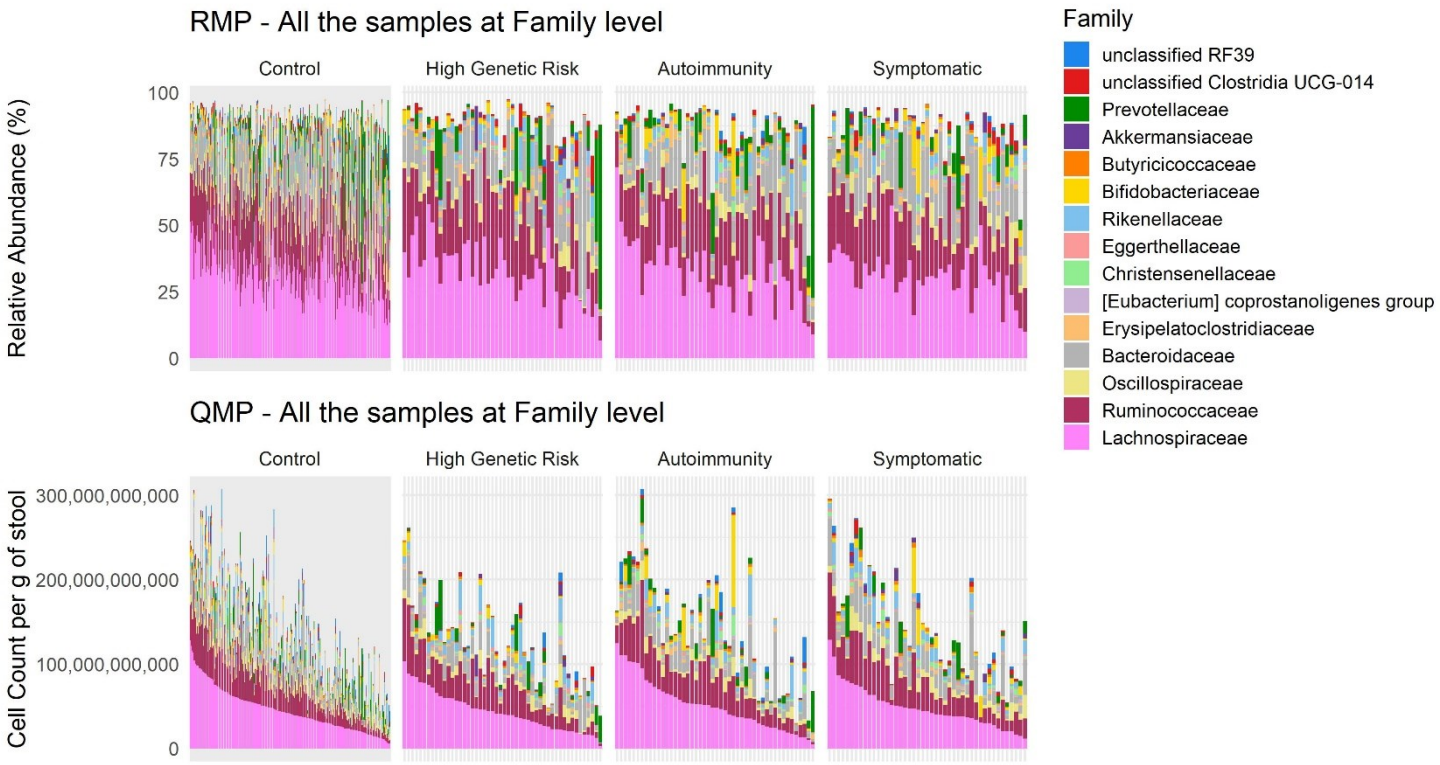


Figure 15: Microbiota profiling at the family taxonomic level, by group. As assessed by 16S sequencing of frozen fecal sample, together with flow-cytometry bacterial cell count. RMP = Relative Microbiota Profiling (provides proportions). QMP = Quantitative Microbiota Profiling (provides estimated cell counts). Each vertical bar is a sample, colored for the most frequent bacterial families found in the data set. Samples are ordered based on the most prevalent family, using the QMP profiling as reference. Each RMP sample sits above its corresponding QMP profile. No significant differences were found between groups (Aldex2, testing at Family level).

Figure 16 is constructed similarly to the previous figure but display only the Prevotellaceae family coloured by the genera that constitute this family. This illustrates that in our dataset, the proportion of Prevotellaceae is generally less than 20%, and about half of the participants do not even have detectable Prevotellaceae (little grey crosses replacing absent bar chart).

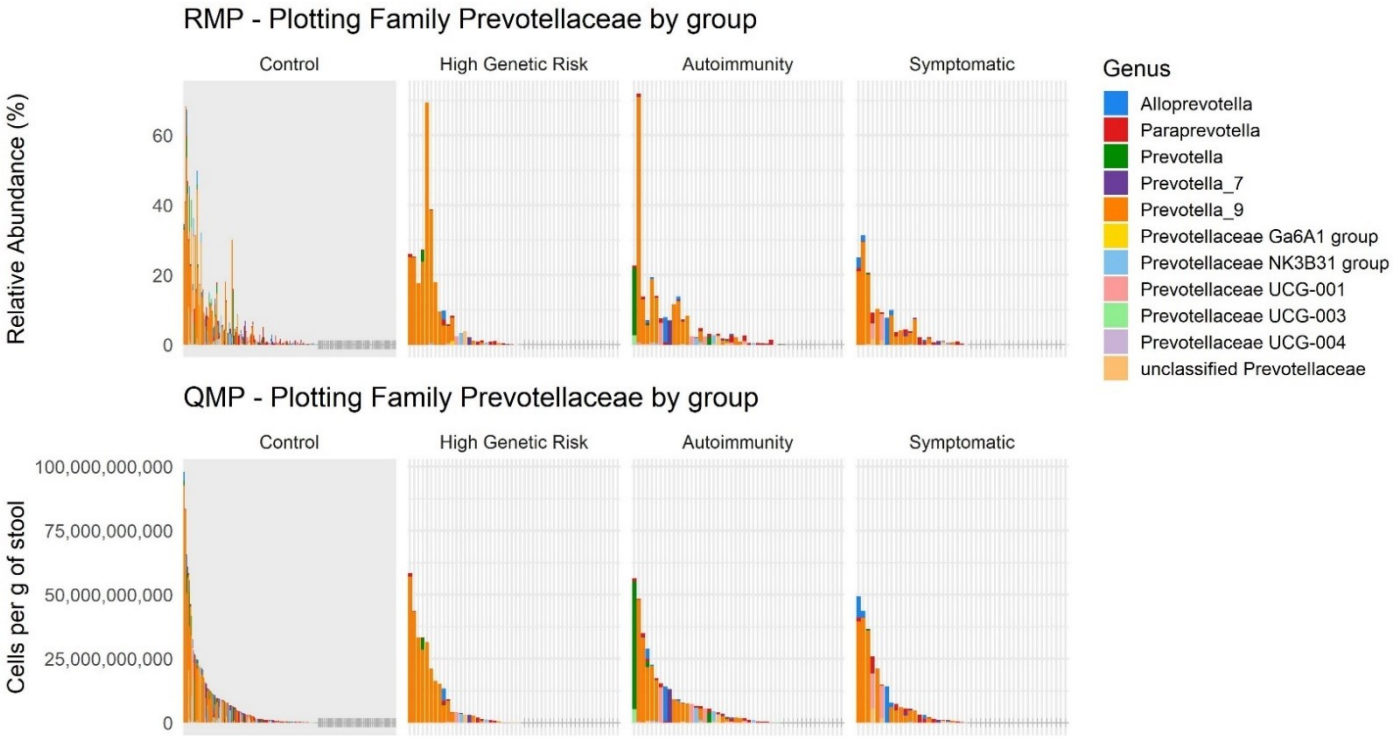


Figure 16: Prevotellaceae abundance by sample, by group. As assessed by 16S sequencing of frozen fecal sample, together with flow-cytometry bacterial cell count. **RMP** = Relative Microbiota Profiling (provides proportions). **QMP** = Quantitative Microbiota Profiling (provides estimated cell counts). Each bar represents one sample, colored by the different genera identified in the Prevotellaceae family. QMP profile sits below its corresponding RMP profile. "Prevotella_9" is the annotation for what is mostly *P. copri*. Overall Kruskal-Wallis p = 0.2879 (on relative abundances).

Figure 17 is further limited to show only the pronounced phenotype individuals. The gradual increase in Prevotellaceae cell counts appears clearly on the bottom line. However, given the small effective, it is hard to say if we were just "lucky" selecting the "right" persons by chance. The permutation test mentioned above assessed this issue; it was quite improbable (max ~4-5%) to obtain such figure only by chance using our dataset (**Figure 20**).

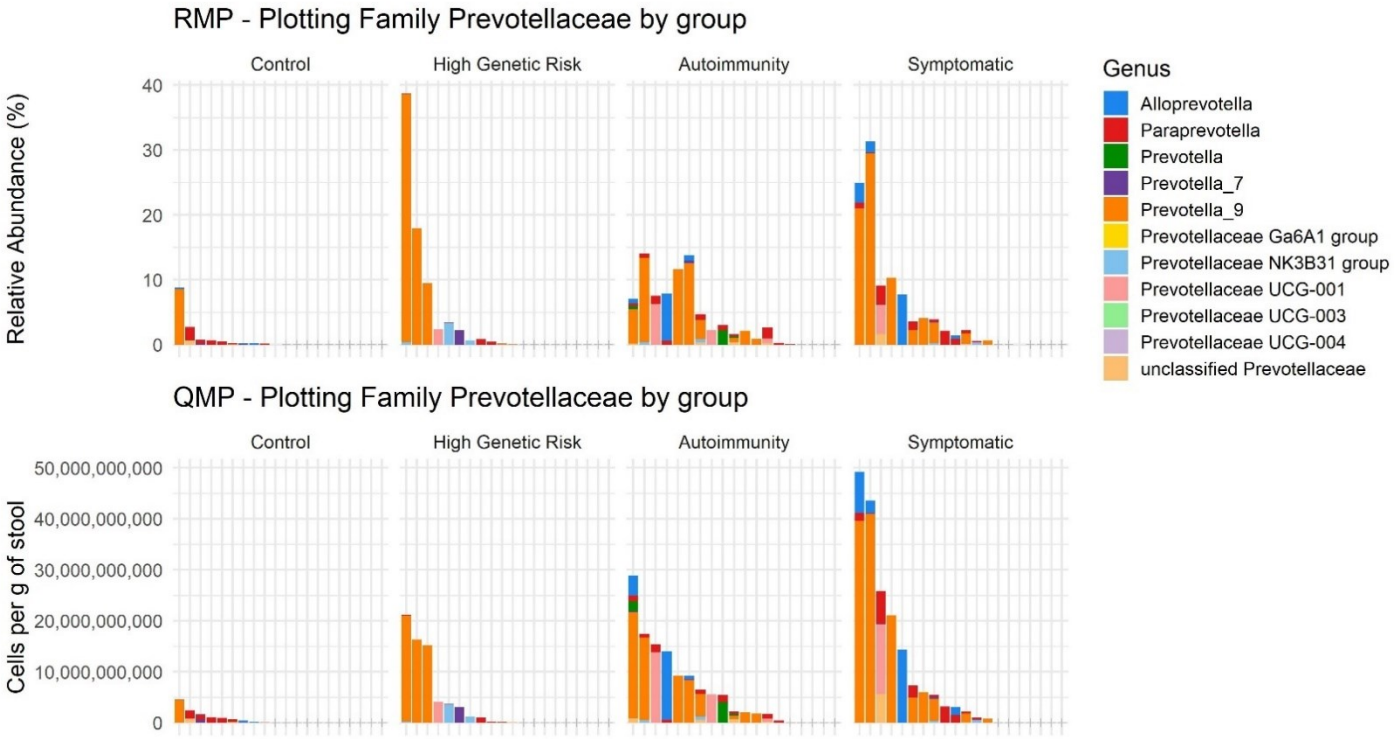


Figure 17: Prevotellaceae abundance by sample, in most pronounced subgroups. This figure shows either proportions or cell counts of fecal Prevotellaceae, based on 16S sequencing of frozen fecal samples, in the most pronounced phenotype subgroups. **RMP** = Relative Microbiota Profiling (provides proportions). **QMP** = Quantitative Microbiota Profiling (provides estimated cell counts). Each bar represents one sample colored by the different genera identified in the Prevotellaceae family. QMP profile sits below its corresponding RMP profile. "Prevotella_9" is the annotation for what is mostly *P. copri*. Overall Kruskal-Wallis p = 0.02074 (on relative abundances). Permutation test: one-sided p-value of 0.054.

Figure 18 is a visualisation of the output of *Aldex2* tool. It looked, in each of our groups, at the sequence counts for every bacterial family and performed a Kruskal-Wallis test, in the results of which a small p-value would mean that at least one group significantly differs from the others.

In blue are the p-values of these tests; in red, the p-values corrected to account for multiple testing. Overall, the family level did not really allow to distinguish between the groups, nor did it for the genus level, not shown here).

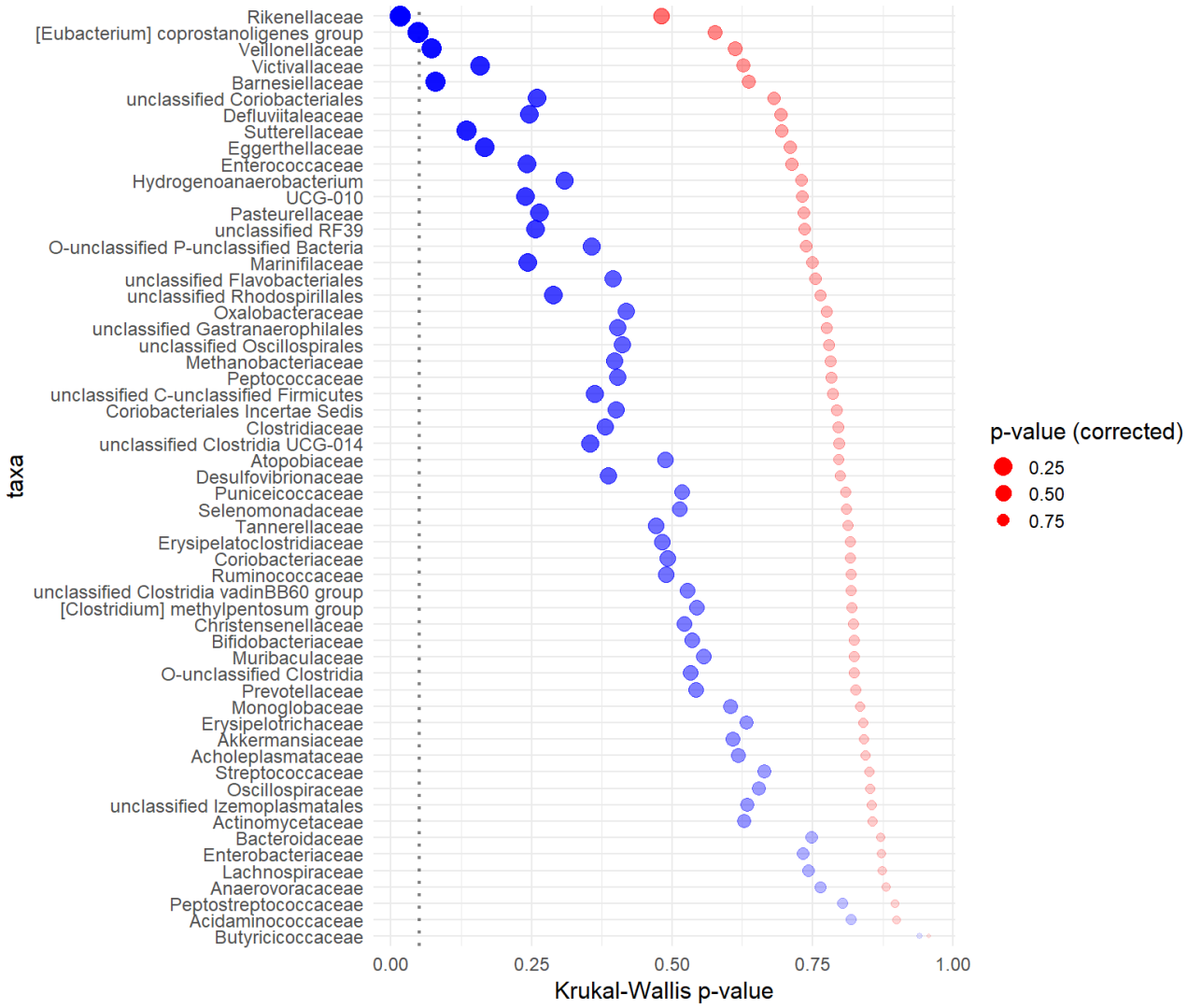


Figure 18: differential abundance analysis performed with Aldex2 (Family level). This analysis is based on 16S data derived from fecal samples. Rare taxa were removed from the dataset, and sequence counts were aggregated at the Family level. Then, *Aldex2* performed a centered-log-ratio transformation, which accounts for data compositionality, and performed serial Kruskal-Walis tests between groups, for every bacterial Family. A small p-value would indicate that the related bacterial family is more prevalent in at least one group. The blue points represent the raw p-values, the red points are the p-values corrected by Benjamini-Hochberg procedure. Vertical dotted line is the significance threshold (0.05). Point size is inversely related to p-value.

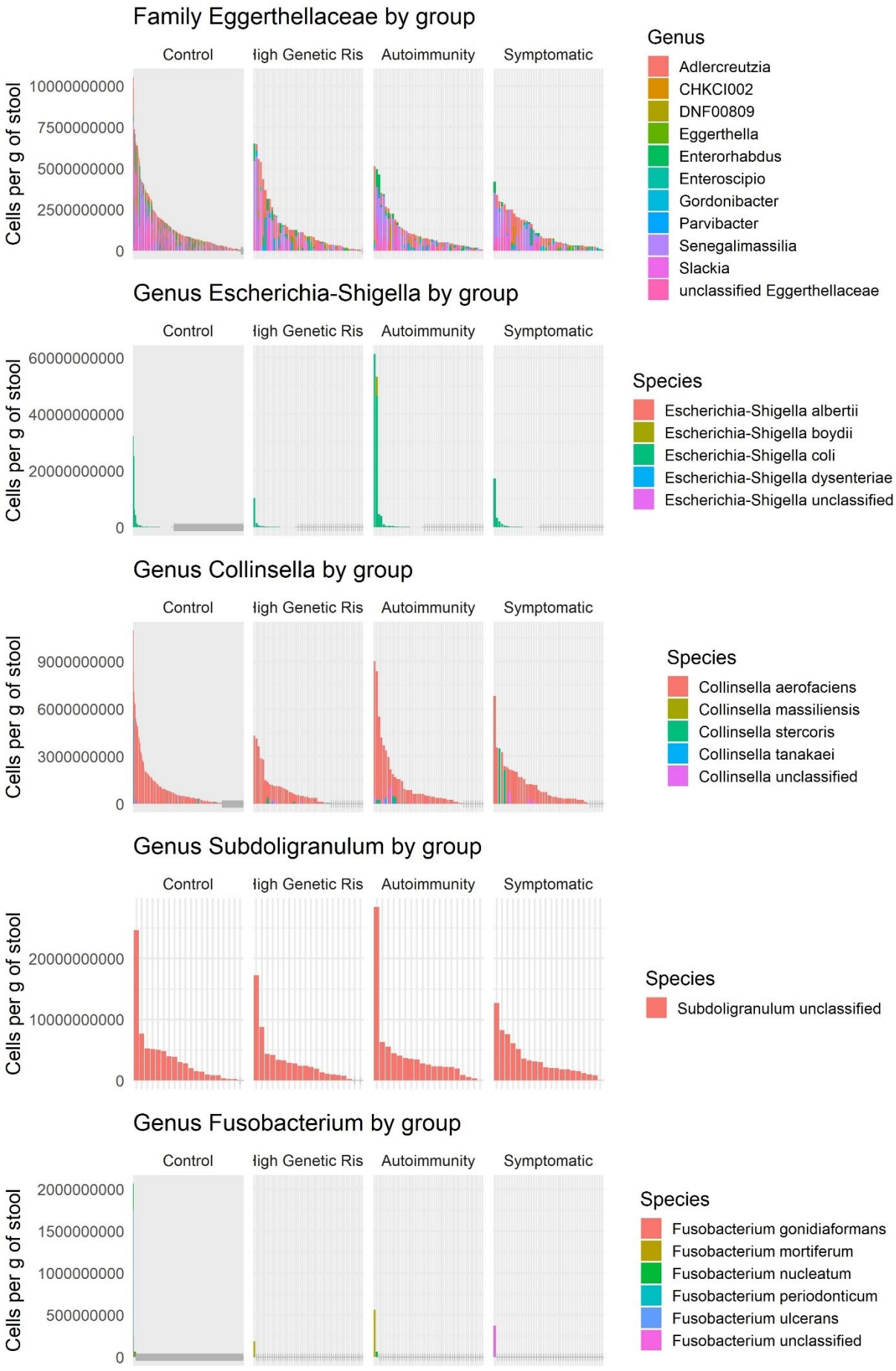

Family Eggerthellaceae by group
Control
High Genetic Ris
Autoimmunity
Symptomatic
Cells per g of stool
10000000000
7500000000
5000000000
2500000000
0
Genus
Adlercreutzia
CHKCI002
DNF00809
Eggerthella
Enterorhabdus
Enteroscipio
Gordonibacter
Parvibacter
Senegalimassilia
Slackia
unclassified Eggerthellaceae
Genus Escherichia-Shigella by group
60000000000
40000000000
20000000000
Species
Escherichia-Shigella albertii
Escherichia-Shigella boydii
Escherichia-Shigella coli
Escherichia-Shigella dysenteriae
Escherichia-Shigella unclassified
Genus Collinsella by group
9000000000
6000000000
3000000000
Species
Collinsella aerofaciens
Collinsella massiliensis
Collinsella stercoris
Collinsella tanakaei
Collinsella unclassified
Genus Subdoligranulum by group
20000000000
10000000000
Species
Subdoligranulum unclassified
Genus Fusobacterium by group
2000000000
1500000000
1000000000
500000000
Species
Fusobacterium gonidiaformans
Fusobacterium mortiferum
Fusobacterium nucleatum
Fusobacterium periodonticum
Fusobacterium ulcerans
Fusobacterium unclassified

Figure 19: Bacterial loads of RA-associated taxa. Estimated bacterial loads in feces samples, based on 16S sequencing and Silva v138 database, by risk group, for bacteria previously reported as associated with RA (and worsening mouse models). Each vertical bar corresponds to one sample. Except for *Fusobacterium*, which was only detectable in a dozen of individuals, other bacteria associated with RA were commonly detected in our population of interest; however, we did not notice significant differences in abundances between groups as assessed using *Aldex2*.

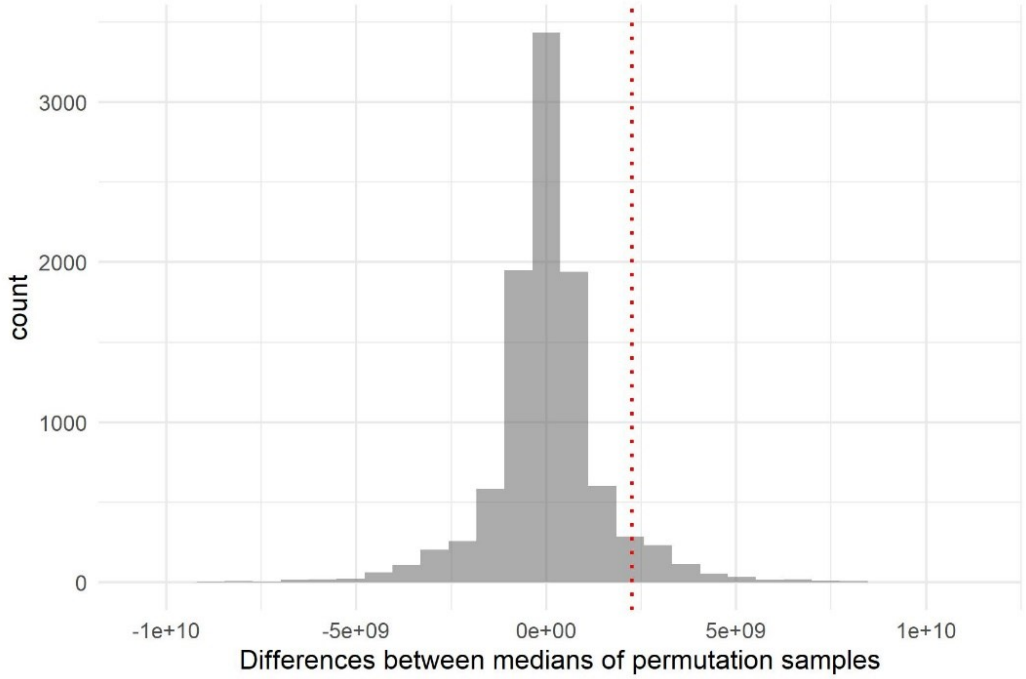


Figure 20 : Permutation test on subgroups analysis. This figure assesses the probability that the results reported in Figure 13 (most pronounced subgroups) are obtained only by chance. To do so, we randomly selected two groups of 20 individuals in the cohort and compared their median Prevotellaceae quantitative abundances (QMP), computing the difference. This figure represents the differences between medians obtained after 10'000 repetitions. The vertical redline is the "real" difference between median Prevotellaceae abundances in control subgroup versus autoimmunity subgroup as presented in Figure 13. Only 5.548 % percents of random sets fell on the right of the red line, meaning that the one-sided p-value for our finding is 0.054 (0.375 if using the RMP data).

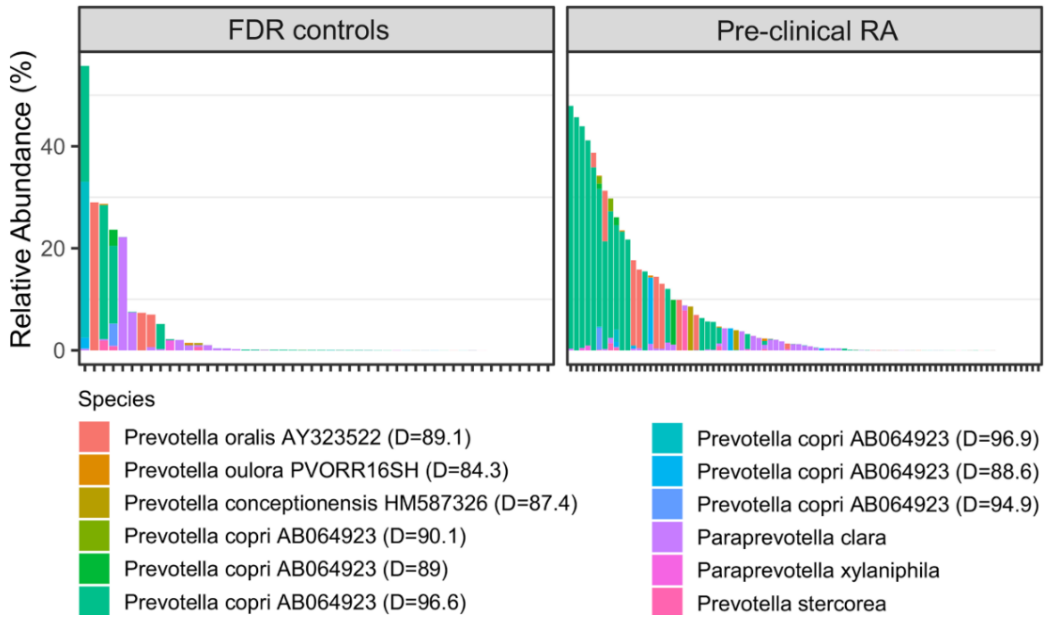


Figure 21 : Prevotellaceae abundance in SCREEN-RA in 2019 (previous data). This figure is the plot obtained by Alpizar et al. in 2019, using SCREEN-RA stool samples from a previous sampling campaign. Each bar represents the percentage of Prevotellaceae in a fecal sample of a participant from the SCREEN-RA cohort, as assessed after 16S-based metagenomics. Non-adjusted linear discriminant analysis found a p-value of 0.040. www.ard.bmj.com/content/78/5/590 (License: author reuse). Compare with the following figure.

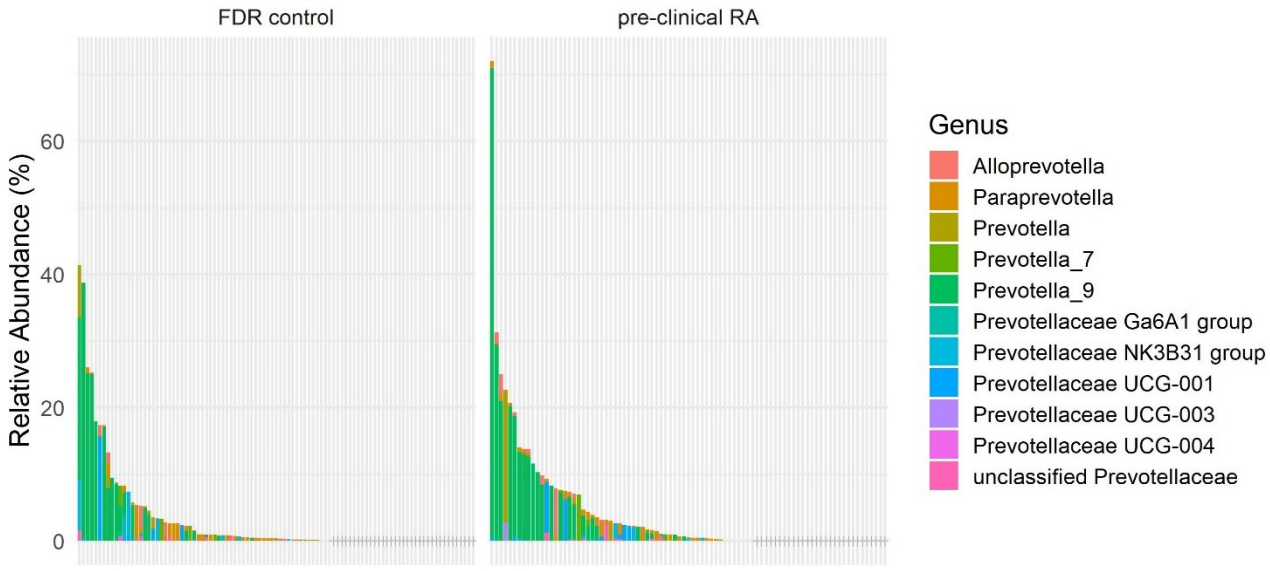


Figure 22 : Prevotellaceae abundance in SCREEN-RA in 2023. This plot shows same data as presented in work-package 1, i.e., the proportion of Prevotellaceae by fecal sample as assessed by 16S-based metagenomics. Here, the layout and grouping match those of Alpizar et al., 2019. We can see that that the difference between groups became less obvious, which is actually non-significant (Wilcoxon p = 0.29).

Discussion

On the one hand, I could say that all the RA-associated bacteria were detected in our population of pre-clinical RA individuals (**Figure 19**). Consequently, it is *plausible* that they could be involved in RA pathogenesis. Also, focusing on the most extreme phenotype subgroups, we could modestly reproduce our previous findings (increased Prevotellaceae abundance in later stages), which is also encouraging.

On the other hand, none of our working hypotheses were confirmed when comparing the full groups. More precisely, the fecal flora composition was highly variable between individuals, and this variability did not correlate with the proposed grouping. Fecal calprotectin was also normal in most participants, and the few individuals with increased values were equally distributed in all groups. Finally, two individuals who developed seronegative RA two years later did not present anything special with regards to the studied parameters (serologies, fecal microbiota and calprotectin) at the time of stool sampling.

Also, 16S-sequencing does not provide information on gene function and metabolic activity of the studied microbiome; and the taxonomic resolution hardly goes beyond the species level, while we explained in section "Loss of tolerance: *Prevotellaceae?*", page 38, how strain-level distinction may matter. We chose 16S to provide findings comparable to most previous studies, and also to limit the burden of bioinformatical analyses, which are much more demanding in case of whole-genome sequencing. Although, it is still possible to use the DNA leftovers to perform an additional whole genome shotgun sequencing, we considered this would be relevant only as a second step if strains of interest were identified in work-package 3, which utilizes shotgun sequencing.

The limitations of our work are further discussed in section "LIMITATIONS", page 109.

Selected publication

A manuscript was submitted to RMD Open journal and is attached to this thesis as "2 – Submitted (RMD Open) - Gut microbiome and intestinal inflammation in preclinical stages of rheumatoid arthritis", page 146.

WORK-PACKAGE 2 – SERUM BIOMARKERS

Rationale

As introduced above (paragraph "Intestinal permeability", page 32), mucosal inflammation and permeabilization of epithelial barrier is thought to be a key step in induction of autoimmunity.(153) In particular, for a local immune response to become systemic, translocation of immunogenic products and dissemination of activated immune cells are necessary.

We initially considered measuring the permeability of the gut mucosal barrier with an "*in vivo*" probe-urinary excretion test, similarly to previous studies that have assessed gut permeability in RA patients.(212,254,255) Such functional tests imply the ingestion of probe molecules that are passively absorbed, and which can subsequently be measured in the urine.

As underlined by Bischoff et al.,(208) the functional tests of gut permeability are logistically complicated and time-consuming as probands have to stay several hours in the hospital and can thus be carried-out only on a limited number of patients. Furthermore, a recent study confirmed that healthy subjects already have baseline ^{12}C-mannitol urinary excretion due to food contamination, which is not ideal and should be replaced by an isotopic version.(351)

Moreover, functional tests of gut permeability present several limitations, such as being influenced by recent NSAID intake, and requiring fasting before the procedure, which may not provide a representative permeability measurement of a "loaded" and active intestine. Also, more pragmatically, we found no established protocol neither within our institution nor at the Lausanne University Hospital, and it would have been beyond the scope of this thesis to set up and validate such tests from scratch.

For these reasons, we assessed the integrity of the intestinal mucosal barrier using serological surrogate markers. We reasoned that better feasibility (less dropouts or protocol-related failures) and significantly larger numbers of patients would offset the loss of precision of serological biomarkers compared to the functional measures of gut permeability. We chose to measure the following markers which were discussed in more detail in paragraph "Intestinal permeability" page 32:

- **Intestinal Fatty Acid Binding Protein** (I-FABP), for this intestinal epithelial tissue damage marker is well established and specific for enterocytes. It has been used in a preliminary study by Matei et al., and we wanted to check such findings with a larger sample.(239)

- **Lipopolysaccharide-Binding Protein** (LBP). At the design stage of our study I have been deceived by the surprisingly recurrent usage of LBP as a pseudo-intestinal permeability biomarker (219–223,262,352–357) even in the context of RA,(239) and also poorly advised by

other colleagues. We later realized that LBP is merely an acute phase protein. Though potentially related to endotoxemia it is certainly not a reliable proxy for LPS blood content.

- **Calprotectin** (serum), which was used as a complementary inflammatory biomarker. Our reasons for assessing serum calprotectin were: 1) it was cheaper than C-reactive protein; 2) it was recently studied as a promising biomarker for RA disease activity, for not being suppressed by DMARDs administration since it depends directly on neutrophilic activity and not on acute phase signaling;(358–363) 3) it could be compared to the fecal calprotectin measure that we also performed.

We did not assess serum zonulin, for not having found updated, reliable commercially available kits. All the available brands seem to be using the primary antibodies from the original manufacturer which were reported to cross react with other compounds,(237) as was checked by obstinate inquiry sent to their customer services.

We expected to find increased levels of serological I-FABP, LBP, and potentially calprotectin, in the autoimmunity and/or symptomatic pre-clinical RA subgroups, compared to the controls. In this case, we planned to analyze the relationship between these biomarkers of mucosal integrity and the presence of RA-associated bacteria, as measured in work-package 1.

Contribution statement

Benoît GILBERT was involved in the study design, online SCREEN-RA interface design (Redcap), data-management of SCREEN-RA cohort and selection of eligible participants. He contacted a few dozens of participants and assessed them at study visits, including clinical examination and serum (same samples as in work-package 1). Benoît GILBERT performed all the ELISA tests, with advice and supervision from Gaby PALMER, Emiliana RODRIGUEZ and Chiraz CHABANE. GILBERT was also in charge of the data analysis and visualization (R coding).

Olivia STUDER, together with study nurses of collaborative centers, contacted most other participants and organized the study visits and serum samplings.

Céline LAMACCHIA was involved in the study design, manuscript revision, data interpretation, oversaw serum sample storage and SCREEN-RA biobank management, and collaboration with HUG central lab for ACPA, anti-Ra33 and RF serologies as part of routine SCREEN-RA workflow.

Till STROWIG and Lena AMEND contributed to study design and data interpretation, as it mirrored some of their experiments. Axel FINCKH (principal investigator) contributed to study design and funding, supervision, and manuscript preparation.

Methods

Study population

In this work-package, the autoimmunity and symptomatic groups were defined similarly to the work-package 1. However, the meticulous reader might notice slight differences in the group-wise effectives, which are due to the following reasons:

- due to the excess number of controls and the limited throughput of ELISA, we reduced the control group to a smaller selection of age- and sex-matched participants. Also, high-genetic risk individuals were not included in this analysis since they were not believed to differ much from other controls regarding the assessed biomarkers.

- The SCREEN-RA database version used for work-package 2 is antecedent to the version used in work-package 1. One or two patients might have had an updated status between the two data extractions, for instance, it was not clear if their symptoms were attributable to a new RA or not – and/or if they omitted to report their new diagnosis in time.

- There were a few cases of technical failures of stool sample processing (WP1), while the serum sample of the same participants were still included in WP2 analysis, or vice versa.

- In work-package 2, one co-author finally asked that we separate the actual new-onset RA from the rest of the symptomatic individuals.

All these technicalities do not affect the overall conclusions.

Serum sample analysis

We used commercially available sandwich DuoSet ELISA kits from R&D Systems (Minneapolis, MN) for LBP (DY870, range 0.78 – 50 ng/ml), calprotectin (DY1820, range 93 - 6000 pg/ml) and I-FABP (DY3078, range 31 - 2000 pg/ml). Samples were randomized and divided into three batches. Each batch was aliquoted in several 96-wells plates at the appropriate dilution ratio. Then, for a given marker to be tested, ELISA tests were run in duplicate, for three consecutive days, according to the manufacturer's instructions (**Figure 23**). For the LBP and calprotectin assays, samples were diluted 1/1000, while for the I-FABP assay, samples were diluted 1/10 in reagent diluent. Due to the preparation procedure, all samples were thawed twice before measurement (i.e., initial freezing, thawing, dilution and aliquoting, re-freezing, final thawing and testing).

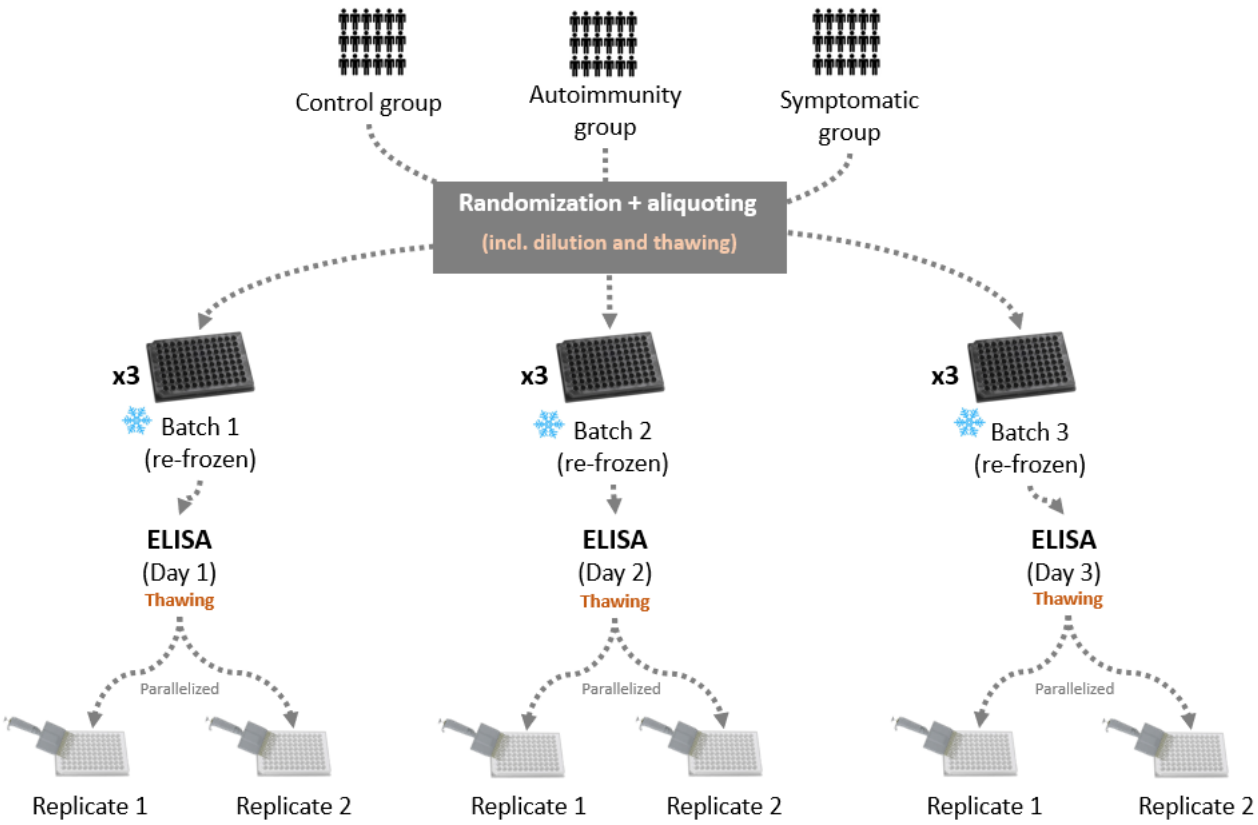


Figure 23: serum sample processing for ELISA testing. This figure illustrates how the serum samples were processed to measure LBP, I-FABP and calprotectin. Frozen serum samples were first randomized into three batches. For each batch, three plates containing serum aliquots at the required dilution were prepared and re-frozen. Finally, for a given serological marker, a plate of each batch was thawed, and ELISA test performed over three consecutive days in duplicate. Reproduced from Gilbert et al., 2023, https://doi.org/10.3389/fimmu.2023.1117742 , (license: author reuse).

Optical density was determined using a LEDETECT 96 automatic reader, set to 450 nm with a correction filter at 570 nm. Finally, for each plate, the standard curve was constructed with R code using the *drm* function from the *drc* package v.3.0-1 to convert optical densities into concentration values. For each duplicated measurement, the inter-assay coefficient of variation (CV) of the two optical densities was computed as standard deviation divided by the mean. Only samples with <10% CV were included in the final analysis. The marker concentration was obtained by averaging the two measured concentrations and multiplying by the dilution factor.

Statistical analysis

For baseline characteristics, continuous variables are expressed as the means and standard deviations (SD), while categorical variables are expressed using percentages. ANOVA, $\chi 2$ test or Fisher's exact test in case of small samples were used to compare baseline characteristics between the groups.

The biomarker concentrations were compared to the low-risk group using two-sided Wilcoxon rank tests. Correlations between the biomarkers were calculated using Spearman coefficient with the related p-value. All statistical analysis was performed using R 2022.02.3 with package *tableone* and *stats*.

Results

We selected 180 individuals: 84 controls (labelled "Low-risk"), 53 autoimmunity participants (labelled "Intermediate-risk") and 38 symptomatic participants (labeled "High-risk"). Five untreated new-onset RA patients were also recruited and sampled at the time of RA diagnosis, prior to antirheumatic treatment initiation. There were no significant differences between the groups in terms of age, gender and BMI (**Table 5**).

Table 5: Baseline characteristics of serum-tested participants

Variable	Groups			
	Low-risk n = 84	Intermediate-risk n = 53	High-risk n = 38	New-Onset RA n = 5
Female	81 %	75 %	87 %	100 %
Age (the mean) (SD)	54 (13)	54 (16)	55 (11)	53 (17)
BMI (the mean) (SD)	25 (5)	24 (4)	24 (5)	26 (6)
ACPA positivity (>1x norm)	0 %	32 %	8 %	60 %
RF serology				
1 to 3x the norm	11 %	8 %	16 %	0 %
>3x the norm	0 %	66 %	10 %	60 %
Anti-Ra33 positivity				
1 to 3x the norm	13 %	21 %	13 %	NA
>3x the norm	0 %	4 %	0 %	
With detectable RA autoimmunity (low threshold >1x norm)	20 %	100 %	42 %	60 %
Shared epitope alleles				
0 alleles	48 %	49 %	55 %	40 %
1 allele	52 %	38 %	39 %	40 %
2 alleles	0 %	13 %	7 %	20 %

Legend

ACPA: Anti-Citrullinated Protein Antibodies. RF: Rheumatoid Factor. Anti-Ra33: anti-Ra33 autoantibodies. CSA: Clinical Suspect Arthralgia according to the EULAR definition.

Low risk = asymptomatic RA-FDR without specific RA- autoimmunity. Intermediate risk = asymptomatic RA-FDR with specific RA-autoimmunity. High risk = symptomatic RA-FDR. New-onset RA are untreated at sampling time. NA = Not Assigned, i.e. the new onset RA were not tested for anti-Ra33 antibodies.

The mean inter-assay coefficient of variation (CV), computed on optical densities, was 1.7% for LBP, 2.2% for I-FABP, and 3% for calprotectin. One sample was excluded from the I-FABP analysis, and 7 samples were excluded from the calprotectin analysis because the difference between the two replicates was too large (CV >10%). Overall, the mean values of the three biomarkers did not differ between the groups (**Table 6**). Outliers were kept in the analysis.

Table 6: Biomarker concentrations per risk group

Variable	Groups			
	Low-risk n = 84	Intermediate-risk n = 53	High-risk n = 38	New-onset RA n = 5
Number of samples analyzed, n				
LBP, n	84	53	38	5
I-FABP, n	84	53	38	4
Calprotectin, n	82	50	36	5
LBP (µg/ml), mean (SD)	10.83 (4.39)	11.07 (4.55)	11.75 (4.27)	12.44 (6.53)
I-FABP (pg/ml), mean (SD)	1746 (1617)	1393 (823)	1438 (965)	1009 (487)
Calprotectin (ng/ml), mean (SD)	2043 (1396)	1860 (1163)	1629 (1114)	1897 (649)

Legend

LBP: Lipopolysaccharide Binding Protein. I-FABP: Intestinal Fatty Acid Binding Protein.

SD: Standard Deviation.

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We found no correlation between LBP and I-FABP levels (Spearman rho -0.06; p = 0.40), nor between I-FABP and calprotectin serum concentrations (Spearman rho -0.07; p = 0. 36). LBP modestly correlated with systemic inflammation, as reflected by serum calprotectin levels (Spearman rho = 0.32; p < 0.001) but not with RF status. In additional experiments we noticed that further thawing cycles reduced detectable protein concentrations for LBP but not for I-FABP and calprotectin.

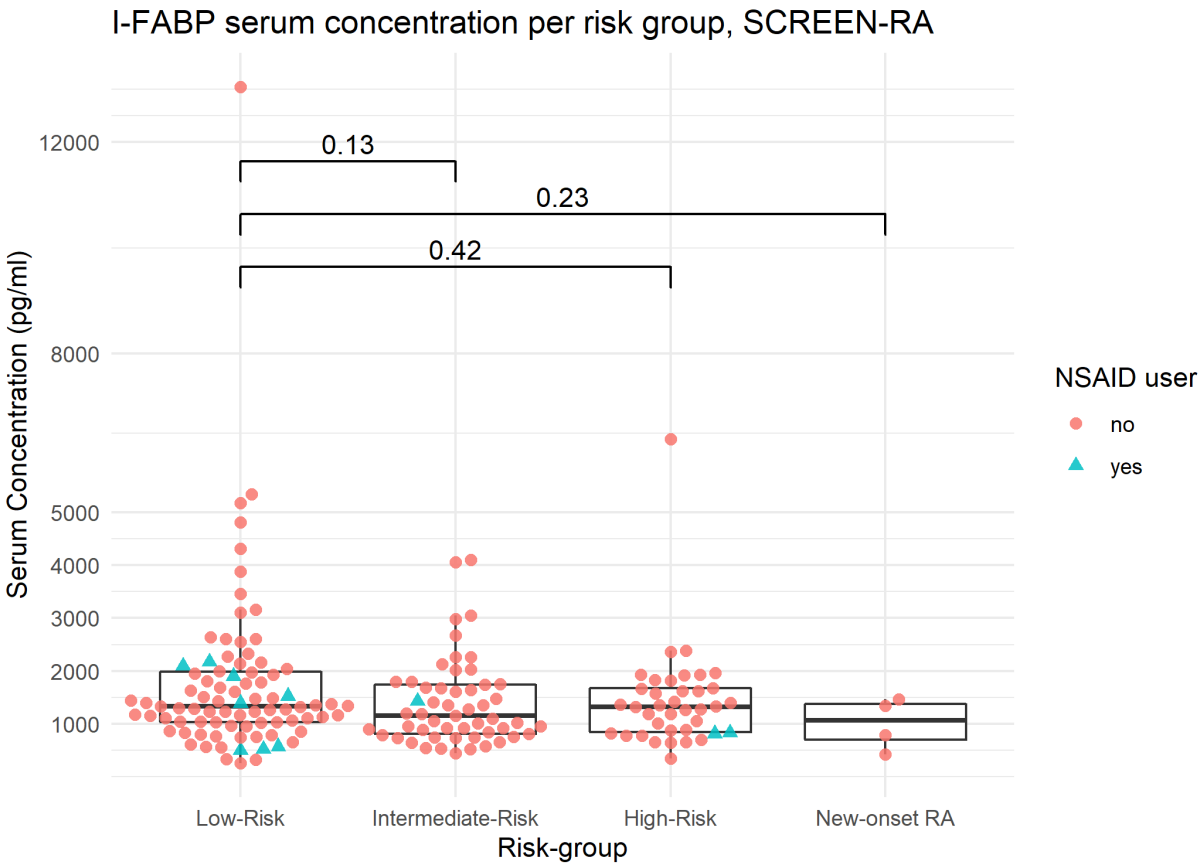


Figure 24: I-FABP serum concentration per risk subgroup, SCREEN-RA. Serum concentrations of I-FABP, as assessed by duplicated ELISA testing (R&D Systems DY3078), in: – Low-risk: asymptomatic seronegative FDR of RA patients. – Intermediate-risk asymptomatic FDR with autoimmunity (ACPA, RF, or Ra33). – High-risk FDR with clinically suspect arthralgia, based on EULAR criteria. – New-onset untreated RA patients. RA: Rheumatoid Arthritis. Outliers are included in the analysis. p-values are displayed (Wilcoxon test). Total n = 179. Reproduced from Gilbert et al., 2023, https://doi.org/10.3389/fimmu.2023.1117742 , (author reuse).

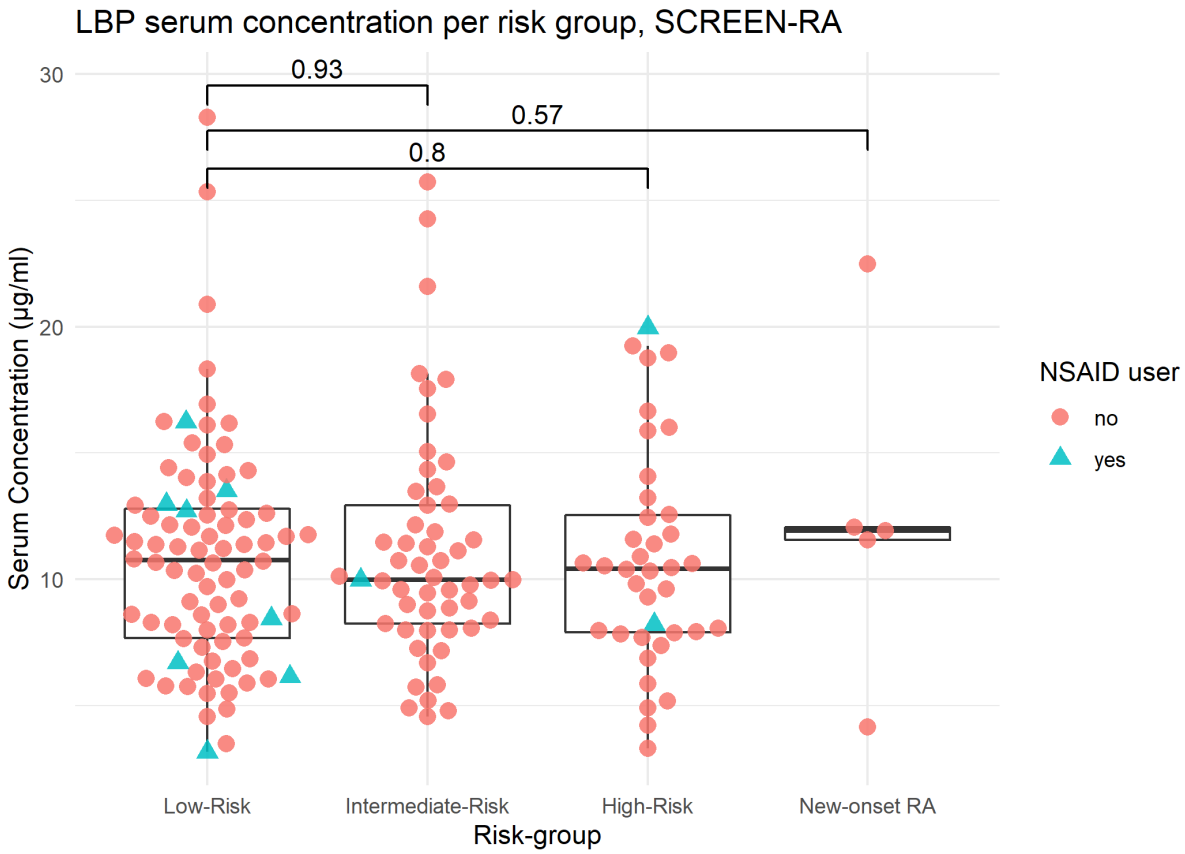


Figure 25: LBP serum concentration per risk subgroup, SCREEN-RA. Serum concentrations of LBP, as assessed by duplicated ELISA testing (R&D Systems DY870), in: – Low-risk: asymptomatic seronegative FDR of RA patients. – Intermediate-risk asymptomatic FDR with autoimmunity (ACPA, RF, or Ra33). – High-risk FDR with clinically suspect arthralgia, based on a combination of EULAR criteria. – New-onset untreated RA patients. RA: Rheumatoid Arthritis. Outliers are included in the analysis. p-values are displayed (Wilcoxon test). Total n = 180. Reproduced from Gilbert et al., 2023, https://doi.org/10.3389/fimmu.2023.1117742 , (author reuse).

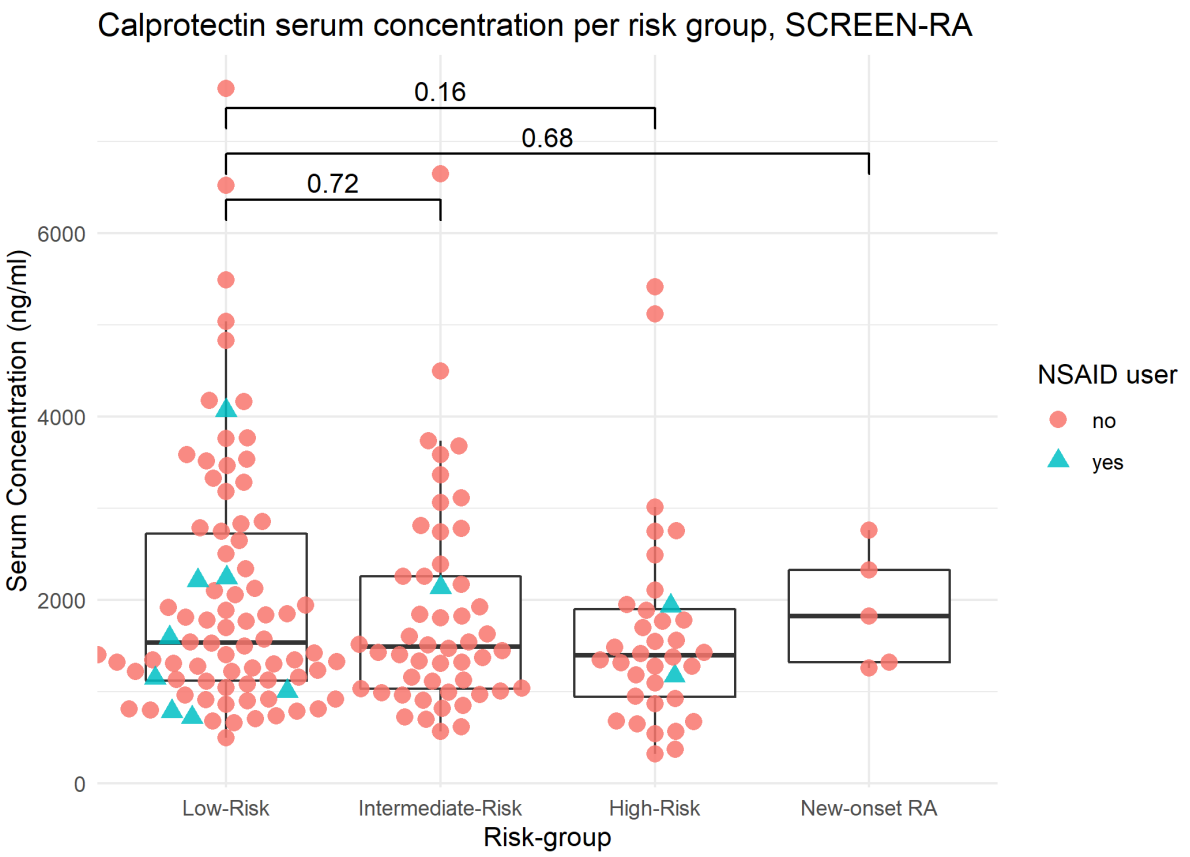


Figure 26: Calprotectin serum concentration per risk subgroup, SCREEN-RA. Serum concentrations of calprotectin, as assessed by duplicated ELISA testing (R&D Systems DY1820,), in: – Low-risk: asymptomatic seronegative FDR of RA patients. – Intermediate-risk asymptomatic FDR with autoimmunity (ACPA, RF, or Ra33). – High-risk FDR with clinically suspect arthralgia, based on EULAR definition. – New-onset untreated RA patients. RA: Rheumatoid Arthritis. Outliers are included in the analysis. p-values are displayed (Wilcoxon test). Total n = 173. Reproduced from Gilbert et al., 2023, https://doi.org/10.3389/fimmu.2023.1117742 , (license: author reuse).

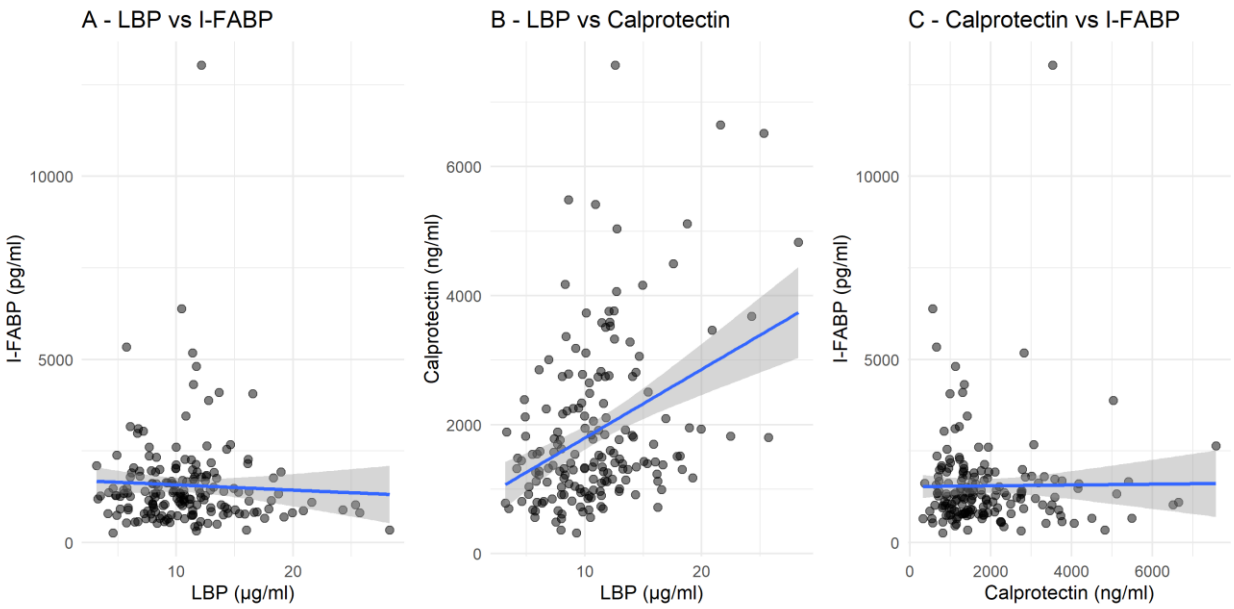


Figure 27: Serum LBP, I-FABP and calprotectin concentrations correlations, SCREEN-RA. Correlation of serum concentrations determined by ELISA for LBP, I-FABP and calprotectin in 180 samples of the SCREEN-RA cohort. The line is a fitted linear regression model. LBP = Lipopolysaccharide Binding Protein. I-FABP = Intestinal Fatty-Acid Binding Protein. Spearman coefficient for LBP versus calprotectin: rho = 0.32; p < 0.001. Reproduced from Gilbert et al., 2023, https://doi.org/10.3389/fimmu.2023.1117742 , (license: author reuse).

Discussion

We found no association between the putative serum biomarkers of intestinal integrity (LBP and I-FABP) and preclinical stages of RA development.

Also, after careful review of the literature, we concluded that the previous findings regarding LBP should be interpreted with caution. It has been known since LBP's discovery that its induction in the liver depends on IL-1beta and IL-6, which makes it an acute-phase protein,(364–366) though extra hepatic secretion by adipocytes has also been documented.(367) In the context of RA, this could explain why LBP correlates with disease activity markers such as erythrocyte sedimentation rate and C-reactive protein.(225,239,368) On the other hand, LPS is by itself a strong pro-inflammatory agent, which might contribute to low-grade systemic inflammation, adding even more confusion to the matter.(218) Thus, it is not clear whether the increased LBP is to be seen as the result of systemic inflammation, LPS-induced endotoxemia, or both. In our study, we noticed a modest correlation between LBP and serum calprotectin, while LBP serum concentrations did not differ between the three studied groups. Similarly, Matei et al. were not able to distinguish healthy controls from the individuals in the pre-clinical stages of RA using LBP.(239)

Selected publication

The related publication is attached as "3 – Frontiers – Brief report: assessment of mucosal barrier integrity using serological biomarkers in preclinical stages of rheumatoid arthritis", page 173.(369)

WORK-PACKAGE 3 – IMMUNE REACTIVITY

Rationale

We believed that some pre-clinical RA patients would have an increased permeability of the gut mucosal barrier, facilitating the systemic spread of bacterial products and inflammatory cells, which may result in the development of adaptative immune responses against commensal bacteria.(153,292,294)

The gut mucosa produces an important amount of IgA antibodies, secreted in the gut lumen and binding to local microbes. Some researchers have suggested that highly IgA-coated intestinal bacteria reflect strong adaptive immune reactions against these particular species indicating that these microorganisms represent a threat to the gut barrier integrity (e.g. *Collinsella aerofaciens,*(284) *or* adherent-invasive *E. coli* (370)). *Collinsella aerofaciens*, a bacteria associated with RA in previous analyses of the microbiota, has been detected in both IBD patients and healthy controls but was highly coated with IgA only in IBD patients.(284) Also, the presence of highly IgA-coated bacteria was associated with the infiltration of the mucus layer by bacteria in IBD mouse models.(284) Similarly, *Prevotella* species have been found invading the mucus layer in IBD models (371) or in human colonic cancer related studies.(372,373) We hypothesized that some *P. copri* strains may be able to colonize the intestinal mucus layer and induce a specific IgA response in pre-clinical RA patients.

IgM can also be secreted in the gut lumen,(374) and coating patterns converge toward IgA-bound microbes;(375) but IgM tends to coat microbes present in the mucus layer.(376) IgG can sometimes opsonize about 1% of gut bacteria, but this presence of luminal IgG might be explained by alteration of epithelial barrier (such as in Crohn's disease patients (377)) rather than by an active secretion. However, IgA and IgG coating of commensal microbes also seem to occur in homeostatic condition.(378,379) It is also possible to assess IgG binding by incubating serum with the autologous feces. During the incubation process there seem to be a convergence between IgG and IgA coating, which target the same bacteria.(380) Serum IgG usually targets only the IgA-coated bacteria, though on a different antigen, and does not bind to the IgA-uncoated microbes, which suggests that the IgG response is a continuation of the IgA response. These patterns also do not seem to depend on microbes' relative abundances.

To identify potentially pathological microbes we initially planned to compare the IgA intestinal coating pattern with the IgG coating patterns by adapting the work of Palm et al. (named "IgA-SEQ") to a subset of representative patients of our study population.(284) However, due do technicalities and time constraints, we performed a study of the IgG and IgA binding pattern but only the IgG fractions of a

subset of samples were sorted and sequenced. We were expecting to find *P. copri* among the highly IgG-coated portion of isolated microbes of high-risk SCREEN-RA participants compared to the asymptomatic controls, together with a systemic immune response against *P. copri* (IgA and/or IgG).

External collaborations: Marianne GAZZANO and Amend et al.

Marianne Gazzano (Paris, Pitié-Salpêtrière) assessed the percentage of the IgA-coated and IgG-coated bacteria in the provided samples.

Additionally, we collaborated with Amend et al. (Helmholtz Centre for Infection Research, Braunschweig, Germany) to assess the humoral response against various strains of *P. copri*.

Contribution statement

Benoît GILBERT was involved in the study design, online SCREEN-RA interface design (Redcap), data-management of SCREEN-RA cohort, selection of eligible participants and sampling (same serum samples as in work-package 2 and their corresponding stool). Benoît GILBERT prepared stool samples with advice from Guy GOROCHOV, Vladimir LAZAREVIC and supervision from Chiraz CHABANE. IgG-seq processing, including sample preparation and cell-sorting, were performed by Benoît GILBERT, with the help and supervision from Marianne GAZZANO, Christophe PARIZOT and Guy GOROCHOV, in Paris (Laboratoire d'immunologie, Pitié-Salpêtrière).

GILBERT also performed DNA extractions; Lejla IMMAMOVIC was in charge of library preparation and shotgun sequencing. The sequencing data was processed by Nadia GAIA, and Benoît GILBERT used the output for statistical analysis and visualizations in R language.

Olivia STUDER, together with the study nurses of collaborative centers, contacted most participants and organized the study visits and serum samplings.

Céline LAMACCHIA was involved in the study design, data interpretation, oversaw serum sample storage and SCREEN-RA biobank management, and collaboration with HUG central lab for ACPA, anti-Ra33 and RF serologies as part of routine SCREEN-RA workflow.

Axel FINCKH and Guy GOROCHOV (principal investigators) contributed to study design and funding, and supervision. Till STROWIG and Lena AMEND contributed a separated set of complementary experiments, which I partly mention below, in particular, serum IgG reactivity against various *P. copri* strains – using, among others, samples we provided from the SCREEN-RA and SCQM cohorts.

Finally, in Paris (Pitié Salpêtrière), Marianne Gazzano used the microbiome suspension and serum samples to perform an anti-IgG and anti-IgA staining, thus assessing the proportions of IgA+ and IgG+ bacteria for each sample.

Methods

Population

Initially, all serum samples from work-package 2 were processed with their corresponding stool sample to prepare them for use in work-package 3 (**Figure 28**).

Nonetheless, only a subset of them were finally included in the IgG-seq experiment as we tried to prioritize either clean control samples or case samples with the RA diagnosis, the highest CSA scores, or auto-antibody titers, similarly to the "most pronounced phenotype" subgroups presented in work-package 1.

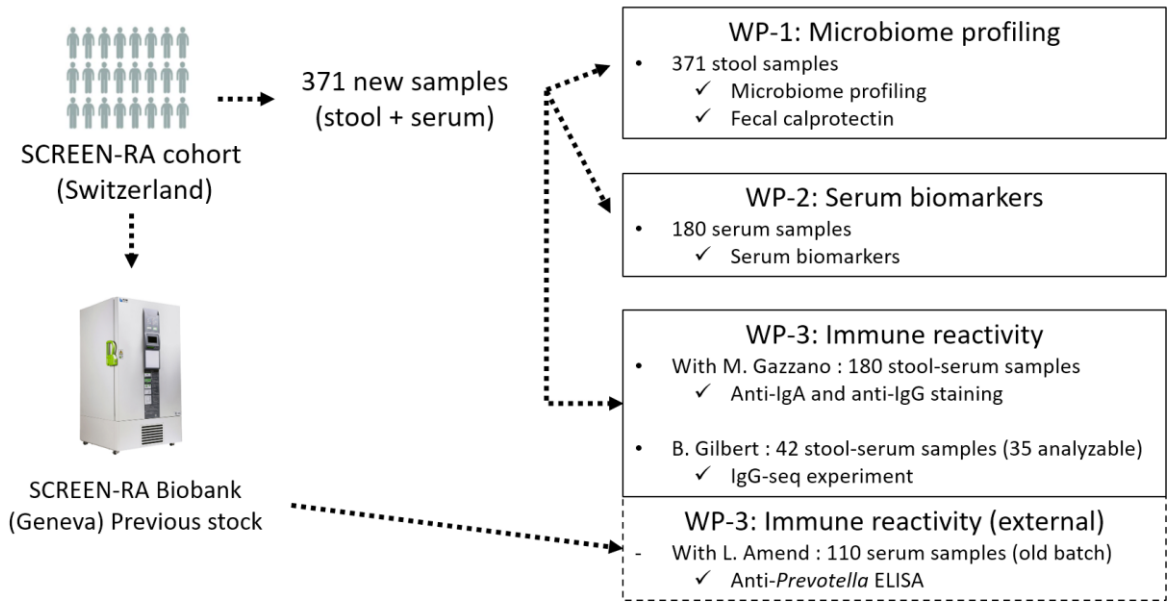


Figure 28: Sample and population by work-package. Most samples analyzed in work-package 3 are derived from the 371 paired stool-serum samples collected for the purpose of this thesis. However, an additional experiment conducted by L. Amend analyses the serum samples collected previously and which sometimes come from different participants of the same cohort.

Sample preparation

For one sample, an aliquot of 200mg of frozen feces was rehydrated for 5 min in 1 mL of Phosphate-Buffer-Saline (PBS). Forty seconds of mild bead beating ensured suspension and fragmentation of macroscopic particles (2.8mm ceramic beads, Vortex Genie 2 speed 6/10). The sample was then centrifuged for 10 min at 150 g, 4°C. The total of 150 µL of supernatant were collected and washed in 500 µL of PBS before pelleting by centrifuging at 8050 g for 4 min at 4°C. The final bacterial pellet was resuspended in 300 µL of PBS with 10% glycerol, divided in two aliquots and stored at -80°C for the cytometry experiment.

Sample processing – Serum incubation

Bovine serum albumin blocking buffer (PBA) was prepared by adding 2% bovine albumin in phosphate buffered saline and sterilized by filtering in 20 µm pores. Total concentrations of IgG, IgA and IgM were determined in an aliquot of the serum samples by nephelometry performed at HUG Central Lab facility. For the IgG-seq another aliquot was diluted at the target concentration of 20 µg/mL of IgG using the information provided by the nephelometry measure.

Then, for a stool-serum pair of samples: in a 96 v-wells plate, 25 µL of patient's serum diluted at 20 µg/mL of IgG concentration was incubated for 30 min at 4°C with 2 µL of thawed microbiome suspension and 23 µL of PBA, for a total volume of 50 µL. A negative control was also prepared by replacing the serum with humanized anti-TNF Infliximab 10 µg/ml, which is an antibody with a humanized Fc part but whose binding groove does not bind bacterial epitopes. After the incubation, 180 µL of PBA were added as a wash in each well before centrifuging at 3500 g for 10 min at 4°C. Supernatant was discarded.

Secondary antibodies were added in the wells (25 µL of goat anti-IgG FITC, diluted at 1/400; or 25 µL of PBA for unstained negative controls) and used to resuspend the hardly visible pellet. After 20 min of incubation at 4°C in the dark, the wells were washed again with 180 µL of PBA, and the plate was centrifuged at 3500 g for 10 min at 4°C. Supernatant was discarded. The final stained pellet was resuspended in PBA and transferred into cytometry tubes.

Bacterial cell-sorting

Stained microbiome samples were loaded on a BIORAD S3e Cell Sorter. Acquisitions were made using Side Scatter Area on an axis, and FITC (which bound to anti-IgG antibodies) fluorescence intensity on the other.

Negative controls were used to determine sorting gates for the IgG negative (IgG-) and the IgG positive (IgG+) fractions. Consequently, each sample had its own controls and sorting settings to determine the threshold above which a particle is considered "IgG+".

At least 1 million events were sorted for each fraction, hence, separate tubes were used for the IgG- and the IgG+ fractions of each sample; the sorted fractions were pelleted by ultracentrifugation in 1.5 mL Eppendorf tubes, for 5 min at 17'000g and frozen at -80°C before the DNA extraction.

DNA extraction and shotgun sequencing

The DNA extractions were performed using a Zymobiomics Micro-Prep kit according to the manufacturer instructions but omitting the optional step 4 and repeating the final elution two times in

15 µL of water each time. The DNA concentrations were estimated in test samples using Q-Bit High Sensitivity quantification kit (fluorescence based).

To obtain the strain-level resolution and to derive functional information from the genomes we opted for a shotgun sequencing that processes all available DNA fragments. It improves taxonomical resolution and allows and allows bioinformatical reconstitution of whole genomes. A next-generation sequencing library preparation was made using miniaturized library preps (1/4 of the reaction volume) with a QIAseq kit (Qiagen). The sequencing was performed on Illimuna NovaSeq (pair reads, 250 bp read length).

Data analysis

The sequencing data was processed according to the following steps:

1- Processing the reads with Trimmomatic-0.36 (SLIDINGWINDOW:10:28 MINLEN:150). Several tests used a window of 10 bases, an average quality of 30 and a min length of 200 but the quality of the reads was not sufficient to stay within these parameters.

2- Removing the replicated sequences with a python script.

3- Removing the low complexity reads with Komplexity.

4- Removing the reads classified as human with Kraken2+Python script.

5- Classifying the reads at species level for bacteria, fungi, parasites and with Kraken2 (ignoring virus and phages reads).

The hereabove pipeline provides taxonomic counts for each sample, with species level resolution (strain-level would require additional processing). The classified reads and taxonomic assignations were manipulated in R using package *phyloseq* and visualized using package *microViz*. Samples with less than 100'000 classified reads in one of their fractions were dismissed, and the rarefaction depth was set at 90% of the minimum sampling depth in the dataset. For PCoA plotting, the read counts were transformed in proportions and samples were compared using Bray-Curtis index. Statistical significance of the PCoA was computed using *Adonis2* function. The IgG-coating index was computed as: $log10(\frac{IgG+taxon\ abundance}{IgG-taxon\ abundance})$ for each identified species.

Ig-opsonized fractions (Marianne Gazzano)

Microbiome samples from individuals included in work-package 2 were incubated with autologous serum as described hereabove. Goat anti-human IgG or goat anti-human IgA, linked to a fluorochrome (fluorescein, FITC or phycoerythrin, PE), were added as secondary antibodies. Isotypic antibodies provided by the same manufacturers were used as negative controls as they are similar to the secondary antibodies but bind to nothing.

Data acquisition was performed on a Beckman Coulter cytoFLEX. For each sample a first gate was applied to stringently select bacteria-like morphology by applying a concomitant staining to distinguish whole bacteria from debris using Cell Proliferation Dye eFluor 450, eBioscience. Then, the negative control was performed with the help of isotypic staining to determine the positivity thresholds, and the percentage of stained events falling in the positivity region (IgA+, IgG+ or IgA+IgG+) were assessed as the main outcome.

Anti-*Prevotella copri* immune reactivity (Amend et al.)

As a parallel experiment, we provided Amend et al. with serum samples from 110 participants of the SCREEN-RA cohort and 45 confirmed (treated) RA cases from the SCQM cohort. For this part only, the analyzed serum samples were not the same as those studied in the rest of the thesis – they came from a previous blood sample, and not all the participants were the same as in work-package 1. However, the concept of the group classification remained identical to the presented above, page 45.

The methods are described in the already published article,(381) but are summarized as follows:

Several *P. copri* strains were isolated from *P. copri* positive donors, while *P. copri* type strain (DSM 18205), *Porphyromonas gingivalis* (DSM 20709), *Prevotella intermedia* (DSM 20706) and *Prevotella melaninogenica* (DSM 7089) were sent to Amend et al. by the Leibniz Institute.

Amend et al. subsequently used these strains to coat ELISA plaque and perform a custom ELISA test using serum as the first antibody and goat anti-human IgG or goat anti-human IgA as the HRP-conjugated secondary antibody.

Results

Ig-opsonized fractions (Marianne Gazzano)

The 180 samples of work-package 2 were analyzed by Marianne Gazzano (+2 samples which were not excluded yet when this experiment was run; hence total n = 182). Cytometric acquisition was first gated on morphology, and then on eFluor signal (which stains DNA), to focus on bacterial cells instead of debris particles (**Figure 29**).

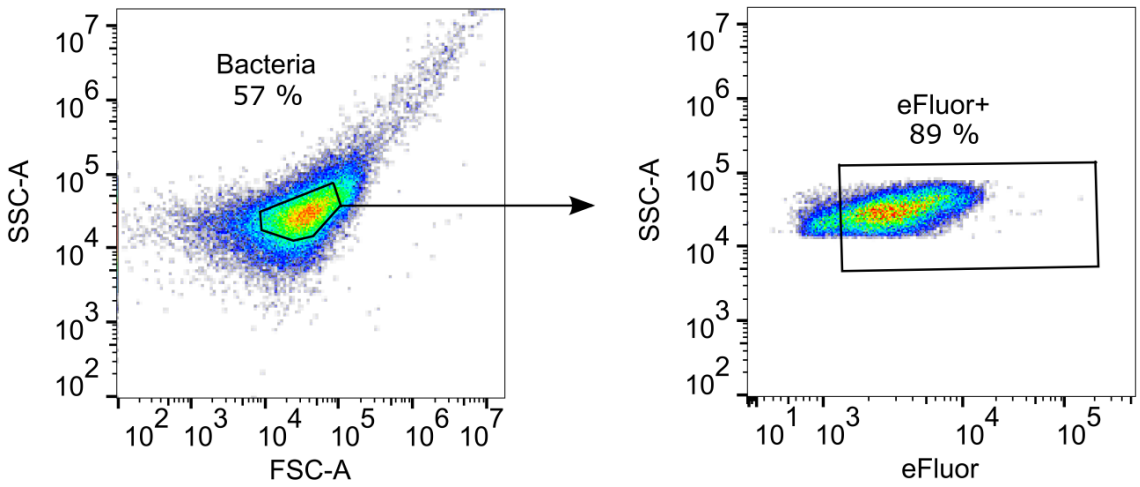


Figure 29: Example of morphological sorting and DNA-staining. Fecal samples were incubated with autologous serum, stained with eFluor, and acquisition was made on a Beckman Coulter cytoFLEX. Panel **A** – shows the raw morphology of particles in sample n°429. A first gating is applied on particle morphology. Panel **B** shows eFluor signal on horizontal axis, which reflects the DNA content. Particle with low signal are dismissed by a second gate.

Then, acquired events were assessed for IgG and IgA staining intensity. Each sample had a negative control with its own positivity thresholds (**Figure 30**).

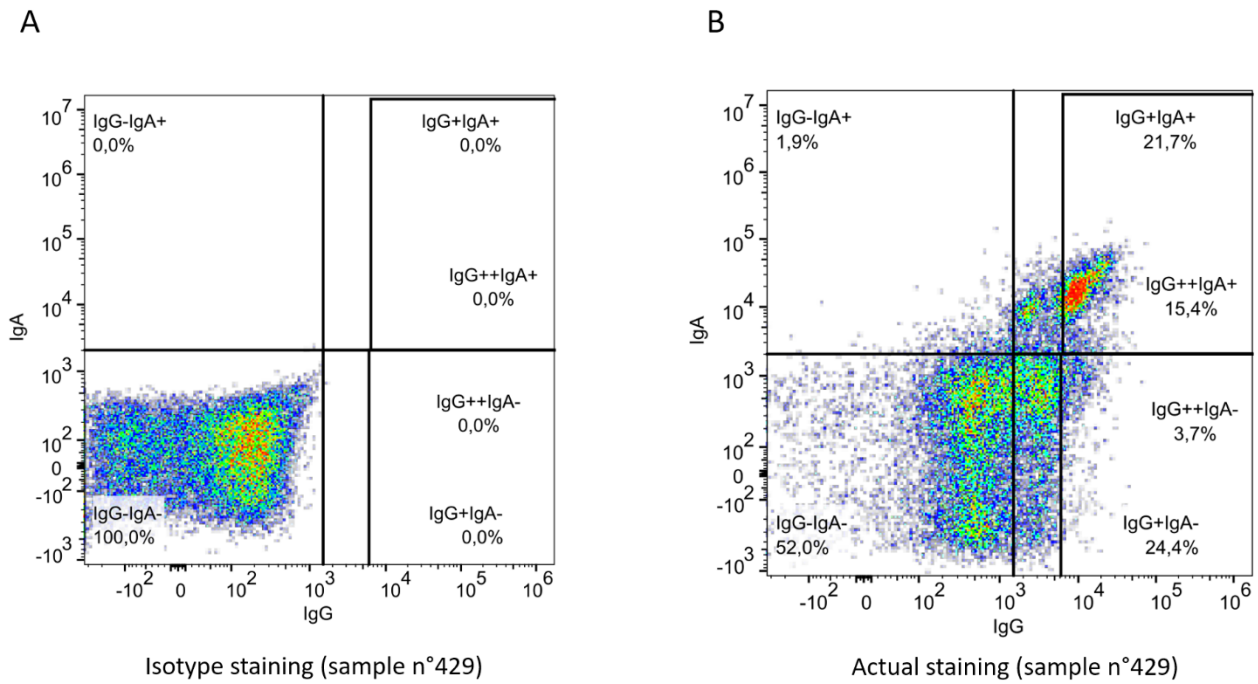
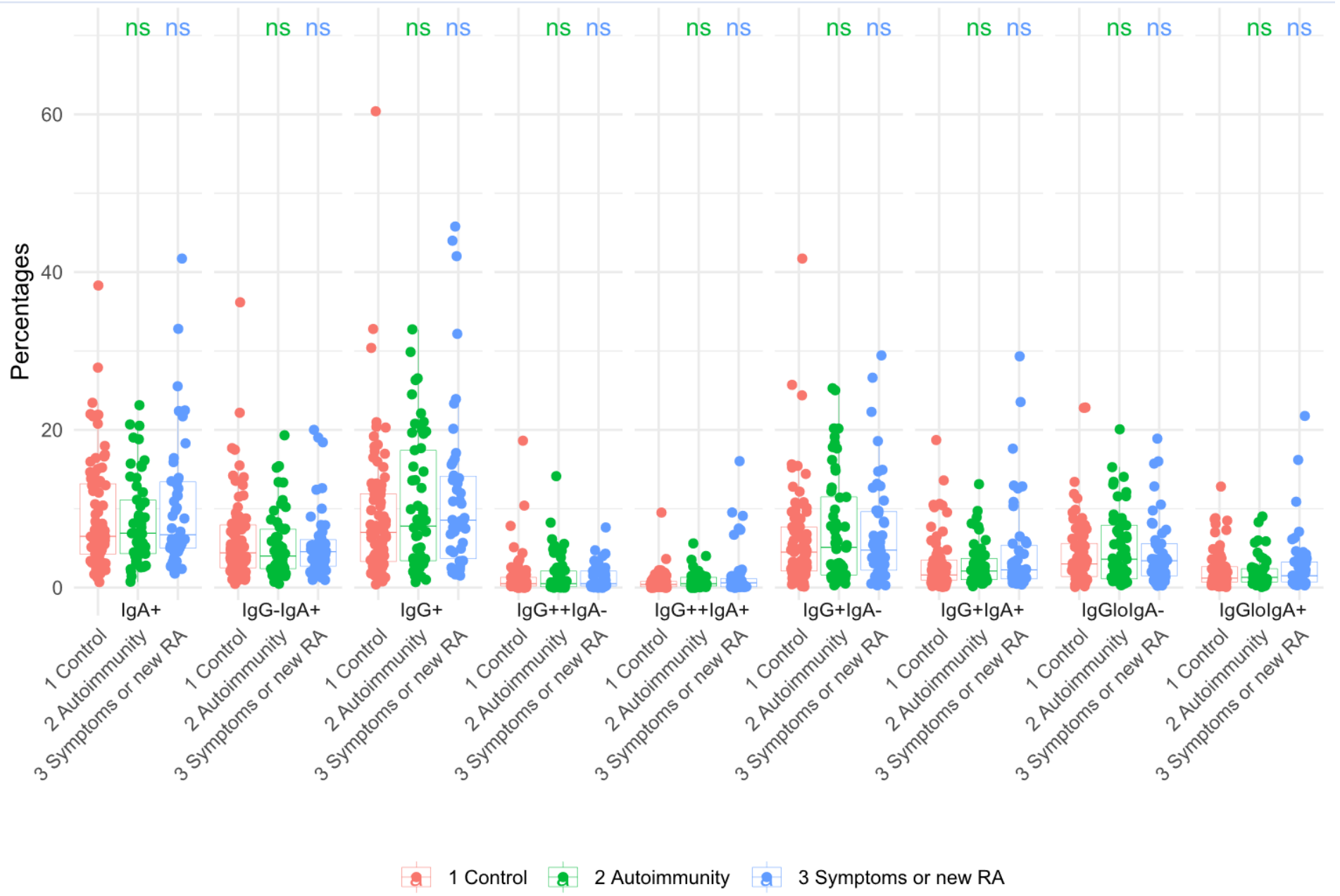


Figure 30 (previous page): anti-Ig staining of sample n°429. This figure shows, as an example, the cytometry data of fecal sample n°429, incubated with autologous serum and stained with anti-IgA and anti-IgG secondary antibodies after a first gating on morphology (previous figure). Acquisition is made on a Beckman Coulter cytoFLEX. **A** – on this panel, the sample is stained with the isotypic control secondary antibodies. The data is used to setup the positivity thresholds, and each sample has its own negative control. **B** – this panel shows the actual anti-IgA and anti-IgG staining. Events falling above the thresholds are counted, being IgG+, IgA+ or both.

Overall, the percentage of events positively stained, even though sometimes surprisingly different from one sample to another, did not differ between the groups (**Figure 31**).

Figure 31 (next page): Ig-opsonized fractions per group (SCREEN-RA). All fecal samples (n = 180) from the SCREEN-RA participants were incubated with autologous serum and stained with eFluor, anti-IgA and anti-IgG secondary antibodies. After a first gating on morphology and DNA content, IgA+ and IgG+ particles were counted on a Beckman Coulter cytoFLEX. This figure shows the percentage of events positively stained, for each fraction, for each sample, by group. ns = not significant (Wilcoxon).


Percentages
0
20
40
60
ns ns
IgA+
IgG-IgA+
IgG+
IgG++IgA-
IgG++IgA+
IgG+IgA-
IgG+IgA+
IgGloIgA-
IgGloIgA+
1 Control
2 Autoimmunity
3 Symptoms or new RA

IgG-seq experiment

Figure 32 exemplifies the gating strategy for the IgG-seq sorting procedure.

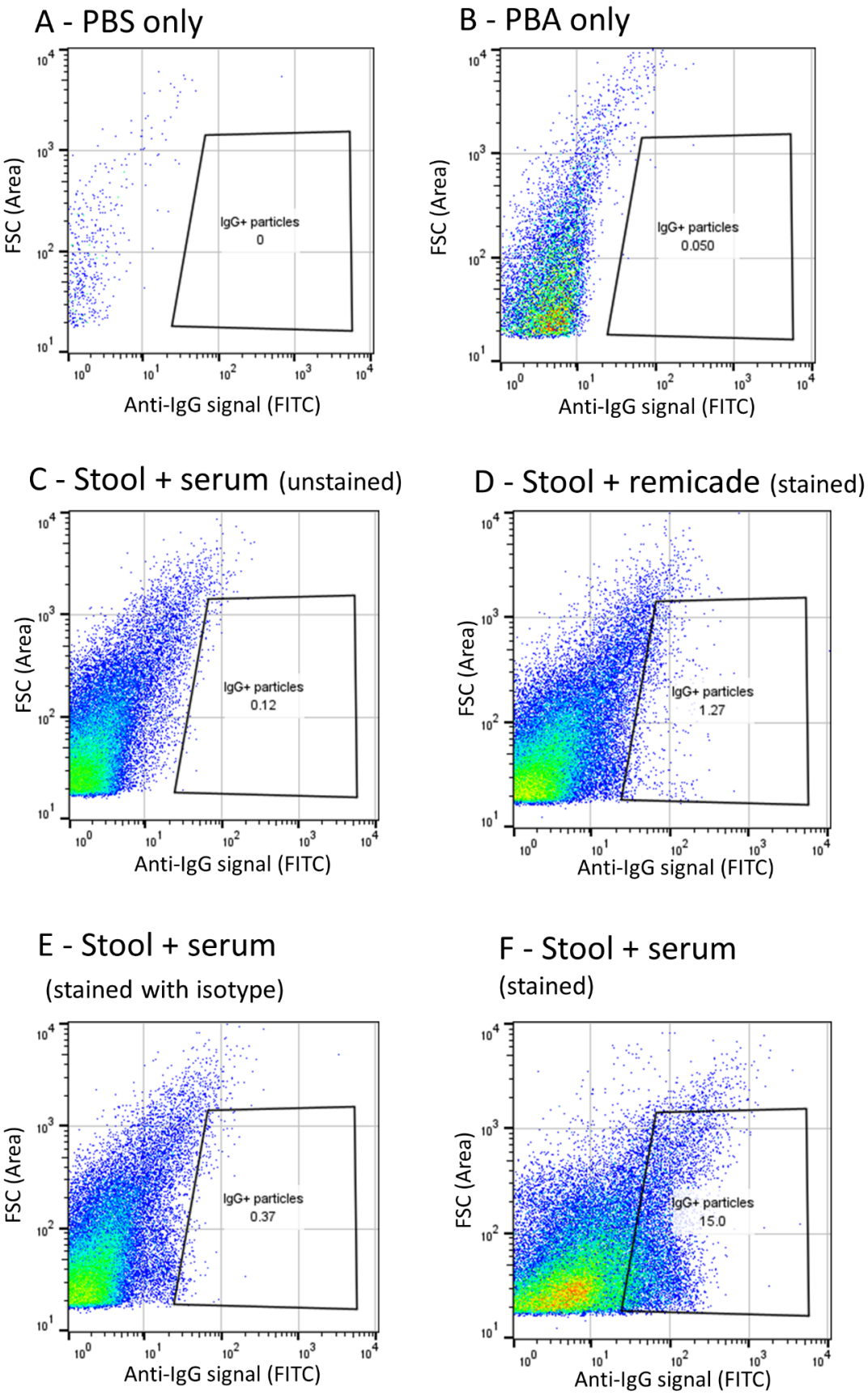


Figure 32: IgG-Seq sorting strategy (sample n°292). Stool microbiome was suspended, incubated with autologous serum and stained with anti-IgG secondary antibodies. Negative controls were prepared simultaneously. The Biorad S3e cell-sorter was setup with an axis for FSC and an axis on the FITC signal. **A** and **B** – only carrier fluid was passed in the cytometer (hence points are background noise due to small particles in PBS, artefacts or bovine albumin in PBA. **C** – Stool sample incubated with serum, but without secondary antibodies. Shows native fluorescence. **D** – Stool sample incubated with Infliximab and stained with anti-IgG-Fc goat-IgG-FITC. It is used to setup the sorting-gate for IgG positivity, so that the possible false positive rate is ~1% (Infliximab binding estimates the non-specific binding of IgG, through Fc part). **E** – Stool sample incubated with serum and stained with control isotype (negative control goat-IgG-FITC that binds to nothing). **F** – Stool incubated with serum and stained with anti-IgG-Fc goat-IgG-FITC. Positive particles were sorted in a separated tube (here, 15% of particles were IgG+).

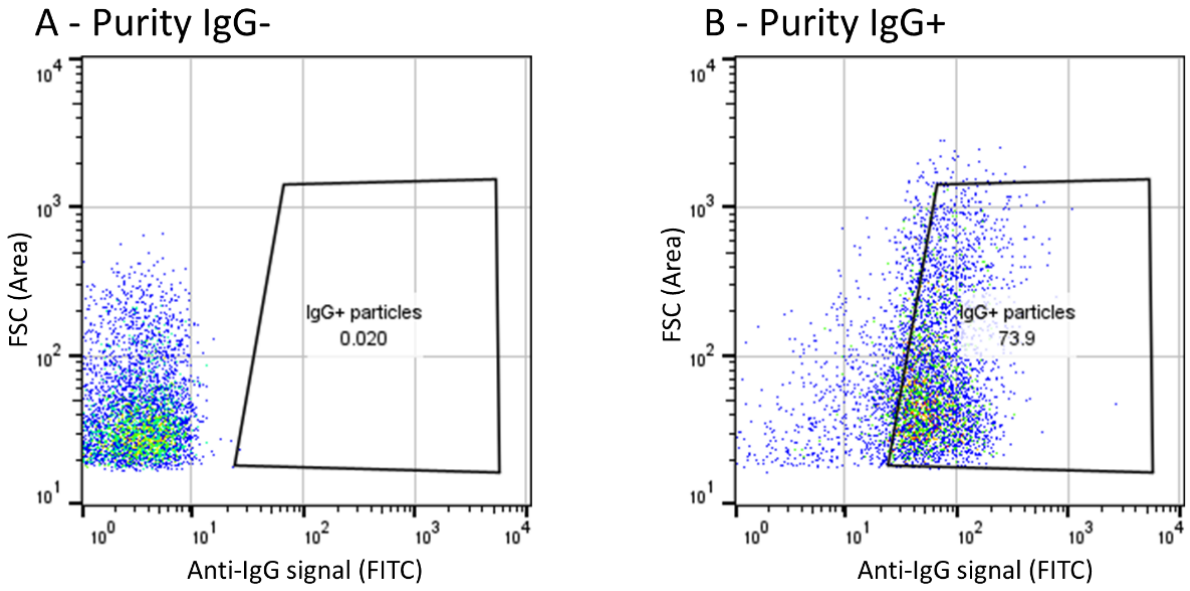


Figure 33: Purity control of sorted fractions (sample n°292). Same setup as previous figure. Once sorted, IgG- and IgG+ fractions were re-acquired in the Biorad S3e to check purity. Most of the sorted particles of the IgG+ fraction fall again in the corresponding sorting gate. Nb: as being exited a second time, the fluorescence intensity of FITC is slightly lower, which explains the shift to the left on panel B.

The quantity of retrieved DNA was extremely low (~20ng/mL per IgG fraction) but after 37 PCR-cycles of the 16S gene the negative controls (DNA extraction kit) remained negative for bacterial DNA, contrary to sorted fractions (**Figure 34**).

A total of 42 samples were processed, of which 35 were analyzable as they had enough DNA material in both the IgG- and the IgG+ fractions.

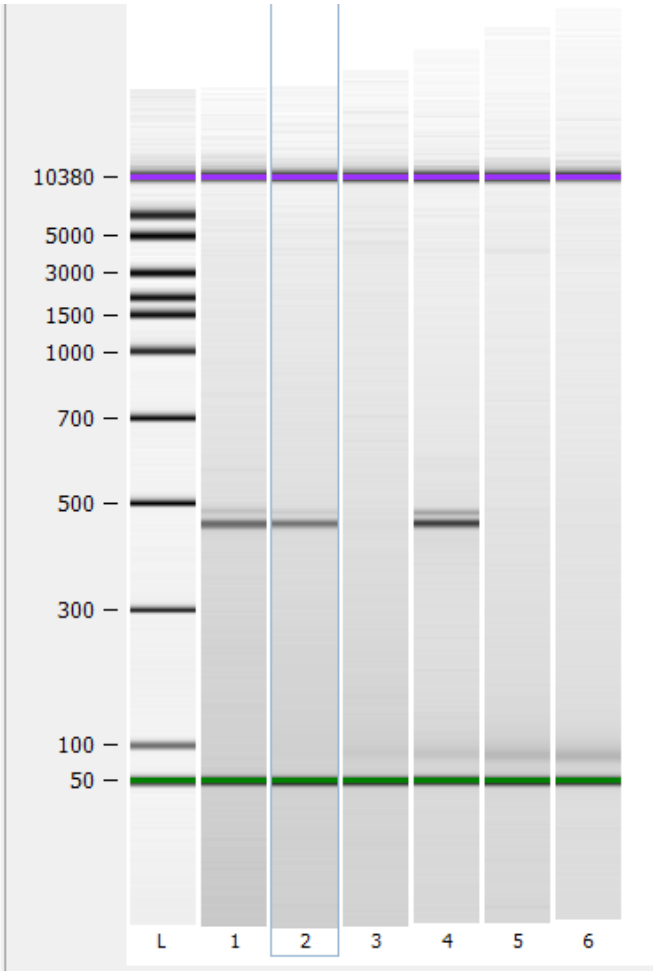


Figure 34 : 16S V3-V4 PCR with usual protocol, 37 cycles and 5 ul of sample input. As a quality control, Ig+ or Ig- fractions of 5 test samples were sorted on a Biorad S3e cell-sorter. After the DNA extraction, a 37-cycle 16S PCR was made, and the PCR products were loaded on a micro-electrophoresis gel (Agilent Bioanalyzer) to check for non-contamination as well as presence of bacterial DNA. **1**: test sample n°140- (800k sorted events); **2**: test sample n°140+ (800k sorted events); **3**: test sample n°423- (600-700k sorted events); **4**: test sample n°423+ (600-700k sorted events); **5**: Negative control (process through pipeline); **6**: Negative control (PBS only). Below the ~500 bp tracer are the line corresponding to the 16S amplification; sample n°423- and negative controls were still negative, after the 37 PCR cycles.

As a control for the procedure reproducibility the IgG-positive fraction of sample n°175 was sorted and sequenced two times. The final microbiome profiles suggest good reproducibility (**Figure 35**); more reproducibility controls will take place in 2024.

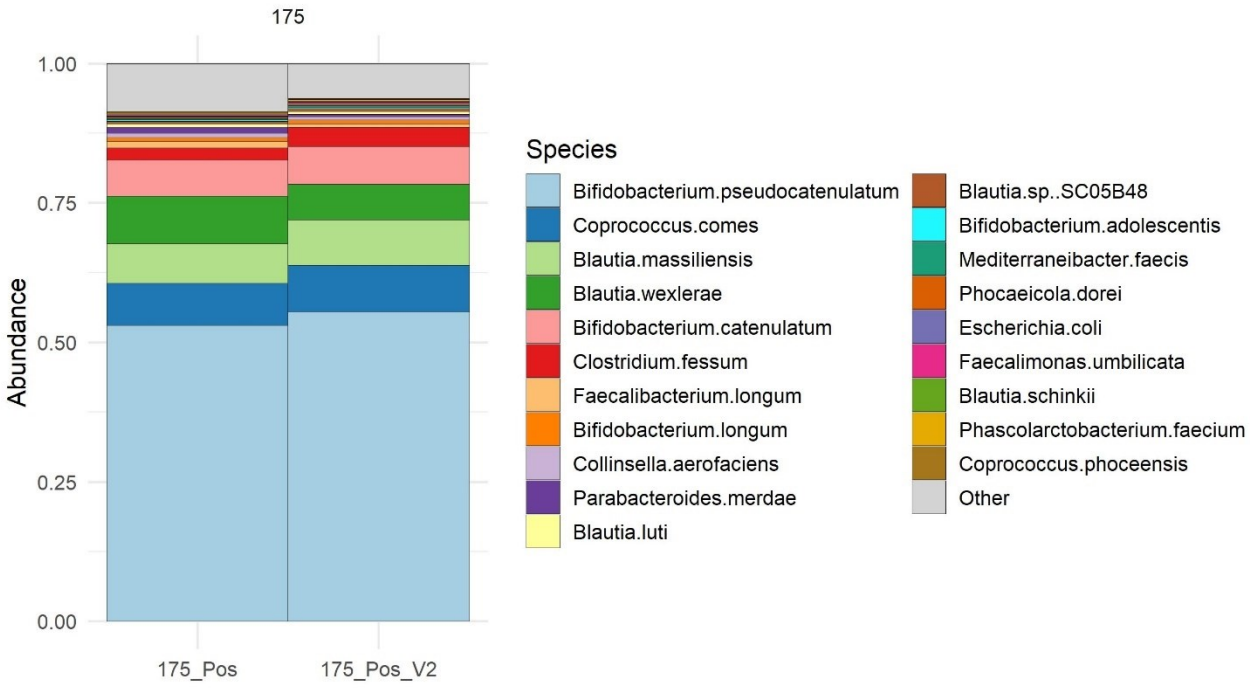


Figure 35: Two replicates of sample 175 IgG+ fraction (reproducibility control). Stool sample n°175 was suspended, incubated with autologous serum, and stained with secondary anti-IgG antibodies, before sorting IgG+ and IgG- fractions on a Biorad S3e cell-sorter. The procedure was repeated two consecutive days. Hence, sample n°175 had its IgG+ fraction sorted two times and sequenced two times. The figure shows, after DNA extraction and shotgun sequencing, the resulting microbiome profiles of the two duplicates. It suggests a satisfying reproducibility of the whole pipeline. Each vertical bar is one duplicate of the IgG+ fraction n°175, colored by bacterial species identified in it (as a fraction of the total number of classified reads).

Three samples were also sequenced in duplicates to account for the variability in the sequencing and bioinformatical pipeline (**Figure 36**).

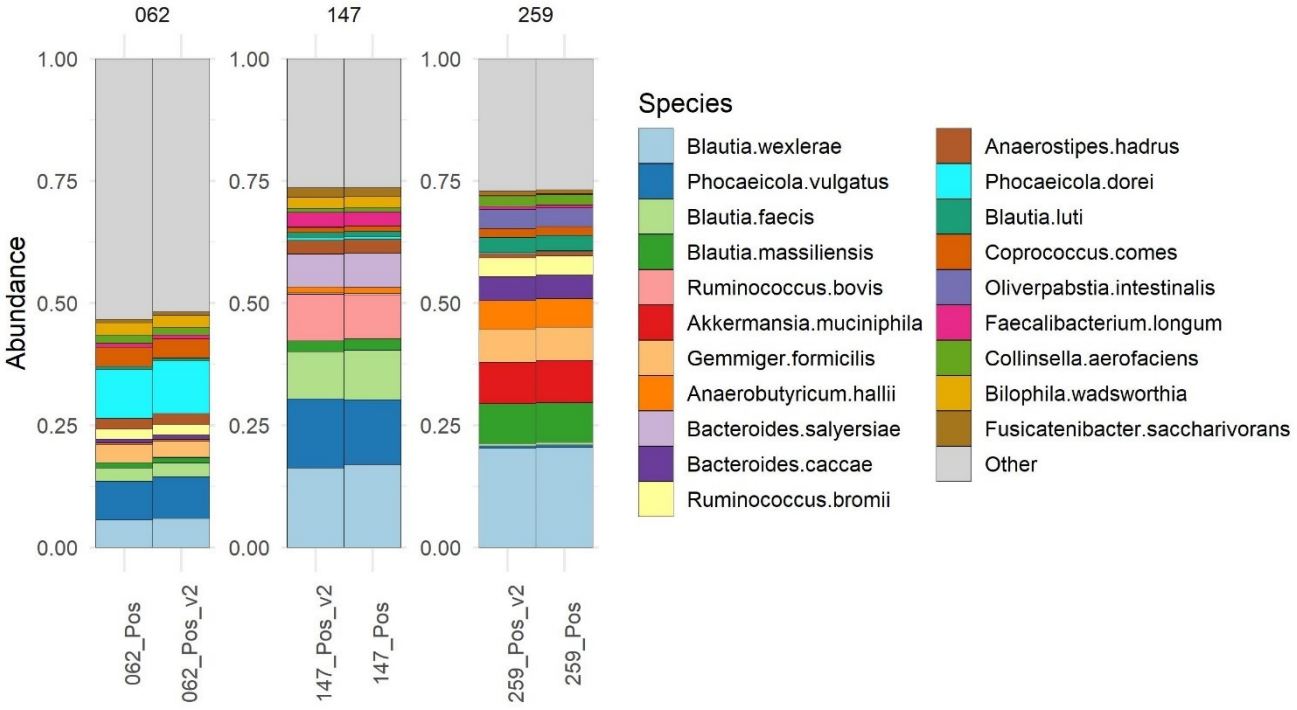


Figure 36: IgG-sorted fractions sequencing duplicates (quality control). Stool samples n°062, 147 and 259 were suspended in PBA, incubated with autologous serum and stained with secondary anti-IgG antibodies before sorting of the IgG+ and IgG- fractions on a Biorad S3e cell-sorter. After DNA extraction, the DNA content was loaded two times on the same shotgun sequencing run (Illimuna NovaSeq) to assess intra-batch variability. The resulting microbiome profiles of the two duplicates are shown. Each vertical bar corresponds to a duplicate, colored by the bacterial species identified in it as a fraction of the total number of classified reads.

Characteristics of the 35 studied individuals are provided in **Table 7** below.

Table 7: Characteristics of participants included in IgG-seq experiment

Variable	Control n = 14	Miss.	Autoimmunity n = 8	Miss.	Symptomatic n = 13	Miss.	p value
	n % of total in group Otherwise: Mean (SD)						
Female	79 %		100 %		100 %		0.085
Age	46 (10)		67 (10)		54 (17)		0.005
BMI	24 (4)		26 (5)		25 (4)		0.528
Share epitope copies							
0	100 %		50 %		54 %		<0.093
1	0 %		38 %		8 %		
2	0 %		12 %		38 %		
RA autoimmunity	0 %		100 %		54 %		<0.001
ACPA							
Negative	100 %		0 %		69 %		<0.001
Low	0 %		13 %		8 %		
High	0 %		87 %		23 %		
RF							
Negative	93 %		63 %		38 %		<0.052
Low	7 %		13 %		23 %		
High	0 %		25 %		38 %		
Anti-Ra33							
Negative	43 %	57 %	50 %	25 %	54 %	38 %	0.262
Low	0 %		25 %		8 %		
High	0 %		0 %		0 %		
Clinically Suspect Arthralgia (CSA)							
No	100 %		100 %		8 %	8 %	<0.001
Yes	0 %		0 %		85 %		
CSA score (detail)							
1	100 %		75 %		0 %		
2	0 %		25 %		8 %		
3	0 %		0 %		8 %	8 %	<0.001
4	0 %		0 %		54 %		
5	0 %		0 %		15%		
6	0 %		0 %		8 %		
Antibiotics (past 2 months)	0 %		0 %		15 %		0.166
Probiotics (past month)	0 %		0 %		8 %		0.419
Surgery (past 2 months)	0 %		13 %		8 %		0.443
Travel outside Europe (past month)	0 %		13 %		0 %		0.176

SD = Standard Deviation. BMI = Body Mass Index. RA = Rheumatoid Arthritis.
ACPA = Anti-citrullinated Peptide Antibodies. RF = Rheumatoid Factor. CSA = Clinically Suspect Arthralgia.
Notably, 4 patients with new-onset RA included in "symptomatic" group due to their diagnosis, however, did not meet threshold for "CSA" because of either missing data in questionnaires or not having obvious symptoms at the study visit (symptoms can fluctuate and regress between flares).

Among the 35 samples included (= 70 DNA fractions, one IgG- and one IgG+ per sample), the average number of classified reads (at the species level) per fraction was 2'294'966 (**Figure 37**).

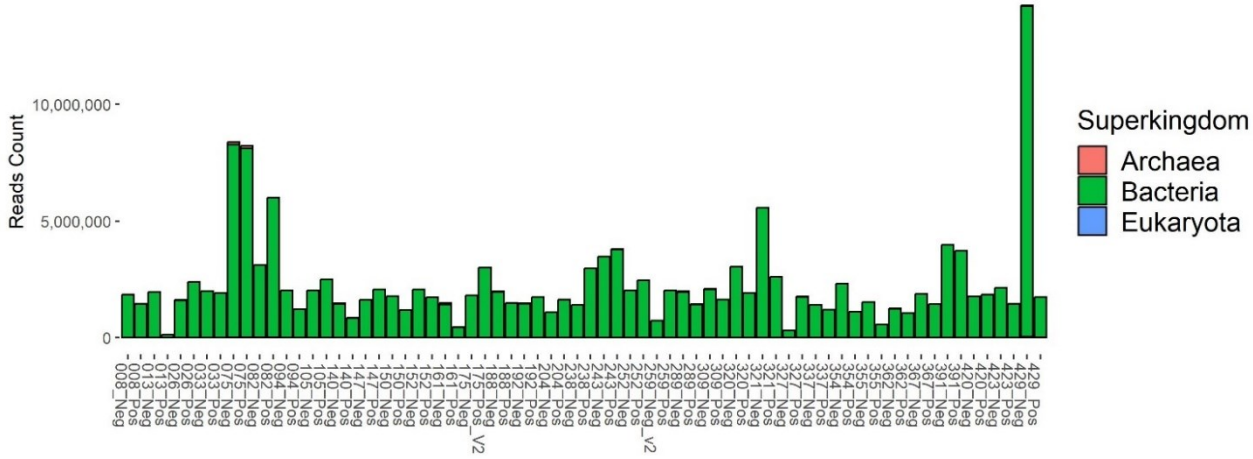


Figure 37: Read counts per sample. After performing the IgG-seq experiments, the IgG+ and IgG- fractions were processed through Zymobiomics MicroPrep DNA extraction kit. Shotgun sequencing of the DNA was performed on a Illimuna NovaSeq platform. This figure represents the number of reads per sorted IgG fraction which were successfully assigned to a taxon at the species level, when processed using our bioinformatical pipeline.

Based on the species-level taxonomy, expressed as relative abundance, a PCoA (using Bray-Curtis index) could be built. Overall, IgG- fractions were significantly different from IgG+ fractions (PERMANOVA $p < 0.001$; **Figure 38**).

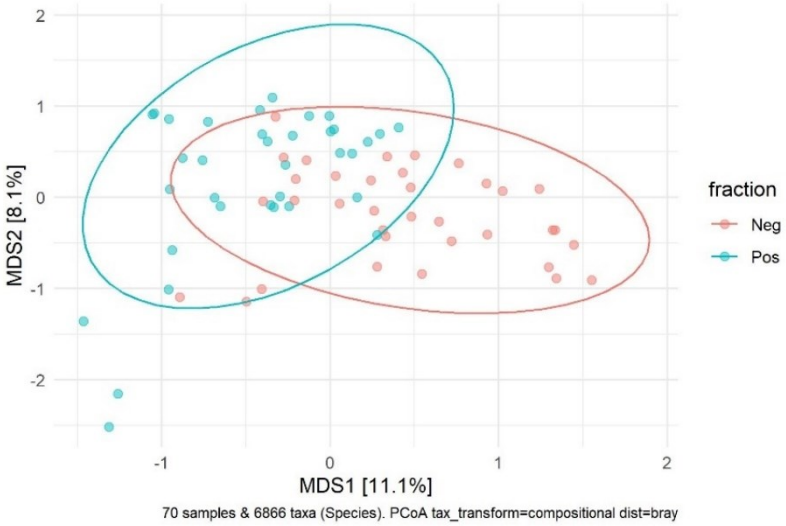


Figure 38: PCoA of the sorted IgG fraction. This figure is a principal coordinate analysis, i.e. the distance between points reflects their compositional difference. We see that IgG- and IgG+ fractions tend to respectively cluster together and be different. PERMANOVA $p < 0.001$.

After rarefaction, the dataset still contained 6875 taxa (species level). As a gross assessment, it is possible to merge the samples together, to represent the “average” composition of IgG- and IgG+ fractions per group.

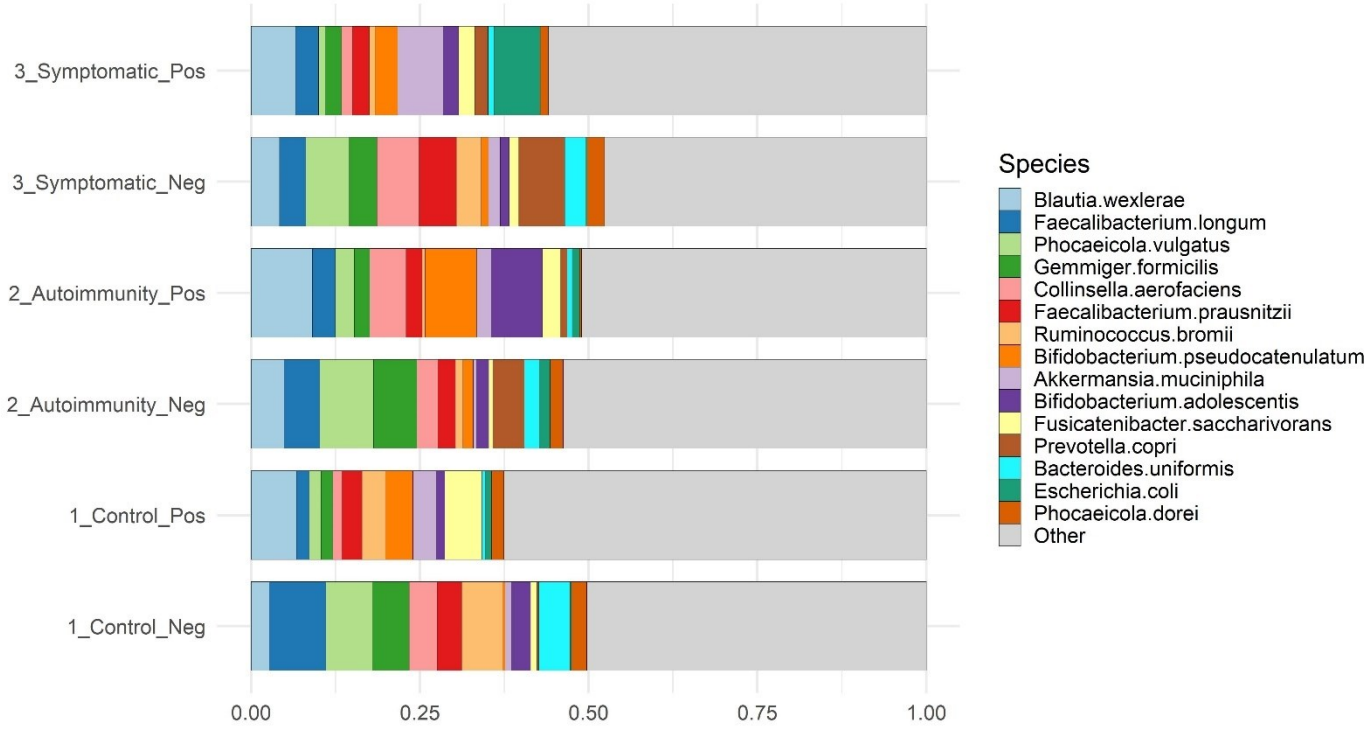


Figure 39: Species-level composition, by fraction (grouped). Fecal samples were incubated with autologous serum, stained with anti-IgG antibodies, and IgG+/IgG- fractions were separated using a Biorad S3e cell-sorter. After DNA extraction and shotgun sequencing, microbiome profiling of the IgG+ and IgG- fractions was done. This plot represents the “average” composition of the IgG+ and IgG- fractions, by group. Many species of lesser abundance could not be represented and are merged together in the “Other” label.

Prevotella copri could be detected in the data set, and by testing genome assembly we could identify several different strains with different numbers of genes (**Figure 40**). However, *Prevotella copri* was only present in a minority of samples with a low number of reads and mostly in the IgG- fractions. This does not suggest a particular IgG reactivity against *P. copri* in the assessed individuals.

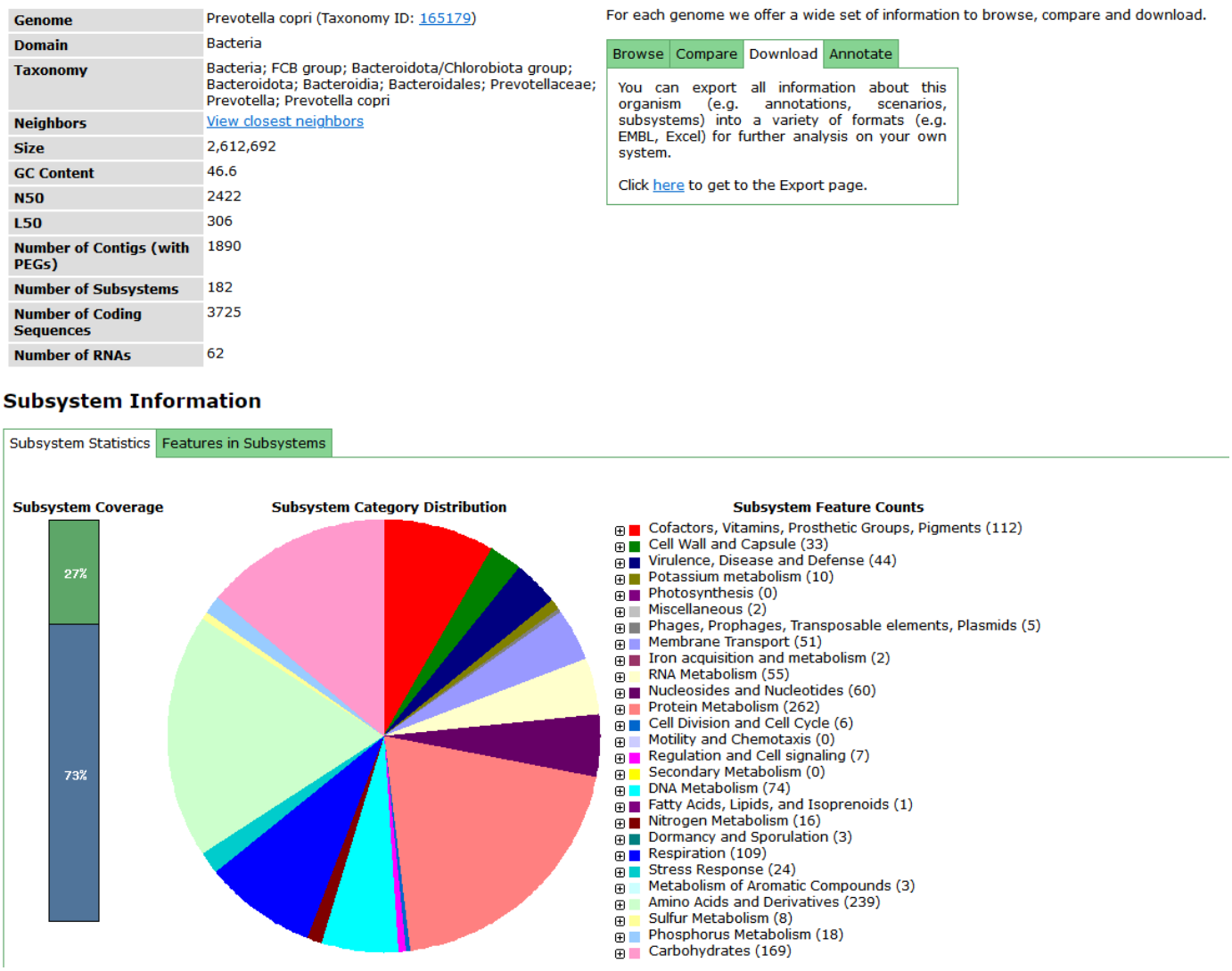


Figure 40: Genomic profile of a typical *Prevotella copri* strain. Fecal samples were incubated with autologous serum, stained with anti-IgG antibodies, and IgG+/IgG- fractions were separated using a Biorad S3e cell-sorter. After DNA extraction and shotgun sequencing, microbiome profiling of the IgG+ and IgG- fractions was done. Shotgun sequencing allows reconstructing whole genomes and therefore identify bacteria at the strain level. This figure represents the genomic characteristics of a typical *P. copri* strain from our dataset reconstructed from sample n°423 (IgG- fraction).

Figures below provide a detailed representation of the relative composition of the IgG- and IgG+ fractions of each sample.

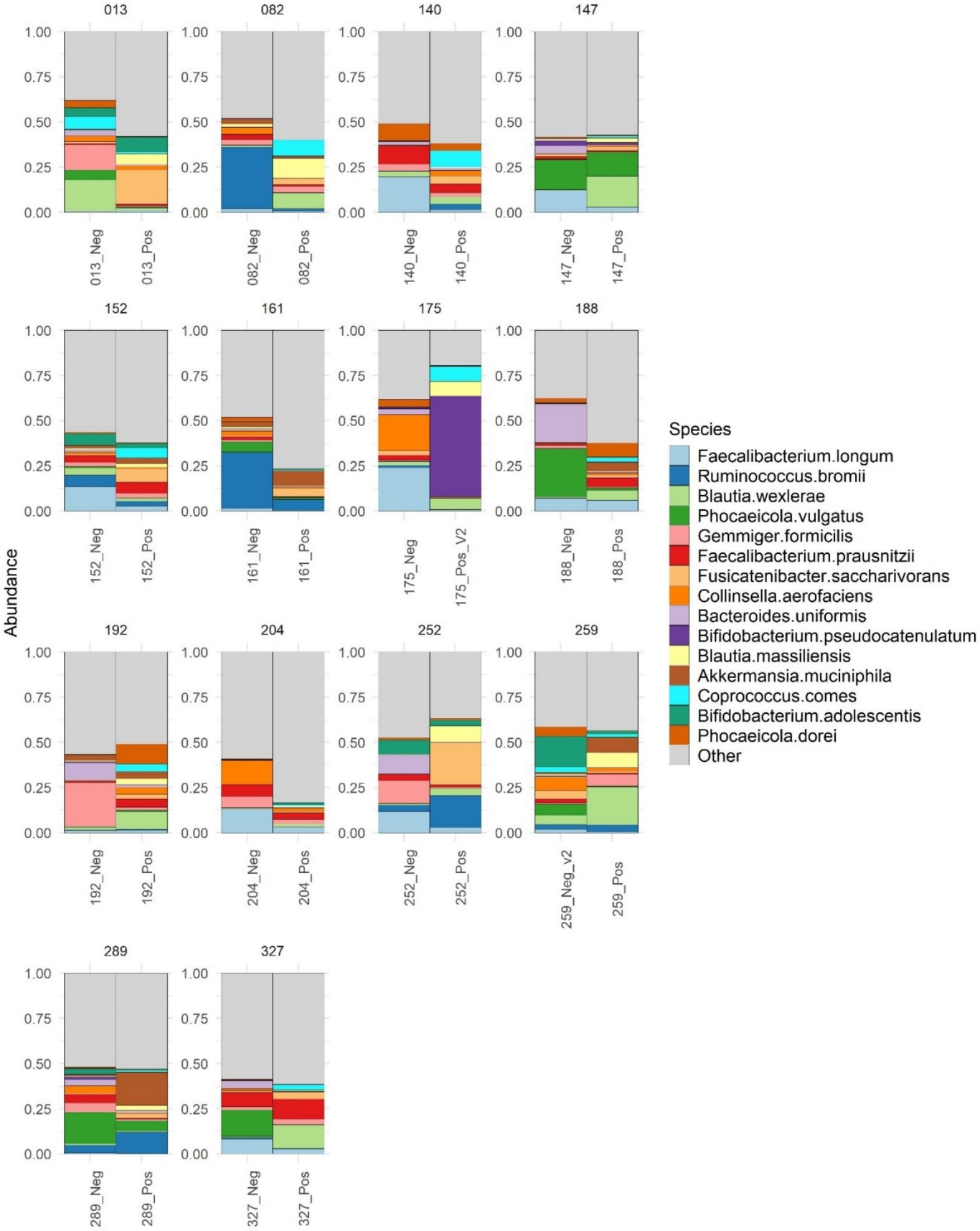


Figure 41: IgG+ and IgG- fractions composition (control group). Fecal samples were incubated with autologous serum stained with anti-IgG antibodies, and IgG+/IgG- fractions were separated using a Biorad S3e cell-sorter. After the DNA extraction and shotgun sequencing, microbiome profiling of the IgG+ and IgG- fractions was done. This figure shows the detailed microbiome profile at the species level of the IgG+ and IgG- fractions in the control group. Each pair of vertical bars is one sample with both IgG- and IgG+ fractions. An enrichment in the IgG+ fraction of a bacterial specie is to be interpreted as an IgG coating by autologous serum.

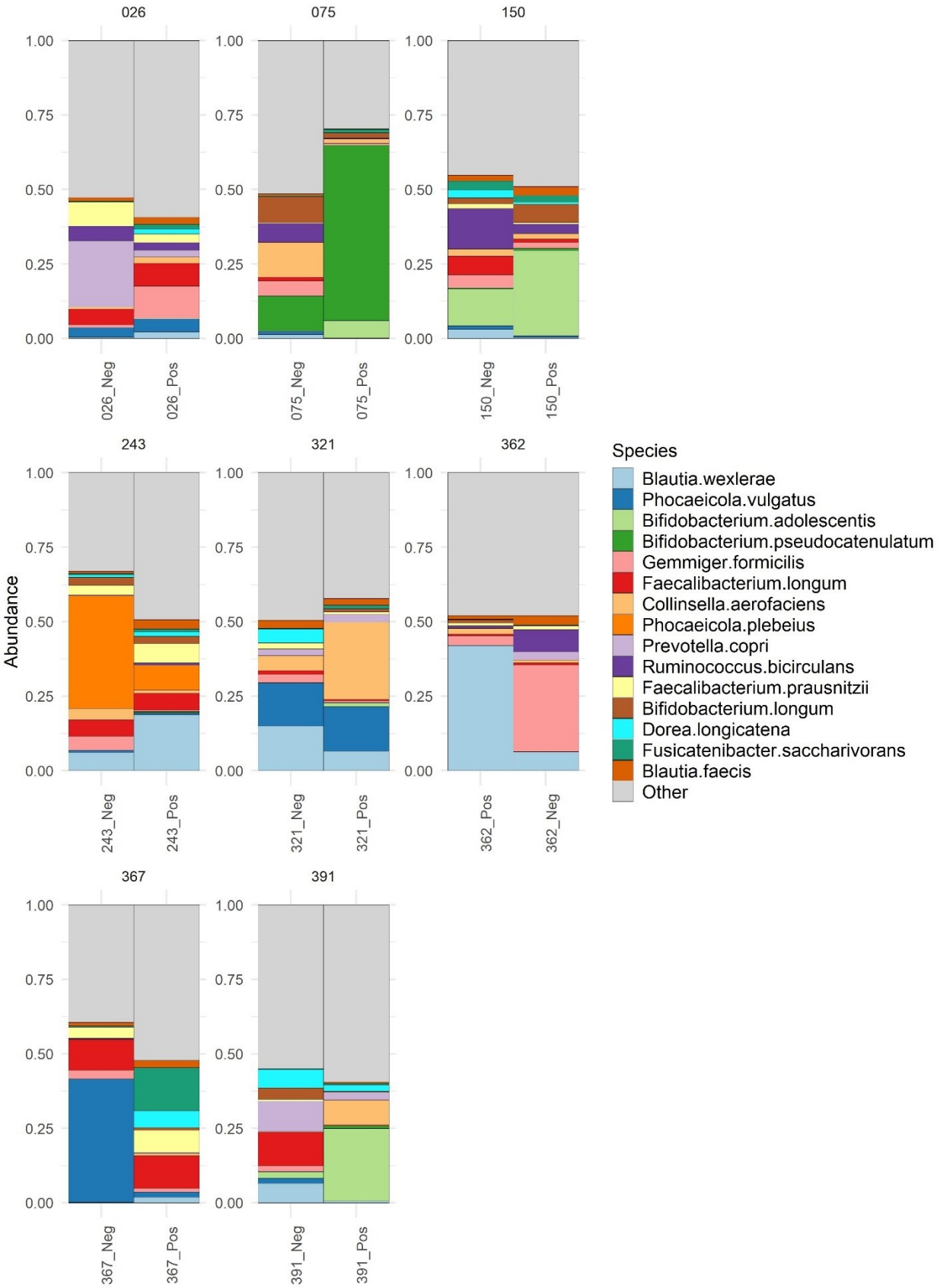


Figure 42: IgG+ and IgG- fractions composition (autoimmunity group). Same as in the previous figure, but for the autoimmunity group, i.e. SCREEN-RA participants with detectable RA auto-antibodies.

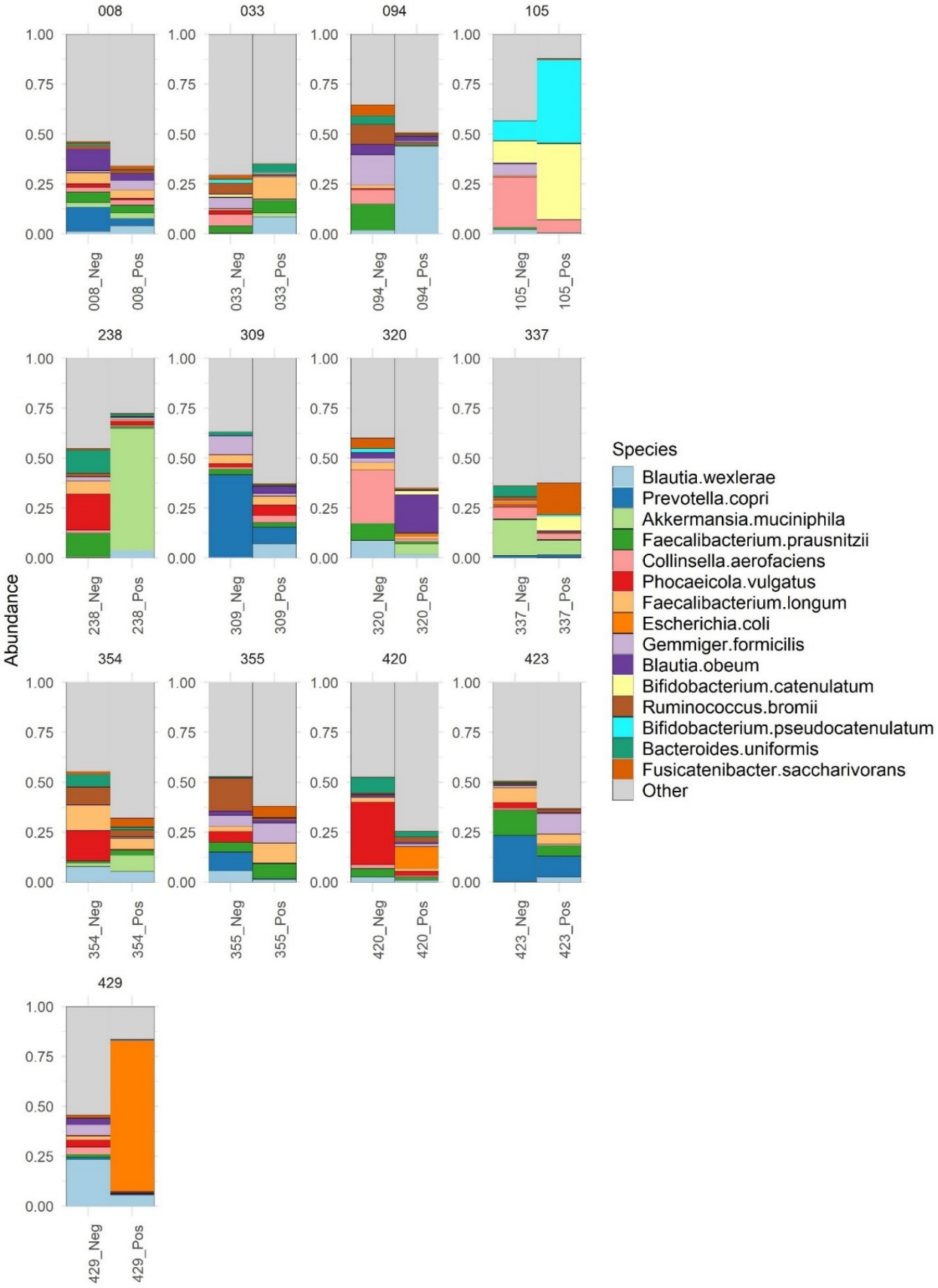


Figure 43: IgG+ and IgG- fractions composition (symptomatic group). Same as in the previous figure, but for the symptomatic group, i.e. SCREEN-RA participants with clinically suspect arthralgia.

Species enriched in the IgG+ fraction were compared to the IgG- as supposedly those targeted by serum IgG antibodies. To determine the most IgG-opsonized microbes we computed "coating index" as: $log10(\frac{IgG+taxon\ abundance}{IgG-taxon\ abundance})$.

To focus on the potentially most interesting taxa we looked for species that had a high coating index in at least two different individuals from the autoimmunity or the symptomatic groups. Then, we retained only the species that did not have the high IgG coating index in the control group (**Table 8**).

Apart from the known pathogens or antigenic microbes, such as *Klebsiella pneumoniae*, we found it surprising that several species of *Bifidobacterium* were highly IgG-coated in the case groups, in particular *Bifidobacterium adolescentis*), while they are usually reported as commensal bacteria.

Table 8 : Remarkably IgG-coated bacteria in autoimmunity and symptomatic groups.

Taxa	Control	Autoimmunity	Symptomatic
Agathobaculum butyriciproducens	0	1	2
Anaerotignum faecicola	0	1	1
Bifidobacterium adolescentis	0	3	0
Bifidobacterium dentium	0	1	1
Butyricicoccus faecihominis	0	1	1
Ellagibacter isourolithinifaciens	0	1	3
Eubacterium coprostanoligenes	0	1	1
Gemmiger formicilis	0	1	1
Holdemanella porci	0	0	3
Klebsiella pneumoniae	0	1	1
Tractidigestivibacter scatoligenes	0	1	1

This table shows the number of individuals for which the reported taxa has a high IgG coating index only in the autoimmunity or symptomatic group.

The **Figure 44** below shows and example of how *Bifidobacterium adolescentis* was differentially abundant in the IgG- and IgG+ fractions.

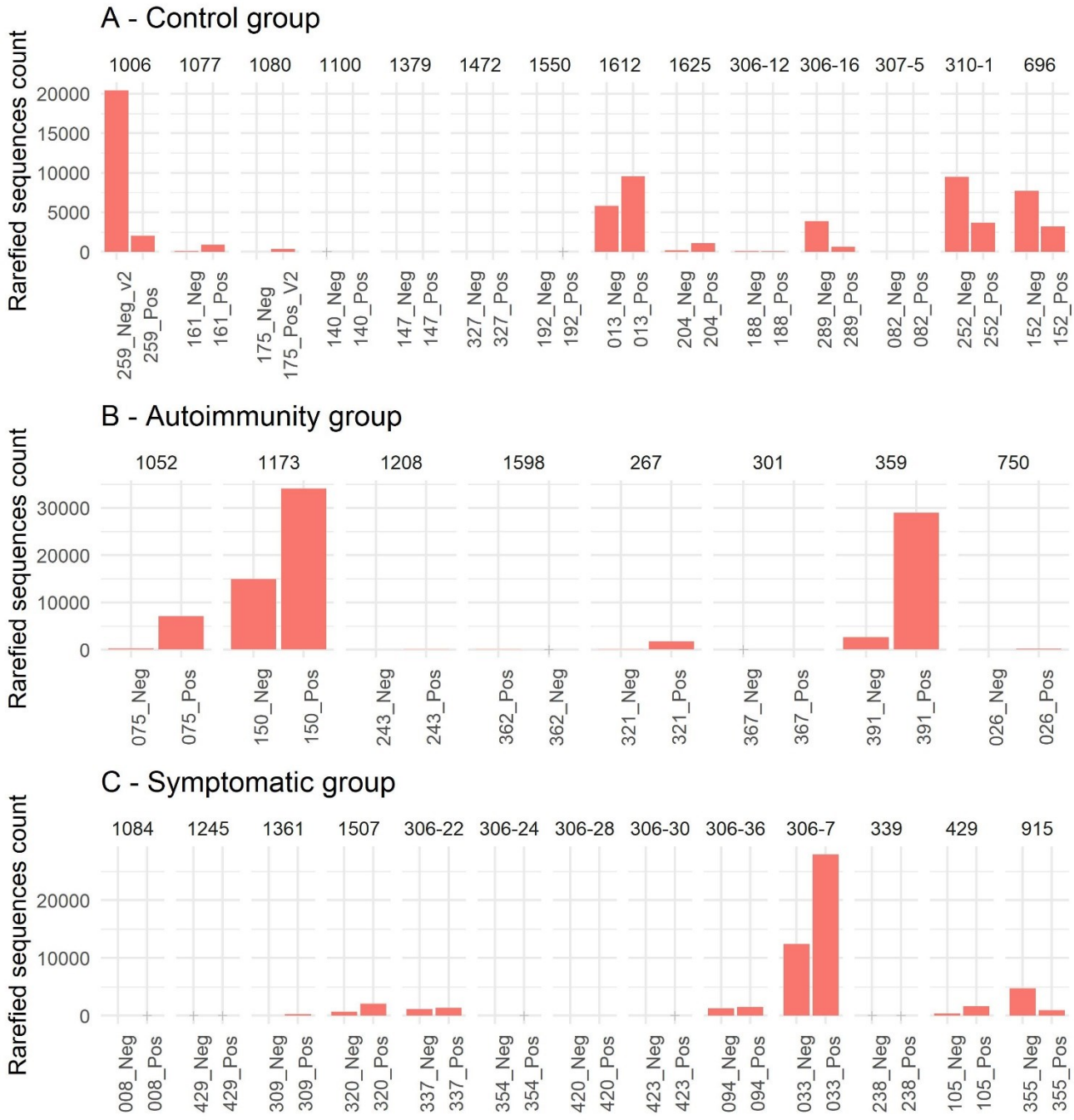


Figure 44: *Bifidobacterium adolescentis* sequence counts, per fraction per sample. Fecal samples were incubated with autologous serum stained with anti-IgG antibodies, and IgG+/IgG- fractions were separated using a Biorad S3e cell-sorter. After the DNA extraction and shotgun sequencing, microbiome profiling of the IgG+ and IgG- fractions was done. This figure shows the sequence counts of *B. adolescentis* after rarefaction of the data (total ~120'000 read per fraction). We see that the symptomatic (**B**) and autoimmunity (**C**) individuals who host detectable *B. adolescentis* tend to have it in the IgG+ fraction, suggesting an ongoing IgG immune reaction which is not seen in the control group (**C**).

Serum reactivity against *P. copri* (Amend et al.)

Using previous serum samples of the SCREEN-RA cohort, Amend et al. studied the IgG response of different pre-clinical RA subgroups and different patient groups against bacteria relevant to RA. Overall, no differences were found between the tested groups (**Figure 45**, **Figure 46**)

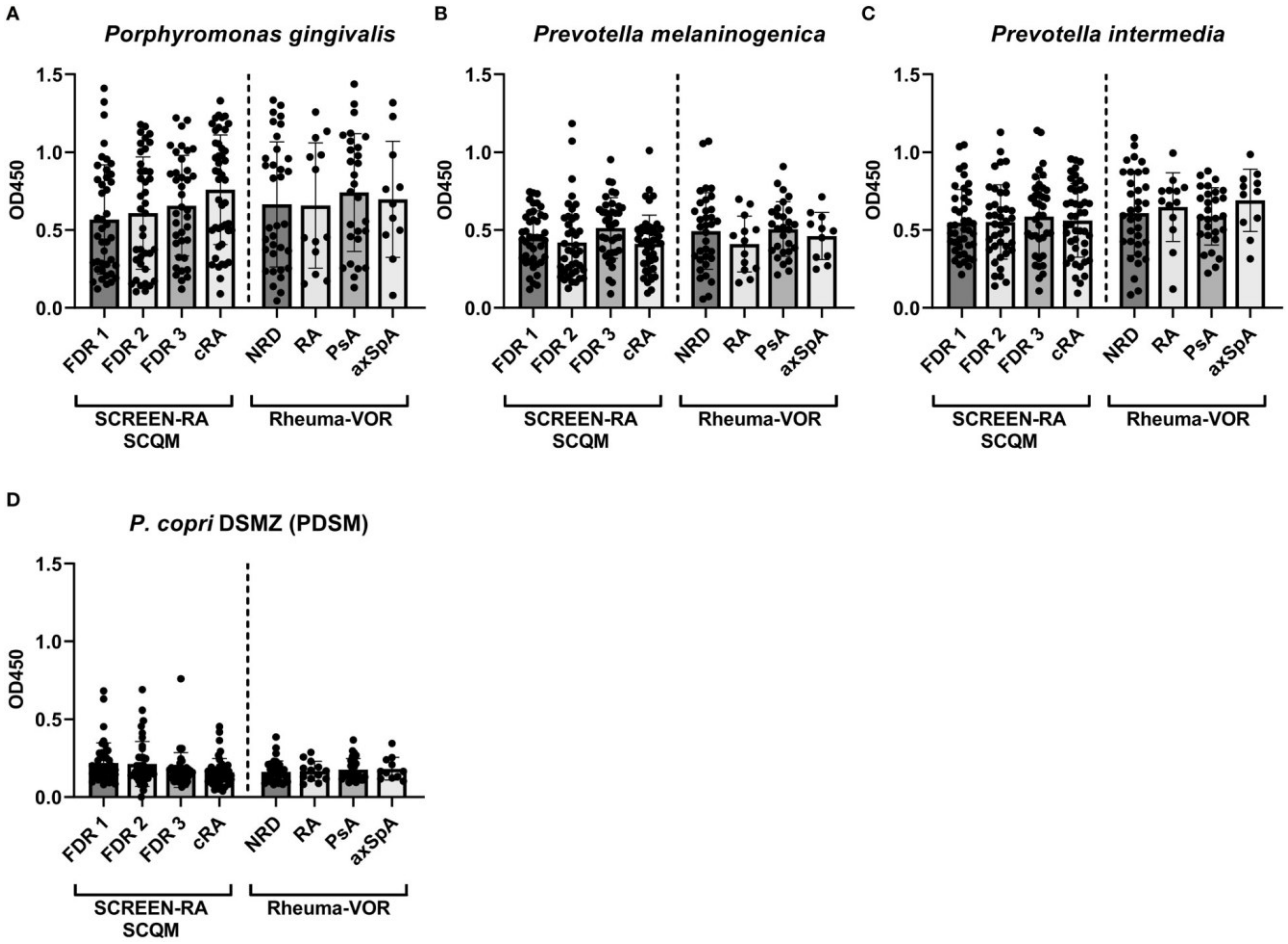


Figure 45: Serum IgG responses against oral pathobionts and the *P. copri* type strain in individuals at risk of RA, new onset rheumatic patients and patients with chronic RA (Amend et al.). IgG responses against the oral bacterial species *Porphyromonas gingivalis* (**A**), *Prevotella melaninogenica* (**B**), *Prevotella intermedia* (**C**) and against the *P. copri* type strain (PDSM) (**D**) in control group ("FDR 1", n=42), autoimmunity group ("FDR 2", n=40), symptomatic group ("FDR 3", n=38) and chronic treated RA patients ("cRA", n=45), as well as in patients with non-rheumatic disease ("NRD", n=34) or new onset RA ("RA", n=13), psoriatic arthritis ("PsA", n=28), and axial spondyloarthitis ("axSpA", n=11). Data is shown as the mean ± SD, differences are not statistically significant if not so indicated. Overall, there were no differences between groups. Figure reproduced from Amend et al., 2022, Front. Cell. Infect. Microbiol., https://doi.org/10.3389/fcimb.2022.1096211 , (license: author reuse).

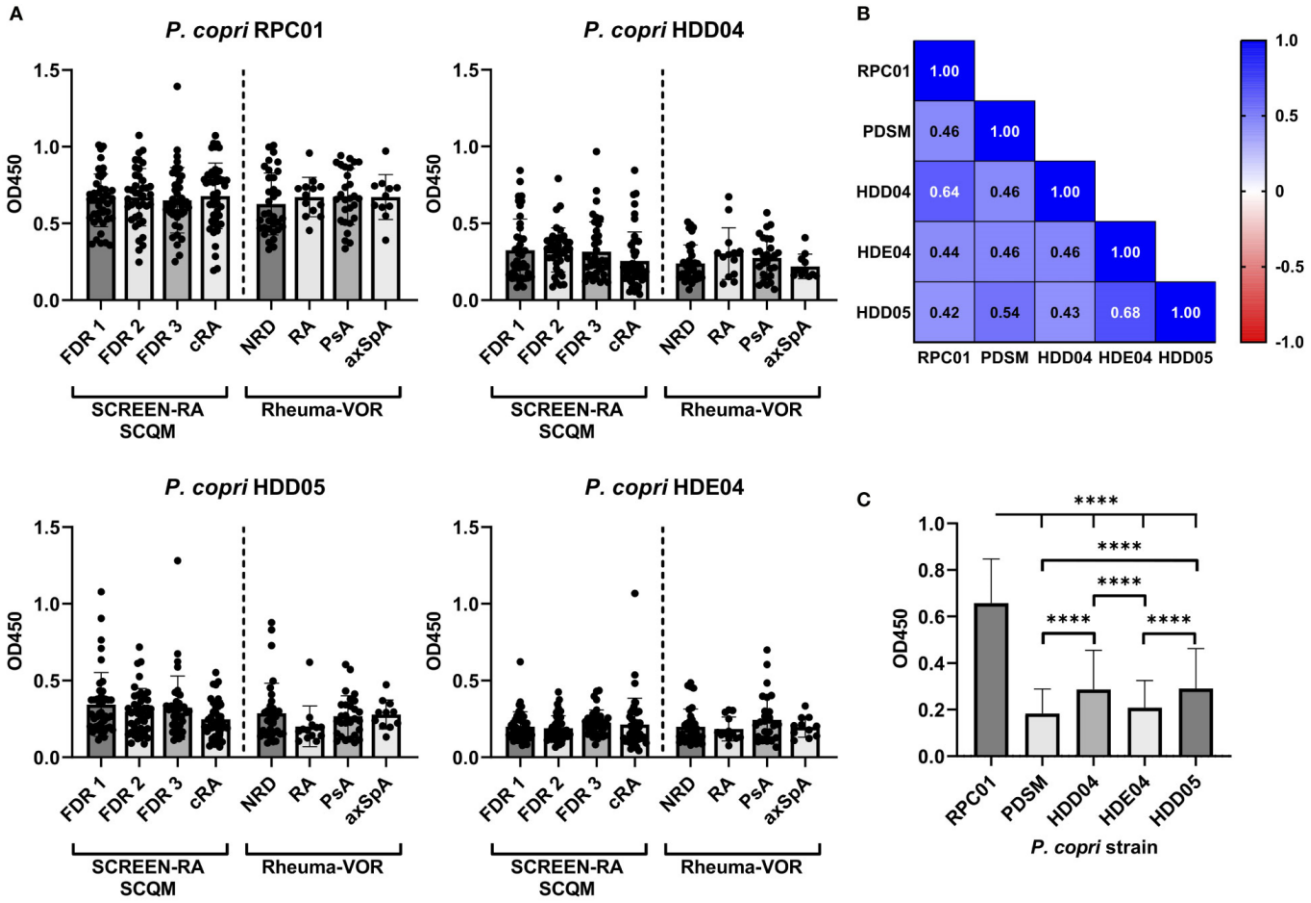


Figure 46 : Serum IgG responses against distinct, genetically different *P. copri* strains in individuals at risk of RA, new onset rheumatic patients and patients with chronic RA. (**A**) IgG responses against the different *P. copri* strains RPC01, HDD04, HDE04, and HDD05 in serum samples of control group ("FDR 1", n=42), autoimmunity group ("FDR 2", n=40), symptomatic group ("FDR 3", n=38) and chronic treated RA patients ("cRA", n=45), as well as in patients with non-rheumatic disease ("NRD", n=34) or new onset RA ("RA", n=13), psoriatic arthritis ("PsA", n=28), and axial spondylarthritis ("axSpA", n=11). (**B**) Correlation matrix showing Spearman's correlation coefficient (r) of serum IgG responses against the *P. copri* strains RPC01, PDSM, HDD04, HDE04, and HDD05, p-value of all comparisons < 0.0001 (****). (**C**) Averaged serum IgG responses against *P. copri* strains, independent of disease grouping, data analyzed using Friedman test. Data indicates mean ± SD, ****p < 0.0001, not significant if not further indicated. One of the four *P. copri* strains seems to have a particular immunogenicity, for there were overall higher IgG titers against it. Figure reproduced from Amend et al., 2022, Front. Cell. Infect. Microbiol., https://doi.org/10.3389/fcimb.2022.1096211 , (author reuse).

Discussion

This section presents preliminary evidence which shall be interpreted with caution. In particular, due to the way "IgG-coating" index was computed, the DNA with very low prevalence and thus being a potential background noise or contamination could still be identified as "highly coated." For instance, if 100 reads were found in the IgG+ fraction and 1 in the IgG- fraction the IgG-coating index would of log10(100). To minimize this problem we 1) removed samples with less than ~100'000 species-level classified reads in at least one of the fractions, 2) rarefied the sequence counts, which also results in filtering out low-prevalent taxa, 3) showed highly IgG-coated bacteria for which at least 500 rarefied reads were identified in the IgG+ fraction, in at least two different case individuals and none in the controls.

Overall, it seems that in all individuals IgG mostly targets specific microbes, hence, the IgG- and IgG+ fractions are significantly different, though, the composition of the IgG+ fraction is highly variable from a participant to another. Also, among the few individuals with autoimmunity or symptoms, who had detectable *P. copri* in their IgG fractions, *P. copri* was more detected in the IgG- fraction. This suggests the absence of particular IgG response towards *P. copri*, which is in line with the work of Amend et al.(381) Finally, when trying to determine the most IgG-coated microbes in the autoimmunity and symptomatic groups, we surprisingly found several species of *Bifidobacterium*. This could suggest a loss of tolerance towards the commensal flora, however, we should further verify the data and cross-link it with the additional experiments before confirming this assumption. Analyzing only 35 pairs of samples, while assessing up to 7'000 different bacterial species, creates a strong probability of false positive findings, which is not properly accounted for in this preliminary analysis.

Amend et al. findings corroborate ours as they were not able to demonstrate neither significantly increased titers of anti-*P. copri* IgG or the IgA in the serum or RA patients or in high-risk SCREEN-RA participants. Assuming no technical inconsistency in the custom ELISA of Amend et al., our results contradict the reports of Pianta et al.(292,294) as well as Seifert et al. from 2023, though they focused on an immune response only against the Pc-p27 protein and not on whole *P. copri* as we did.(852) One could argue that the latter publications might be influenced by the absence of normalization of serum Ig concentrations. Indeed, some patients with RA can have higher than normal circulating Ig, in particular IgA, which could have biased the reports of Pianta et al. and Seifert et al.(382,383) On the other hand, Amend et al. did not normalize Ig concentrations either (they focused on IgG and found similar total IgG concentrations across their study groups).(381) Even though Amend et al. noticed a small tendency for higher anti-*P. copri* response when patients had higher IgG titers, this trend was not significant in their dataset.(381) Regarding our IgG-Seq experiment the serum samples were all diluted at the same IgG concentration based on a measure performed by nephelometry at HUG Central Lab.

Last but not least, we found conceptual difficulties in interpreting microbe-specific serological IgG titers because, on one hand, such IgG response is likely to be initiated as a consequence of gut inflammation or bacterial translocation. On the other hand, once initiated, this response prevents bacterial translocation.(380) It is known, for instance, that intra-venous IgG treatment reduces bacterial translocation,(384) but the current literature interprets IgG coating as a sign of bacterial translocation.(385)

Finally, the preliminary data of Marianne GAZZANO did not suggest that individuals with autoimmunity or clinically suspect arthralgia had an altered overall pattern of IgA nor IgG anti-gut-microbe reactivity.

Forthcoming publication

Apart from the published work of Amend et al. the data outlined above are still preliminary. Results will be further expanded and are planned to be included in a publication together with the experiments of Marianne GAZZANO on the same set of samples in 2024.

III. COMMON DISCUSSION

LIMITATIONS

In this section, I will discuss the main limitations of my work. Given that I did my best to work with the same standards as other researchers in the field, the technicalities discussed below actually apply to most of the “microbiota-research” in RA.(386)

The press and editorial policies

Theoretically, the editorial decision to publish a scientific article should not be influenced by financial, political, ideological or any other unscientific motives. Punctual cases of such conflicts of interest regularly raise debates which are beyond the scope of the present thesis.(387–392) Apart from such direct corruptive interferences, I believe the scientific writing and editorial policies are also influenced in a more indirect manner, from the mainstream unscientific press shaping public opinion. It is not part of my work to demonstrate this conjecture here. However, I realized that the microbiome research is certainly subjected to such distortive processes in several ways:

- 1 - Many unscientific journals and magazines have broadcasted the idea that the gut “was the second brain”, that “microbiota” was a main driver of health and disease, etc., often oversimplifying or even distorting uncertain preliminary findings.
- 2 - The matter has thus become “trendy” also among the scientists, who may also read some of the popular press.
- 3 - Due to the growing appeal of this subject, the editors of scientific journal started searching for more content to publish, even if it meant being less stringent in their reviewing or decisions.

This resulted in two major issues:

- 4 - Methodologically unsatisfying research has been published in this field.
- 5 - Countless literature reviews have been published, acting as a sounding board for findings that were only preliminary or uncertain.

Consequently, most “microbiome” and especially the human-derived findings should be,(393) in my opinion, interpreted more cautiously.

RA definition – seropositivity framework

In medicine, a disease's name is to be conceived as a box into which patients are put if they fulfil a set of more or less objective criteria. Hence, such a group of patients are named with the same label because they have the same clinical and biological phenotype. And often we assume that this common phenotype is the result of common causes, and that is why we will treat them in a similar fashion. The classification relies heavily on the idea that a given phenotype is the result of certain causes, and, conversely, that this set of causes usually results in the same phenotype. But this is not as obvious as it seems.

Discussing the history of RA classification is instructive.(394) What we call RA today was previously classified together with gout and pseudo-gout. It is because the articular phenotype, from what could be observed at that time, was indeed similar: painful, non-infectious inflammation of joints, sometimes in a symmetrical manner.

It would be naïve to imagine that today's classifications are definitive. What we call RA could still be a mix of distinct pathophysiological pathways leading to apparently similar phenotypes. This question has become increasingly relevant in RA, with an ongoing discussion whether or not seronegative arthritis shall still be considered part of RA. Alternatively, cellular "pathotypes" are also discussed.(395,396)

In the present study, both seropositive and seronegative RA were considered (5/8 RA cases were seropositive), while another pre-clinical group includes an "autoimmunity" group, which corresponds to the framework of seropositive RA. Obviously, an early seronegative RA will not fall in our "autoimmunity" group; these individuals will jump directly from being "control" to "symptomatic" – as it happened to be the case for two of the participants after the study was completed.

The study design: pre-RA and cross-sectional design

As explained in section "RATIONALE AND OBJECTIVES" page 41, because we hypothesize microbiome to play a causal role in RA development, we aimed to study what happens before RA fully developed. The phases preceding clinical RA are termed "pre-clinical" phases of RA.(80) However, there is a recurrent confusion in this terminology. Strictly speaking, "pre-clinical RA" is **not** synonym with "pre-RA"; the latter designates individuals who are known to have subsequently developed RA. Studying samples of individuals X years before diagnosis and comparing them with matched controls would have been the ideal study design.

As I am writing, only two samples from work-package 1 have newly developed RA (about two years after sampling). Those are the only two actual "pre-RA" samples at our disposal. Ironically, they are

both seronegative RA and were asymptomatic at the time of stool sampling, two years ago, and therefore, assigned to the control group in my experiments!

Regarding other participants, it is important to keep in mind that we have absolutely no idea if "autoimmunity" or "symptomatic" individuals will subsequently effectively develop RA; we only assumed that having signs and symptoms associated with RA would increase their future risk of developing RA. This assumption is based on external studies performed within other cohorts.

Also, the problem of comparing humans is that they are phenotypically diverse; as our study is not randomized, we had no control over participant's diet, smoking habits, stress, pollutants exposure, etc. For work-package 1 subgroup analysis and work-package 2 we did our best to select the controls in such a way that they are comparable at least regarding sex and age with the case groups. However, we cannot exclude that some other environmental variables could be differentially present in the groups and bias our findings. In particular, food frequency questionnaires were in fact filled by all participants, but we have not been able to process this valuable metadata yet. Also, one could hypothesize circular causality : it would be possible that patients with joint pain would modify their eating habits in an attempt to cope with their state, i.e. eating more fibers and, therefore, selecting fiber-degrading taxa such as Prevotellaceae.

Exposure of interest: group assignment

In the absence of sufficient "pre-RA" samples, we used "pre-clinical stages" of RA development as our exposure of interest. A corner stone of our study design is thus the definition of "pre-clinical stages". Our strategy was to use all the collected samples. Consequently, we had to assign each person to a group. This implied defining cutoffs; however imperfect they can be. Although the group placement algorithm is already represented in section "Exposure of interest (case and control definition)", page 45, I would like to point-out a few consequences of this methodology.

- We did not consider slightly increased levels of RF, between 1 to 3 times the upper limit of the norm (ULN), as something clinically relevant. This is mostly based on expert opinion, and similar cutoffs have been used in comparable studies to increase the specificity of classification algorithms using RF. However, it is also debatable. For the little we know, it is unusual to be seropositive for RF under the age of 85.(397) Yet, 10% of our control participants were mildly seropositive for RF (i.e., between 1x and 3x the ULN), but were not classified as having 'autoimmunity associated with RA'.
- The same comment applies to anti-Ra33 serologies. Furthermore, due to technical reasons, anti-Ra33 titers were measured only one time. Consequently, 50% of controls have unknown

anti-Ra33 serology as they did not have a recent sample with available measurement. In other words, in the present study we assumed current anti-Ra33 serology based on serological measures obtained months to years before the stool sampling of interest, which explains why we only considered high titers as clinically significant. We preferred using all the available information, even if imperfect, rather than ignoring available anti-Ra33 serologies.

- Since the cutoff for having "clinically suspect arthralgias" (CSA) was defined as a score of at least 4, or 3 in case of one or two missing items or concomitant autoimmunity, 33% of control individuals (out of total 226) had a CSA score of 2 or 3. The latter means that, despite being labeled "control", they could have mild arthralgia, however they did not meet more than one or two criteria of the CSA score. Although this is not strictly synonym of being totally asymptomatic. These persons were preferentially removed from work-package 2 and other subgroup analyses, where we attempted to focus only on particularly extreme phenotypes.

Finally, as mentioned in section "COMMON METHODS", several different kits may have been used to assess ACPA serology, and most participant have had previous samples in the SCREEN-RA biobank. Even though it happens rarely, it is theoretically possible that a participant was positive using a given kit on a previous sample, but then negative using another kit on the sample provided for this thesis (see the various ELISA kits page 44). In case of two discordant recent results on two different ELISA kits the situation becomes blurry. Our algorithm would still consider the previous positive value as relevant and classify the participant in the "autoimmunity" group.

This participant classification was designed and defined before obtaining the microbiome analyses and biomarkers. As a result of these classification efforts and assessment issues is that our groups of interest are phenotypically quite close to each other, making any statistical signal even less prominent (issues of non-differential misclassification). Even if alternative groupings or subgroupings could have been tried, we decided not to report them to avoid spurious results and "fishing expeditions".

Assessor and patient-derived data collection

Other potential sources of misclassification bias are:

- The subjectivity of pain and articular assessment by various study nurses and myself. Assessor may not always agree on what a "tender" or "swollen" joint is, in particular, when joints are deformed by chronic osteoarthritis (arthrosis) they may sometimes have been erroneously labelled as swollen.

- Some items of the CSA score rely on patient's answers to his follow-up questionnaire (for instance, morning stiffness). There were 4% undermined CSA scores in the control group

because of missing data. This is also the case for one new-onset RA, who was assigned to the "symptomatic" group though having a missing CSA score.

On phenotype fluctuations

As mentioned in section "Fluctuations and seronegative rheumatoid arthritis", page 19, it is known from a few cohort studies that ACPA and RF seropositivity are sometimes reversible, especially when present at low titers.(95–98) We have also noticed this phenomenon in the SCREEN-RA cohort, and it adds an additional layer of complexity and mis-classification.

If someone has had mild titers of ACPA, which later become negative, we were unsure if we should still consider this person a "clean control". The supposition is that having had ACPAs in the past could induce susceptibility to autoimmunity in the future, for instance if ACPA-specific memory B-cells were generated in the process. Our "control" group might include such participants.

Even after RA diagnosis, the disease still manifests itself through recurrent flares alternating with calm periods. In the "symptomatic" group, 4/8 RA patients did not fulfill the criteria for CSA because:

- Being in between two crises and having used NSAID, they had no pain the day of the assessment (subjects n° 306-5 and 306-22).
- Having been on NSAID and glucocorticoid regimen a few weeks before, as their diagnosis was still being discussed at that time (n°1363).
- Having joint involvement but not having reported the morning stiffness and other CSA questions; this could have happened because the participants might have misunderstood the questions) (n° 306-31).

In the latter cases, the RA diagnosis was retained for group assignment despite the CSA scores.

The rational of stool sampling

As mentioned in "COMMON METHODS", page 43, we assessed intestinal microbiome using fecal samples mostly because of practical considerations. However, biologically speaking, nothing guaranties that the stool represents correctly what the immune system is facing at the mucosal level.(398) Furthermore, even at the intestinal mucosal level the microbiome varies all along the digestive tract.(399) Feces are probably the most representative of sigmoid/rectum microbiome, which, for the little we know, may not be exactly similar to the colonic content, nor to the actual mucosal microbiome.(206,400,401) Nonetheless, the majority of the intestinal immune system is located in Peyer's patches in the **small** intestine or the appendix. Consequently, we cannot be sure if the increased

fecal abundance of Prevotellaceae truly means that the mucosal immune system is exposed to pathogenic loads of such bacteria.

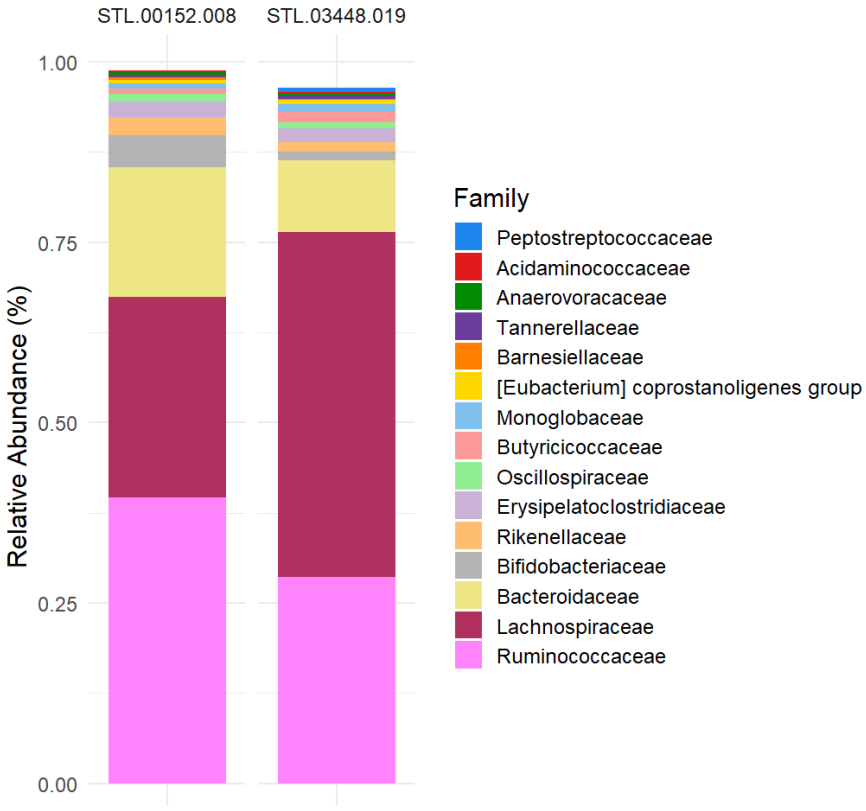


Figure 47: repeated stool sampling, subject n°923. This plot shows the relative composition at the Family level of the two stool samples mistakenly provided by subject n°923.

Actually, certain *Prevotella* species have been found invading the mucus layer in IBD models,(371) or in human colonic cancer related studies.(372,373) We regret it was not possible to obtain intestinal biopsy samples from the SCREEN-RA cohort; which would have been the only way to properly assess if mucus invasion also happens in the context of RA and preclinical RA.

It is not clear if microbiota profiling is representative of the long-term gut flora composition. In work-package 1, subject n°923 mistakenly provided stool samples two consecutive years; even if not exactly similar, their microbiota profile resembles each other (**Figure 47**).

Few studies have formally assessed the intra-individual variability of feces composition in case of longitudinal repeated sampling, which seems to be largely underestimated.(402)

Sample collection

One may ask if the sampling procedure itself could have generated biases. I believe that in this regard the SCREEN-RA cohort's sampling was satisfactory and fulfilled our needs. We provided the operating procedures and supervision to all of the recruitment centers, and the serum sampling and storing system was already ongoing for 10 years. Dividing serum in several aliquot also greatly minimized the impact of thawing cycles during later processing.

However, regarding stool sampling, feces were not always collected on the same day as the corresponding serum sample. The average time difference between the two was 2.55 days, with a standard deviation of 13.6 days due to a small number of outliers. As the immunoglobulin contents should not fluctuate much in such a short time interval,(403) we believe that the collected serum is fairly representative of the participant's status on the day of stool sampling. Lastly, the stool samples were divided in several aliquots before freezing by the participant. Each aliquot was later used for analyzing for only one parameter. It is theoretically possible that the feces material was not homogenous enough, so that small compositional differences may exist between the aliquots. We have not formally tested that, but the Lab of Prof. Raes where I performed the microbiota analysis was aware of the phenomenon (unpublished data).

Sample processing

Work-package 1

Within the context of work-package 1, the stool samples were handled mostly on a robotized platform with well established procedures by the RAES Lab (Leuven, Belgium); the technical biases introduced were as low as possible given the current technology. Multiple standardized DNA preparations as well as some SCREEN-RA samples served as quality controls and were loaded repeatedly across the several sequencing batches. Still, one can easily notice minor differences between the SCREEN-RA quality control duplicates (paired below). Consequently, because of the inherent imprecision of sequencers, I am not sure how to interpret minor numerical differences in the final output.

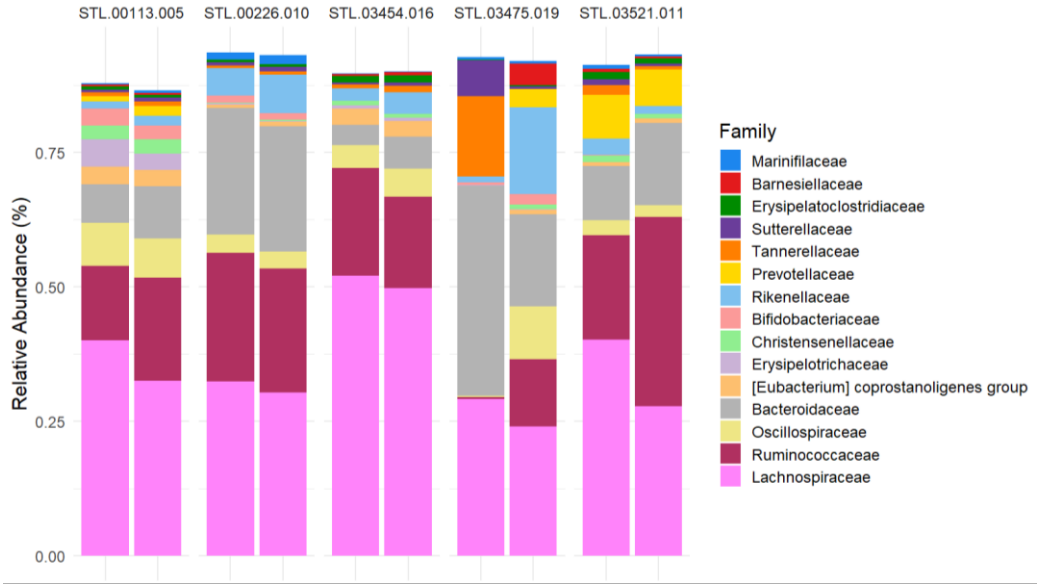


Figure 48: Quality control duplicates of work-package 1. This figure shows the relative composition of stool samples which were analyzed twice, as inter-batch quality controls.

Work package 2

For work-package 2, I have randomized samples between several ELISA batches and performed the measurements in duplicates. To obtain more precise ELISA tests, it would have been better to use a robot, however, it was not available for the desired protocols. Still, the coefficients of variability that I obtained were very acceptable. I even tried to assess the impact of thawing cycles by repeating measures for a third time after an additional tawing. The correlations between pre- and post-thawing were acceptable, and we remarked that freezing cycles tended to lower the concentrations of measured proteins. Hence, we could have slightly underestimated serum I-FABP, calprotectin and LBP concentrations in our study as the samples had to be thawed twice during our protocol.

Work-package 3

We probably faced the most technical limitations when conducting work-package 3.

First, because of the temporal and technical constraints, I had to prepare the microbiome suspensions in Geneva and freeze them again for later use in cytometry, which was done in Paris. Hence, the freezing cycle, together with the aerobic homogenization process, was likely to have lysed a significant proportion of the bacteria – the magnitude of the latter was not thoroughly investigated; but an important number of debris were certainly accompanying the whole bacteria in the PBS suspension.

Also, the fecal pellets were of homogenous sizes, but due to inter-individual variation of the fecal material it was not possible to standardize the number of bacterial cells in each preparation since the density could vary greatly.

Then, the flow-cytometry experiments were perturbed by the substantial number of debris which were perceived by the cytometers as “events”. Coloring all particles with SYBER-Green (which stains DNA) allowed distinguishing the debris from the whole bacteria, which comprise only ~40% of the events (**Figure 49**).

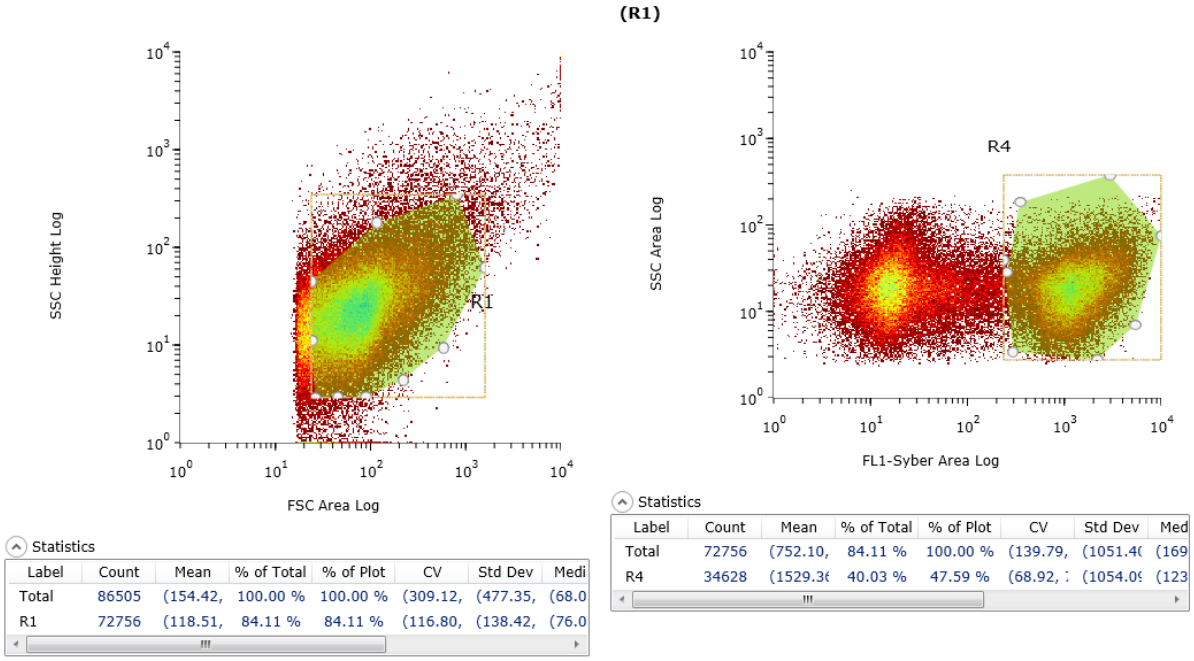


Figure 49: Cell-sorter acquisition of test microbiome sample. This figure is a screen shot of flowing the test sample n°423 through the BioRad S3e cell-sorter used in work-package 3. **Left panel**: forward- and side-scatter of suspended particles. **Right panel**: separation between bacteria and debris after staining DNA with SYBR-Green, on the horizontal axis. The DNA-containing particles, which are mostly bacteria, only represent ~40% of the suspended material.

When performing the IgG sorting I could not use the DNA stain since the FITC secondary antibodies needed the same color canal, and the other available lasers were suspected to not work properly – consequently, I was not able to distinguish the sorted bacteria from sorted debris, which made it difficult to ensure that enough bacteria and DNA were sorted in each fraction, in particular for the IgG-fractions. The latter explains why some samples were finally not analyzable due to low DNA input.

In addition, a recurrent issue with Ig-Seq experiment is the non-specific binding of primary antibodies due to serum IgG or IgA antibodies binding bacteria with their Fc-fragment. We tried to address this limitation by using Infliximab instead of the participant's serum as part of the negative controls. Infliximab is a humanized anti-TNF antibody; hence, if binding the bacteria, it must do it through its Fc-fragment. In such a case, the sorting gates were adapted to try to avoid the particles representative of those bound by Infliximab, which was not a perfectly clean workaround.

Last but not least, the sorted fractions, as outputted by the cytometer (~10 mL), needed to be concentrated and pelleted by ultracentrifugation for storage at -80°C (frozen again); loss in the DNA material might again have occurred at this step.

Overall, the IgG-Seq pipeline, even though conducted as meticulously as possible, could still largely be improved, in particular by optimizing the stool sample preparation and separation of the bacteria from the stool debris. I also believe that, despite the fact that the negative controls were quite satisfying, more positive controls should have been designed and included to quantify the accuracy and reproducibility of the method – the only previously available data was obtained on clean bacterial cultures,(404) which might not be representative of the conditions I have worked with.

The problem of biomarkers

The problem of biomarker is that, even without the technical limitations, they are still only approximations of the true outcome of interest. I have already discussed in work-package 2 how LBP was misleadingly used as a marker of intestinal permeability.

A few more words on permeability markers and serum calprotectin.

Gut permeability measurement

Some authors argued that serum biomarkers, such as zonulin, could be used to approximate the results of a lactulose-mannitol urinary excretion test. For instance, Sapone et al. have made such a claim.(232) However, the claimed correlation is derived from a linear regression improperly applied to a dataset largely driven by 4 outliers, visible on their figure 2C (reproduced below).

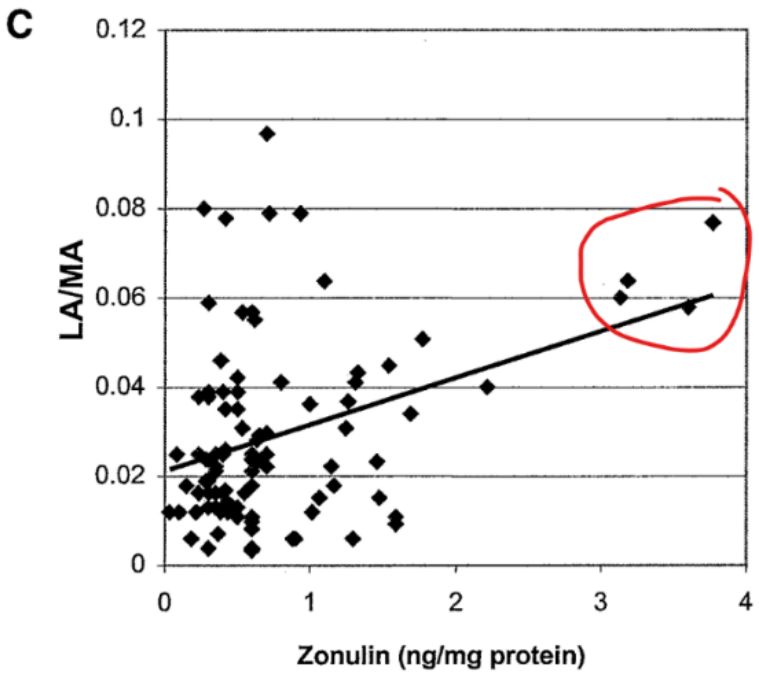


Figure 50: Zonulin levels versus lactulose/mannitol urinary excretion ratio. Measured in in type 1 diabetic patients (n = 36), their relatives (n = 56), and healthy control subjects (n = 43). Reproduced from Anna Sapone et al., Diabetes, 2006; https://doi.org/10.2337/db05-1593 , under license n°5602420660185 .

I decided to further review the published correlations between alleged biomarkers and such *in vivo* intestinal permeability tests. The findings, summarized in **Table 9** below, were deceiving. Not only do the functional permeability tests have their own limitations (discussed in work-package 2), but the proxy biomarkers seem completely inconsistent with the functional tests. I think our results of serum I-FABP are valuable, but it is unclear to me if higher serum I-FABP would have resulted in higher urinary excretion of lactulose or mannitol, had we simultaneously done such *in vivo* tests. In other words, after re-reviewing the literature, I finally found no data to support the idea that lactulose/mannitol tests measure the same thing as serum I-FABP.

Table 9 : Review of the association between oral permeability tests and surrogate biomarkers

Context	Oral test	Zonulin	LBP	sCD14	I-FABP	IL-6	Ref.
Type 1 diabetes: 36 Relative: 56 Healthy controls: 43	LMR	**Correlated** (Driven by 4 outliers)	-	-	-	-	Sapone et al. 2006 (232)
Crohn disease with (16) or without (12) high GM-CSF Auto-Ab, and healthy controls (15)	LMR	-	**Not correlated**	-	-	-	Nylund et al., 2011 (405)
Healthy young males: 20 Crossover, several measures	LMR	**Not correlated** (Data not shown)	-	-	-	-	Russo et al 2012 (406)
Obese: 12 Normal: 12 3x samples (longitudinal)	LMR	**Not correlated**	**Not correlated**	-	-	-	Kuzma et al, 2016 (407,408)
Cirrhosis patients: 46 Healthy controls: 16	LMR	-	**Not correlated**	-	**Not correlated**	**Correlated** (Modestly)	Vogt et al. 2016 (409)
Migraine patients: 63	LMR	**Not correlated** (Data not shown)	-	-	-	-	De Roos et al. 2017 (410)
Type 2 diabetes: 32 Controls healthy: 30	52Cr-EDTA	-	**Not correlated**	**Not correlated**	-	-	Pedersen et al. 2018 (411)
Crohn disease patients: 60	52Cr-EDTA	-	-	-	-	-	Von Martels et al. 2019 (412)
Human and mice experiments	LMR	**Not reported** (let's ask them?)	-	-	-	-	Tajik et al. 2020 (235)
Healthy: 51 Obese: 27	LMR	**Not correlated**	**Correlated** (Independent of age, BMI and sex)	-	**Not correlated**	-	Seethaler et al. 2021 (354)
Children in Indonesia	LMR	-	**Not correlated**	-	**Not correlated**	-	Amaruddin et al. 2022 (220)

LMR = urinary Lactulose Mannitol Ratio, dosed after oral challenge. IL-6 is more a systemic inflammation marker, but I still report it, for it was correlated with LMR. Reproduced from supp. of Gilbert et al., 2023, https://doi.org/10.3389/fimmu.2023.1117742 , (license: author reuse).

Serum Calprotectin

While we were conducting our study, the first report of serum calprotectin assessment in a pre-RA population was published.(413) Though ACPA and RF seropositivity were the best predictors of RA diagnosis, Bettner et al. suggested that serum calprotectin improved the predictive value of these parameters. They hypothesized that neutrophil activation and NETosis may be increased for some individuals during the pre-RA period.(358)

Our findings, both regarding fecal calprotectin and serum calprotectin, may seem to contradict their hypothesis. However, the studied populations of interest differ: the actual pre-RA samples studied by Bettner et al. all came from patients who later developed RA. It is not the case in our study. Also, our results regarding serum calprotectin further confirm what we obtained in collaboration with Amend et al. : chronic or new-onset RA patients due to their inflammatory disease have notably elevated serum calprotectin; while such a change is not visible in pre-clinical RA populations.(381)

The problem of commercial ELISA

As I explained in section “Intestinal permeability”, page 32, several commercially available ELISA kits measuring serum zonulin have been found untrustworthy. Somme investigators (such as colleagues Amend et al.) still decided to use theses kits, showing higher “zonulin” levels in new-onset RA but not in other rheumatic diseases.(381) The specificity of this finding makes it interesting but it is still unclear what the ELISA test has really measured.

Regarding the biomarkers assessed in work-package 2, we acquired kits from a trusted supplier (R&D Systems) and used the provided recombinant positive controls. I did not notice major inconsistencies while using these kits; an outlier I-FABP value was even reverified several months later using a new serum sample. However, I admit we did not check the ELISA kit performances further, for instance by producing our own recombinant I-FABP or LBP positive controls, etc., which was beyond the scope of the thesis.

The data analysis

A common issue with 16S-based analysis is that certain bacteria have multiple copies of the 16S gene, and thus can be overestimated. We tried to address this by using RasperGade16S in our pipeline,(343) which is a new tool that utilizes a model for predicting 16S rRNA genome copies and provides confidence estimates for the predictions.

Another common problem are the multiple comparisons performed during the analysis, giving falsely positive findings. We tried to avoid this mistake by:

- Grouping patients before obtaining the outcome data.
- Predefining as much as possible the analyses.
- Using Benjamini-Hochberg methods to compute adjusted p-values in the exploratory analysis or multiple comparisons, for instance when assessing differential abundance of bacteria other than the pre-planned Prevotellaceae analysis.

Regarding the pronounced phenotype subgroups analysis in work-package 1, this analysis was pre-defined. However, I need to mention that during the analysis I realized that I forgot to match the controls with the symptomatic individuals for sex and age. Thus, the matching algorithm was re-run post-hoc to refine the selection of control individuals. This did not have any impact on the conclusions.

Finally, microbiome analyses generally do not allow adjusting for relevant covariates because it does not work as a regression model, where I could add several covariables. For instance, *Aldex2* identified 15 bacterial families, which were expressed differentially depending on fecal moisture in our dataset. This meant that the water content of the feces was strongly correlated with the presence of absence of these bacteria. Nevertheless, the analysis of the results does not allow any correction or adjustment for potential confounding factors. Prevotellaceae did not seem to be significantly affected by moisture content in our cohort (after correction for multiple testing).

The analysis presented in work-package 3 is only preliminary and has a potential risk of false-positivity given the uncertainty and lack of consensus about the proper statistical methods used.

The problem of too much code

I estimate that this thesis relies on about 7000 – 8000 lines of R code. I would like to illustrate how this could be a source of typographic mistakes that sometimes have great repercussions.

In 2019, we provided to Wells et al. the first set of SCREEN-RA microbiome data derived from the preliminary study.(349) The aim of this collaboration was to validate a finding from a British cohort. P. Wells et al. showed that healthy individuals genetically at risk for RA hosted an increased proportion of *Prevotella* species, but that an expansion of *P. copri* was only seen in pre-clinical RA stages.(350) The article is today cited more than 50 times, leading to many discussions about whether or not certain alleles of the MHC-II gene could predispose individuals for preferentially hosting certain types of bacteria.

Back in 2019 I expressed some concerns to the main author regarding the reported link between SE alleles and Prevotellaceae abundance. After re-checking the data analysis she however confirmed her confidence with the findings, which we accepted. It took me two years to learn the coding skills allowing to re-run completely the analysis. Based on Wells et al., "Prevotella_7 was associated with HLA-DRB1 shared-epitope risk alleles for rheumatoid arthritis in the SCREEN-RA cohort (n=133; q=0·035".(350) The **Figure 51**, which is generated with the original dataset, basically shows the opposite :

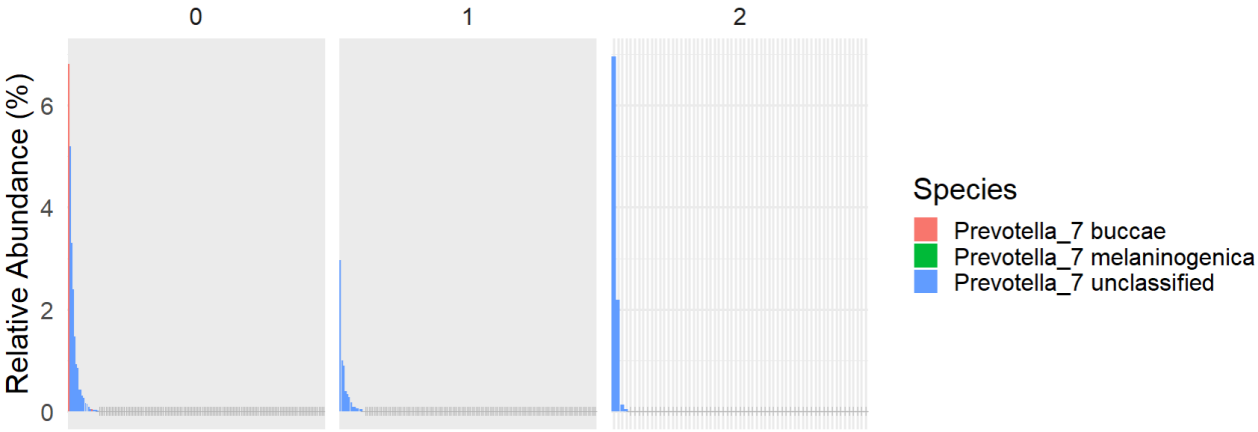


Figure 51: Prevotella_7 proportions per number of shared epitope alleles. This plot was generated using the data from Alpizar et al., 2019, processed with DADA2 pipeline and Silva 138v database. Proportions are expressed as %. Each vertical bar is a sample. Hence, most samples did not even have detectable Prevotella_7.

We have made the main author aware of the issue, which was caused by a single typographic mistake that has inverted grouping. Consequently, there is no consistency in the article as the findings from the British cohort were the exact opposite of those from the SCREEN-RA cohort. But so far it has not been retracted and continues to mislead readers.

In this thesis, we were careful to avoid such mistakes by always generating high quality visualizations of the data, to ensure congruence between raw data and statistical output. Still, it is beyond my ability to certify the perfectness of so much code, – which probably is not an uncommon issue in research involving heavy bioinformatic processing.

In addition, the data generated in work-package 1 rather confirmed that Prevotellaceae, including the genus *Prevotella_7*, do not associated with the number of shared epitope alleles (**Figure 52**).

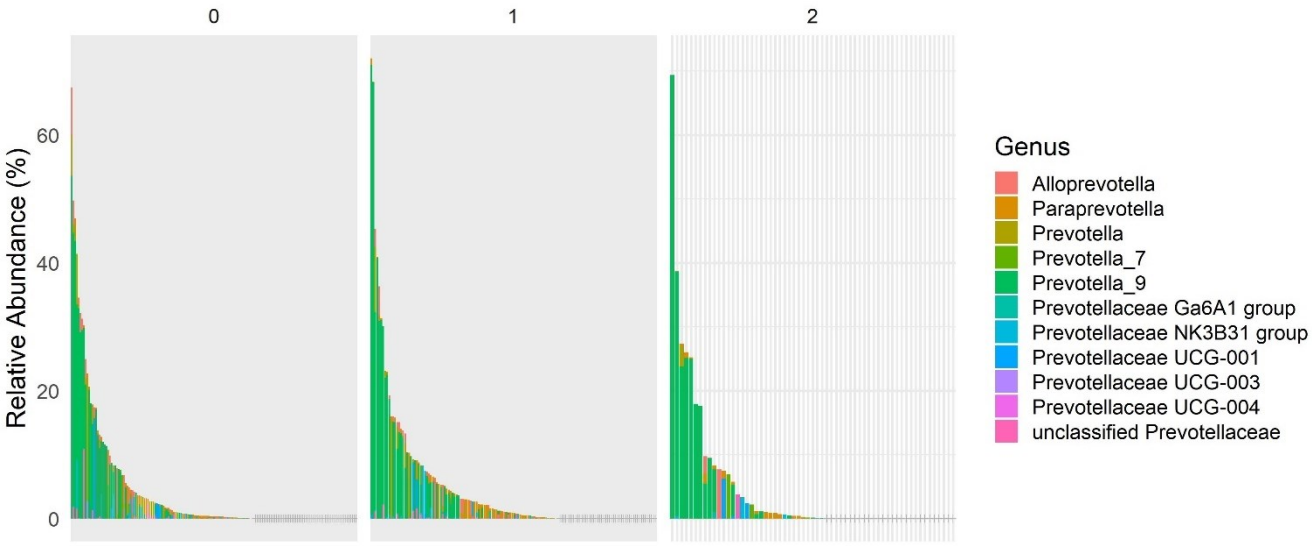


Figure 52: Prevotellaceae proportions per number of shared epitope alleles. This plot was generated using the data presented in work-package 1. Each vertical bar is a sample. About 40% samples did not have detectable Prevotellaceae, hence the vertical bar is absent and replaced by a gray cross. Samples are grouped by copy-numbers of shared epitope regardless of the other RA preclinical stage risk groups. Kruska-Wallis p = 0.251.

Reproducibility crisis

At a closer look, the heterogeneity of the reports published on the gut microbiome in RA generate confusion. In most such reports, the results are not validated in replication cohorts. Part of our work in the SCREEN-RA cohort was an attempt to properly replicate previous findings, which largely was not achieved (**Figure 21** and **Figure 22**, page 67).

Also, in collaboration with Amend et al., we aimed to demonstrate an increased anti-Prevotella IgG response in serum from RA patients of SCREEN-RA participants; and the results did not concur with previous findings,(381) contradicting the results of Pianta et al.(292,294) Despite their optimistic conclusions, Seifert et al. were not much successful when testing IgA and IgG reactivity of RA patients against *P. copri* protein Pc-p27 (**Figure 53** below is the ELISA test they report; their main result relies on a minority of outlier points, which might also, in my opinion, simply be the results of a higher overall serum Ig concentration in some diseased individuals).(414)

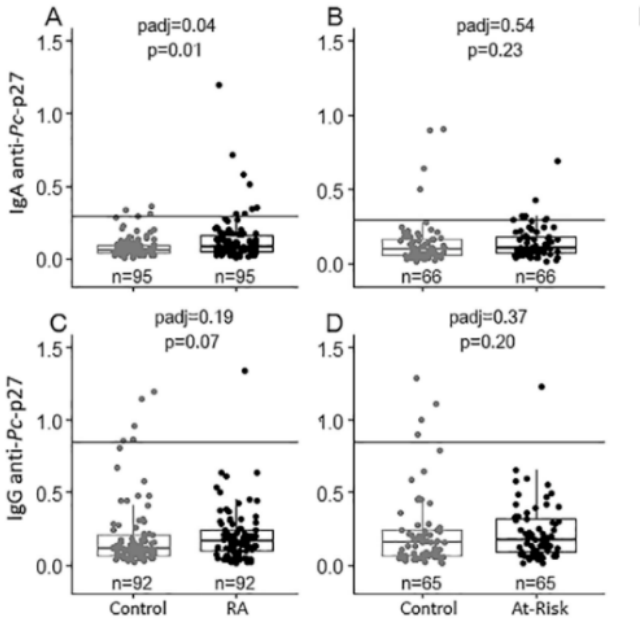


Figure 53: serum level of anti-Pcp27 IgG by group. **A** - Significantly higher IgA anti–Pc-p27 levels in RA patients (median 0.08 [interquartile range (IQR) 0.05–0.17]) versus matched controls (median 0.06 [IQR 0.04–0.10]). **B** - No significant difference in IgA anti–Pc-p27 levels in at-risk participants (median 0.11 [IQR 0.07–0.18]) versus matched controls (median 0.10 [IQR 0.05–0.17]). **C** - No significant difference in IgG anti–Pc-p27 levels in RA patients (median 0.17 [IQR 0.10–0.24]) versus matched controls (median 0.11 [IQR 0.07–0.21]). **D** - No significant difference in IgG anti–Pc-p27 levels in at-risk participants (median 0.18 [IQR 0.09–0.32]) versus matched controls (median 0.16 [IQR 0.06–0.24]). Reproduced from Seifert et al., Arthritis Rheumatol, https://doi.org/10.1002/art.42370 , under license n°5602421112675.

And as stated above (“The problem of too much code”, page 122), our data also contradicts Wells et al. publication.(350)

In 2005, writing a controversial paper, Ioannidis has warned that most research findings are probably false, especially in underpowered studies, or when implying subgrouping and multiple testing or “data mining”.(415) His opinion is partly why we preferred, for this thesis, reporting mostly negative findings, rather than “torturing” the data until finding the p-values we desired. Though, the low reproducibility of results might partly discredit the microbiome field.

FUTURE DIRECTIONS

Future investigation in the area of the intestinal health could focus either on searching for new biomarkers or on developing new interventions that would complement the current therapies in early RA. The potential research interest in each of these areas is briefly discussed below.

NOVEL BIOMARKERS?

Using microbiome

Taking into consideration the results of our research as well as the heterogeneity of previous findings, I believe that:

- we cannot hypothesize anymore that “one particular bacterial strain” would be the universal cause of RA.
- many perfectly healthy persons host within their intestine microbes that worsened or triggered arthritis in mouse models. Consequently, it does not seem logical to use the simple presence or absence of these taxa as an indicator of imminent RA or autoimmunity.

Future research could, however:

- focus on the strain-level identification, in particular when *Prevotella copri* is involved. This is actually already planned in the scope of future experiments with the SCREEN-RA cohort.
- move to a more “functional” profiling, which would disregard the taxonomy and rather list various enzymatic operations that a given microbiome is able to do. It seems also doable using whole genome sequencing approaches similar to the strain-level identification methods.
- determine what the microbes that worsen mice arthritis have in common, such as surface glycan, antigen sequence mimicking host structures, mucus invasion capabilities, toxin or antigenic outer membrane vesicles secretion, etc.

Anti-glycan antibodies

A “glycan” is a generic name for a molecule with glycosidic bonds, such as sugars that are polysaccharides or carbohydrates. Glycans are expressed on the surface of bacteria, and the immune reactivity against these structures is still poorly studied.

A project extending my thesis will test serum reactivity of different groups and subgroups of patients against a large panel of both human and bacterial glycans, as these are structures potentially involved in cross-reactivity.

Others (gut permeability, metabolomics, …)

Three other categories of potential biomarkers could be considered.

- First, as no protocol currently exists in Geneva to functionally assess intestinal permeability, I would be happy to contribute in the future to setting one up. Current methods could probably be improved by using isotopic tracers such as ^{13}C lactulose or mannitol and/or taking advantage of MRI to track the diffusion of tracers directly in the gut and local circulation. Also, the differences between an intestine loaded with food and a fasting intestine have never been studied to my knowledge. Once a reliable measure is found or designed, I believe it would be worthwhile to assess such permeability in the context of new-onset RA or autoimmunity.
- Second, as adaptative immune reactions underlie the onset of autoimmunity, using TCR or BCR sequences from blood samples might be of interest to detect autoimmunity onset. A preliminary study was already conducted in the SCREEN-RA cohort regarding TCR clones.(416) We possess multiple whole-blood RNA-seq data from 173 RA-FDRs in the SCREEN-RA cohort and a group of RA patients form the SCQM cohort. A future research objective for us will be to reconstitute TCR and BCR sequences and assess if recognizable clones correlate with autoantibody production. This research is not per say related to the gut, but the underlying hypothesis is that part of the autoreactive clones originate from the GALT, which will be a later axis to explore.
- Third, our group is in the process of creating a partnership to investigate metabolomic analysis of serum samples in the SCREEN-RA and SCQM cohorts. I am a bit reluctant to perform metabolomics because it typically represents a statistically inappropriate analysis involving outrageously repeated testing. However, we could circumscribe *a priori* a list of metabolites related to relevant pathways or detrimental bacterial species. In such a context it would seem reasonable to correlate metabolite presence with clinical phenotypes.

MUCOSA-ORIENTED INTERVENTIONS?

Below I list the interventions that, in my opinion, could be considered in clinical practice to complement conventional therapies in the context of early arthritis or RA. From a pragmatic perspective, one could even want to combine them all, for instance, as an experimental therapeutic "mucosal-protocol" for individuals at risk with clinically suspect arthralgia and RA-autoantibodies, but who do not yet meet ACR/EULAR classification criteria for RA. We have drafted a protocol for such a study (not yet published).

Diet, lifestyle and RA: shaping the microbiota?

I doubt that purposely "shaping" the microbiota by choosing one or the other food type is something realistically doable based on the current knowledge because:

- We know very little about the "good" and "bad" bacteria that should be promoted or depleted in rheumatic patients.
- We know even less on how a diet can selectively promote or deplete the taxa of interest.

In order to design a possibly beneficial diet, we are left with three complementary reasonings:

- Review the dietary risk and protective factors for RA, as evidenced by epidemiological studies, and try to avoid or include them in a dietary interventional protocol (which we did in section "Risk-factors for rheumatoid arthritis", page 12).

- Review the existing dietary interventional literature.

- Propose further interventions based on murine models, for instance avoiding additives susceptible of altering gut epithelial health, even though this was not per say studied from the epidemiological or interventional perspectives.

Badsha et al. previously reviewed the impact of diet on RA and concluded that certain diets may help some groups of patients,(417) but the available evidence did not establish dietary interventions as a substitute for pharmacotherapy in RA. Limited or cyclical fasting, vegan, Mediterranean diets or elimination of dairy and gluten seemed to play a role, despite some contradictory studies, but do not have high adherence on the long run.(418–434) Bustamante et al. even designed an anti-inflammatory diet reprising most of the previous findings, which is surprisingly close to the suggestions of SEIGNALET, and reported encouraging preliminary results even though it was done through an uncontrolled trial.(435)

Mice experiments and other data

Apart from the dietary interventional literature, other sources of knowledge could be taken into consideration when providing guidance for nutrition in the context of RA.

For instance, factors known to perturb gut epithelial integrity may be avoided (reviewed in “Factors influencing intestinal permeability”, page 189). In particular, food additive such as emulsifiers, based on the available preliminary human studies, not only disrupt mucosal health at various levels,(436) but also impact the microbiome composition in a detrimental manner, i.e. less diversity, reduced production of short-chain fatty acids, and potentially increased mucus invasion.(437)

In mice collagen-induced arthritis, curcumin had a synergistic effect with vitamin-D and omega-3 supplementation, delaying disease onset and reducing severity.(438) Also, high fiber diet was able to attenuate collagen induced arthritis due to a modification of gut microbiome and increased production of propionate, which also works when administered alone.(439) These nutrients also relate to the intestinal barrier function (**Table 10**, page 189), which further encourages to consider them when designing a diet.

Regarding probiotics supplementation, Mohammed et al. conducted a meta-analysis including 361 patients from 6 randomized trials; the tested probiotics were : *Lactobacillus* species, *Bifidobacterium bifidum* and *Bacillus coagulans*.(440) The conclusion of the meta-analysis was that probiotic supplementation reduced levels of IL-6 but did not significantly change disease activity.(440) Two recent studies likewise gave equivocal results.(441,442) Alternative interventions may even include helminths.(443) Still, mouse models could suggest new potentially beneficial probiotics. In general, the experiments are conducted in collagen-induced arthritis model and imply oral gavage of mice with the bacteria of interest, compared to various negative controls. Hence, we could list as beneficial in such models : *Lactobacillus casei* (various strains),(444,445) *Lactobacillus helveticus*,(446) *Lactococcus lactis*,(447) *Lactococcocus lactis*,(448) *Bifidobacterium breve,*(449) *Bifidobacterium adolescentis*,(450) *Bifidobacterium pseudocatenulatum*,(451) *Parabacteroides distasonis,*(283) *Lactiplantibacillus plantarum*,(452) *Lactobacillus rhamnosus*.(453)

Fecal microbiome transfer (FMT)?

Fecal microbiome transfer (sometimes called “fecal transplantation”) consists in administering to a patient a “super probiotic” made from fresh fecal material of a healthy donor. I have already discussed this intervention elsewhere.(454)

To our knowledge, there is currently no data on the possible usefulness of FMT in the context of RA. To date (July 2023), three clinical trials involving FMT on RA patients have been registered: one in China (currently ongoing, NCT03944096), one in England (NCT05790356), and one in Denmark (NCT04924270).

The production of microbiome “transplants” is allowed in Switzerland by accredited centers, and the only one that currently exists is at the CHUV, Lausanne and treats recurrent *Clostridium difficile*

infections. However, these “transplants” have been administratively categorized as “drugs”[1] and the regulatory procedures that would accompany any clinical experimentation for new indications are such that there are probably not attainable by academic investigators. Consequently, there is no new human research on FMT ongoing in Switzerland.

I shall mention the FMT placebo-controlled randomized trial done by Kragsnaes et al. (Denmark) in the context of peripheral psoriatic arthritis, for this disease might share some similarities with RA.(455) Even though there were no serious adverse events, FMT worsened disease activity compared to sham procedure.(455) The later at least strongly suggests a causal link between intestinal microbiome and psoriatic arthritis, which should be further investigated.

Periodontitis treatment?

We have already discussed that periodontitis is known as an important risk factor for RA (see “Risk-factors for rheumatoid arthritis”, page 12), and how treating periodontitis was tried to impact RA disease activity.(329,456,457)

More recently, the OPERA trial randomized 60 RA patients with periodontitis to receive immediate or delay periodontal treatment.(458) The study proved to be feasible and acceptable to the patients. Treatment resulted in significant improvement in periodontal disease, and the overall RA disease activity; even though it is unclear how significant these improvements are sine the p-values were not reported.(458)

Mustufvi et al. similarly intervened to treat periodontitis in individuals who are seropositive for ACPA, demonstrating that the approach has no major risk and can improve periodontitis status.(459)

[1] More exactly, “Advanced Therapy Medicinal Products” , see: https://www.swissmedic.ch/swissmedic/en/home/humanarzneimittel/besondere-arzneimittelgruppen--ham-/innovation.html

CONCLUSION

The available evidence strongly suggests that mucosal health and microbiome are relevant for RA autoimmunity. Still the most convincing data relies on mice experiments.

This thesis attempted to recapitulate the recent findings in an observational setting derived from individuals at risk for rheumatoid arthritis (RA). We aimed 1) to assess the correlation between increased “risk of RA” and the presence of RA-associated bacteria in feces; 2) to assess the correlation between increased “risk of RA” and serological biomarkers pertained to intestinal integrity; 3) to assess patient’s IgG reactivity against the RA-relevant microbes or autologous fecal microbiome.

We collected about 380 stool samples, paired with serum samples, in the SCREEN-RA cohort; the individuals were then categorized as controls, controls with high genetic risk, asymptomatic individuals with detectable RA autoimmunity, and individuals with suspect articular symptoms or new-onset RA.

Unexpectedly, we found that:

- The fecal microbiome and the presence of RA-associated bacteria did not significantly differ between the groups we designed; nor did the fecal calprotectin levels.
- The serum biomarkers of intestinal integrity (I-FABP) and systemic inflammation (LBP and calprotectin) did not significantly differ between the groups either.
- Prevotellaceae, in particular *Prevotella copri*, which were the bacteria of interest, did not appear to be significantly more targeted by serum IgG of neither RA patients, nor high-risk participants.
- Still, creating small subgroups of the most pronounced phenotypes and re-comparing them partly retrieved the previous findings regarding increased Prevotellaceae abundance and a possible though very modest increase in intestinal inflammation as assessed by fecal calprotectin.

However, the absence of observation does not necessarily *prove* the absence of the effect, since it is possibly a consequence of technical limitations inherent to our cross-sectional observational study design.

Future studies could consider 1) obtaining gut mucosa samples instead of using unreliable proxies; 2) employing interventional designs by either using the fecal microbiome transfers or extended hygiene-dietetic protocols.

IV. RELATED PUBLICATIONS

1 – BMJ Open - Cohort Profile: SCREEN-RA: design, methods and perspectives of a Swiss cohort study of first-degree relatives of patients with rheumatoid arthritis

BMJ Open

Cohort profile: SCREEN-RA: design, methods and perspectives of a Swiss cohort study of first-degree relatives of patients with rheumatoid arthritis

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ABSTRACT

Purpose Rheumatoid arthritis (RA) is an insidious autoimmune disease, with an immunological onset years before diagnosis. Early interventions in preclinical stages could prevent or minimise the progression towards irreversible joint damage. The SCREEN-RA cohort (Evaluation of a SCREENing strategy for Rheumatoid Arthritis) aims to characterise the preclinical stages of the disease, to identify environmental risk factors, and to discover or validate novel biomarkers predictive for RA development.

Participants SCREEN-RA includes an at-risk population for RA, namely first-degree relatives of patients with established RA.

Findings to date The cohort started in 2009 is composed of mostly asymptomatic healthy individuals (total n=1458, 7262 person-years), with a mean age of 44 years at enrolment, 74% female and 91% Caucasian ethnicity. During the study period, 16 participants have developed RA. All participants provide baseline serum, DNA and RNA samples, and in a subset, stool samples and oral examination are performed for microbiota assessment. At enrolment, 10% of participants had asymptomatic autoimmunity associated with RA (n=147), 10% presented 'clinically suspect arthralgias' (n=143) and 3% reported arthralgias in conjunction with autoimmunity or high genetic risk (n=51). Studies with this cohort have uncovered risk factors for RA development, such as female hormonal factors, poor oral health or intestinal dysbiosis.

Future plans Future directions include immunological and 'multiomics' approaches to discover new biological markers of progression towards RA, as well as testing preventive interventions in 'high-risk' population.

Strengths and limitations of this study

- Long-term follow-up of individuals at risk of rheumatoid arthritis (RA), with physical and biological data collected in a controlled environment, using standard operating procedures.
- Follow-up of at-risk individuals, prior RA diagnosis, which allows better causal inference than case–control studies.
- Opportunity to realise nested studies or validation studies.
- Symptom-related data are partly based on patient self-assessment, which increases risk of outcome misclassification.
- Slow conversion rate to established RA results in few newly diagnosed RA cases, despite enrolment of numerous participants.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease leading to joint destruction and extra-articular manifestations. RA has a rising prevalence[1] of 0.5%–1% in the European and North American population.[2 3] Important risk factors include genetics,[4–6] female hormonal factors[7] and environmental factors such as air pollution,[8] diet and obesity[9–14] or stressful events.[15] The risk of RA is also strikingly associated with smoking,[16–18] but only in conjunction with specific human leukocyte antigen (HLA) alleles (the so-called 'shared epitope'), implying a strong gene–environment interaction.[17 19 20] Recent investigations have suggested a 'mucosal origin' of RA autoimmunity,[21] because of its remarkable association with periodontal disease[22–25] and other mucosal inflammatory conditions, such as chronic intestinal conditions,[26] or chronic pulmonary disorders.[27 28] Underlying dysbiosis is suspected to play a key role in the development of RA,[29–34] even if exact causality still remains to be determined.

The aetiology of RA is believed to result from a multistep process, where environmental factors gradually initiate a pathological activation of the immune system.[20] Overall, the preclinical progression toward RA can be divided into three 'at risk stages'[35]:

1. Genetic and environmental risk: First-degree relatives (FDRs) of patients with

RA have a 3–5 fold increased risk of developing the disease, which is even higher in families with multiple cases of RA.[5] Among susceptibility genes, HLA-DRB1 variants share a common sequence in the third hypervariable region of the MHC II binding site (referred to as the 'shared epitope'), which is involved in the response to extracellular immune ligands.[4] However, the risk associated with established genetic markers, even in longitudinal studies,[5] remains modest. The latter underlines the importance of environmental factors, which are thought to act as 'triggers'.[8 17 19 36–38]

2. Systemic autoimmunity associated with RA*:* During the preclinical phase, circulating autoantibodies (most specifically anticitrullinated peptide antibodies or ACPA) are already present, often several years before the diagnosis.[39–43] The risk of developing RA within 5 years with ACPA positivity is only 5% for individuals without any familial history of RA, but increases up to 69% among FDRs,[39] especially if titers are high.[44] The presence of both ACPA and rheumatoid factors (RF) further increases specificity (99%) for the future development of classifiable RA.[45] Recent research focused on identifying new autoantibodies, such as anti-Ra33 antibodies, anti-carbamylated protein antibodies[46] or anti-PAD4 antibodies.[47]
3. Symptomatic preclinical phases*:* Asymptomatic autoimmunity can evolve over several years, towards inflammatory arthralgias, or undifferentiated arthritis, before finally leading to clinically-apparent RA.[20 35] These symptomatic 'pre-RA' patients can be identified using specific questionnaires and/or physical examination.[48] In particular, the European League Against Rheumatism (EULAR) has proposed clinical characteristics of arthralgias at risk for RA,[49] namely 'clinically suspect arthralgia' (CSA), which increase the risk of developing RA during a 2-year follow-up.[50]

The preclinical phases of RA represent opportunities for preventive interventions,[35 51] which may allow to avert disease development or improve long-term outcomes.[52 53] However, the optimal screening strategy to identify 'at-risk' individuals most likely to benefit from early interventions is still to be established.

To adequately define the specific preclinical phases of RA development, and to identify environmental factors driving progression from one phase to the other, longitudinal studies are required. In this article, we present a cohort study of FDRs of patients with RA, including 1458 participants.

COHORT DESCRIPTION

Study overview

The SCREEN-RA study is a multicentric observational cohort study across Switzerland. It enrols and follows FDRs of patients with RA and was started in 2009 with the support of the Swiss National Science Foundation. The primary objectives are to characterise the different preclinical stages of RA, and to determine the optimal combination of biomarkers to predict the development of RA within 3–5 years. Recruitment methods include emails to patients, presentations at patient conferences, articles in general audience journals, promotion via patient associations, information to patients with RA within the Swiss Clinical Quality Management Rheumatoid Arthritis (SCQM-RA) register, advertising through radio and television and advertisement in pharmacies. Since 2018, campaigns on social networks have also been organised (Facebook, Snapchat, LinkedIn and website www.arthritis-checkup.ch).

Patient and public involvement

Patient and public organisations were involved in the project, including design and management of the study. The Swiss league against rheumatic diseases has been a long-time partner, in particular helping recruiting participants and disseminating results.[54] Also, as future research might involve preventive interventions, a random sample of SCREEN-RA participants were asked in 2016 if they would take a hypothetical treatment or not, depending on varying levels of treatment characteristics.[55] About one-third of the participants would be willing to take a preventive treatment if the hypothetical risk of developing RA was at least 20%.[55] Face-to-face interviews revealed that lifestyle changes and complementary medicine were also considered.[56] Finally, most participants would agree to enrol in a randomised controlled trial to test the efficacy of preventive interventions.[56] We took this feedback into account for our future research, and furthermore we regularly receive input from one of the members of the rheumatology division who is also a patient with RA herself (not named in the article).

Study population

The primary study population is a genetically defined at risk population, namely FDRs of established patients with RA.[57] The study population also comprises a minority of FDRs of patients with lupus or other connective tissue diseases, autoimmune thyroiditis or type 1 diabetes. Indeed, because of shared genetic risk factors with RA, all these conditions increase the risk of RA among FDRs in a similar magnitude.[58] Other inclusion criteria are the absence of clinically apparent active synovitis on examination, and an age of at least 18 years. Exclusion criteria are an established diagnosis of RA, or the presence of active comorbid inflammatory arthritides (ie, patients with psoriatic arthritis, spondylarthritis or known microcrystalline arthritis) to avoid outcome misclassification. After enrolment, all participants are followed using yearly questionnaires to detect new symptoms or signs of the disease (figure 1).

We use a combination of known risk factors for RA and clinical parameters to define groups of 'high-risk' participants (figure 2). These 'high-risk' participants satisfy at least one of the following criteria:

- Having 2 copies of the shared epitope, which doubles the risk of RA compared with having one single copy.[59]

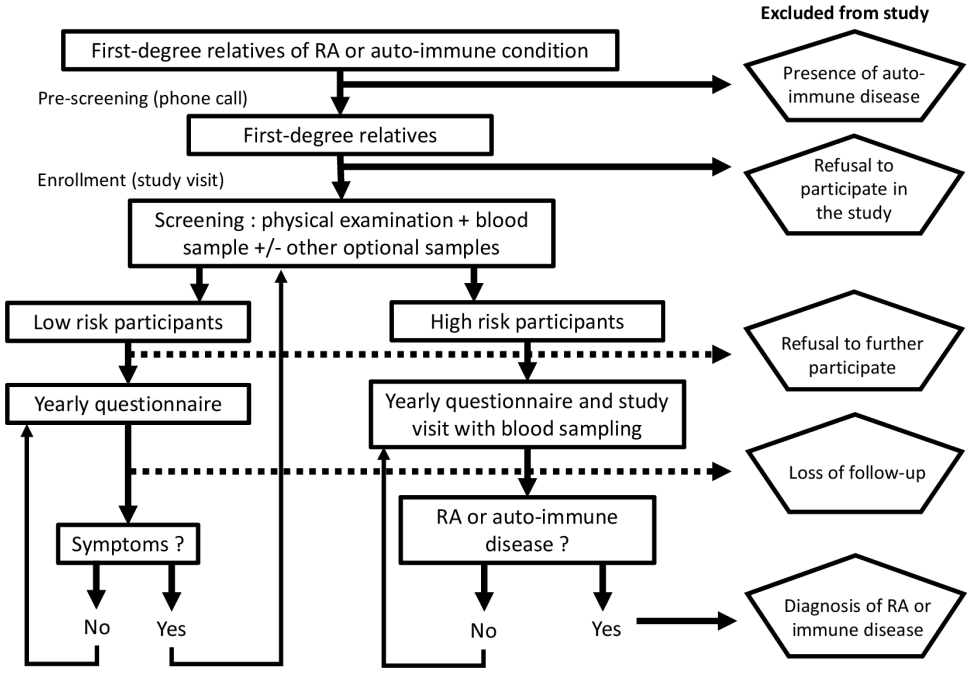

Figure 1 Flow chart of recruitment and follow-up in SCREEN-RA cohort, 2009–2020, Switzerland. RA, rheumatoid arthritis. SCREEN-RA :Evaluation of a SCREENing strategy for Rheumatoid Arthritis.

- Having serological antibodies associated with RA, which strongly increases the risk of developing RA among FDRs[39 60 61] : ACPA seropositivity, or RF levels (either IgA or IgM isotype) three times the upper limit of the norm or anti-Ra33 antibodies three times the upper limit of the norm (IgM, IgG or IgA). The simultaneous presence of several autoantibodies above the upper limit of normal is also considered high risk for future RA development.
- Having 'CSA' defined when satisfying four or more of the seven criteria previously validated by EULAR (ie, symptom duration <1 year, symptoms in metacarpophalangeal joints, morning stiffness duration ≥60 min, most severe symptoms in early morning, being RA-FDR, difficulty with making a fist, and positive squeeze test of metacarpophalangeal joints).[49 50] 'Undifferentiated arthritis' was defined as one or more swollen joints on examination, in conjunction with 'CSA'. Undifferentiated arthritis should not be classifiable as rheumatic disease, nor result from a septic or crystal aetiology. If correctly assessed, subsequent risk for RA development in the following year has been reported as high as 35%.[62]

'High-risk' participants are then followed up more closely in this cohort, with a yearly in person visit and

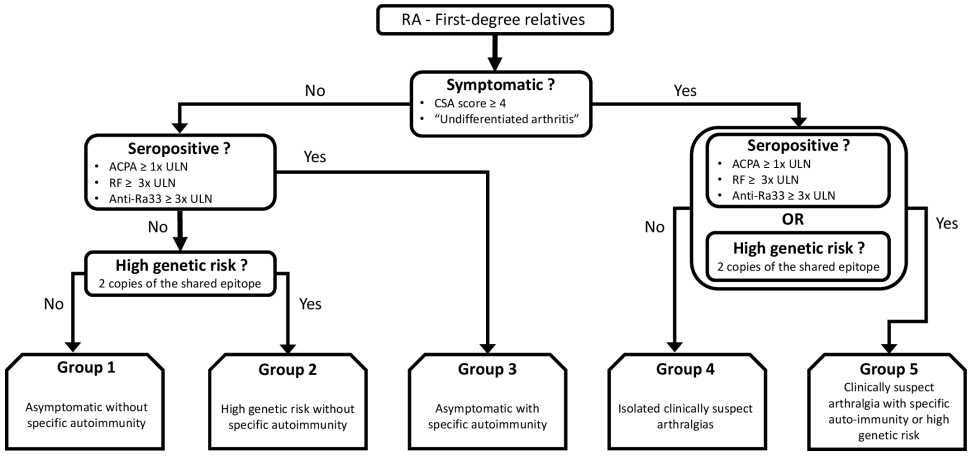

Figure 2 Logogram of risk subgroup classification, SCREEN-RA cohort, Switzerland, 2009–2020. CSA : Clinically Suspect Arthralgia score (calculated using seven items as proposed by EULAR). A given participant will be classified in the highest group for which he or she meets the criteria. ACPA, anticitrullinated peptide antibodies; CSA, clinically suspect arthralgia; EULAR, European League Against Rheumatism; RA, rheumatoid arthritis; RF, rheumatoid factor; ULN, upper limit of the norm. SCREEN-RA :Evaluation of a SCREENing strategy for Rheumatoid Arthritis.

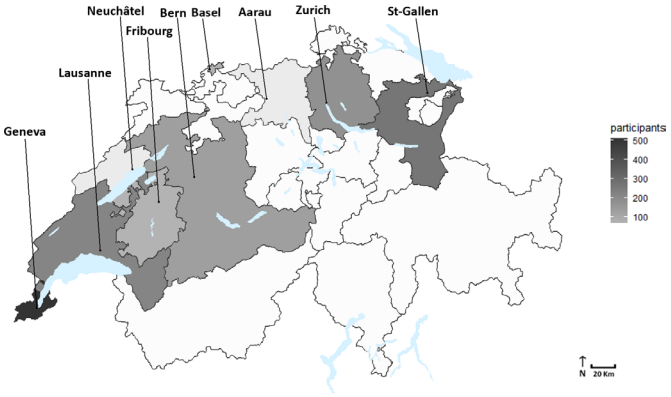

Figure 3 Geographical distribution of participants per Canton of recruitment, SCREEN-RA cohort, Switzerland, 2009–2020. SCREEN-RA :Evaluation of a SCREENing strategy for Rheumatoid Arthritis.

blood sampling, to monitor evolution of serological markers and symptoms over time.

Sample size calculation

The SCREEN-RA sample size was estimated based on the number of FDRs developing RA, to allow predictive modelling of RA in FDRs. We estimated that a minimum of 60 incident cases of RA would be needed to analyse with sufficient discriminative power a predictive model of RA in FDRs. Unaffected FDRs in multiply affected families have an incidence of RA of 8/1000 patient-years (95% CI: 4.2 to 13.6),[5] and lower in families with only a single affected case. Patients develop autoantibodies on average 2–5 years prior to disease onset,[39] which implies that we expect to detect autoimmunity associated with RA in up to 4% of FDRs, which is approximately what has been described in similar populations.[63] With a minimum of 5 years of follow-up, an estimated incidence rate of RA between 0.6 and 1 case/person-year, the sample size required to characterise ~60 patients with RA was estimated to be between 1000 and 2000 individuals. To ensure the feasibility of such a long-term longitudinal follow-up while minimising costs, the study was designed with a yearly follow-up.

Study sites

Enrolment is conducted within 10 collaborative centres, within the following cities: Geneva (Hôpitaux Universitaires de Genève), Lausanne (Centre Hospitalier Universitaire Vaudois), Fribourg (Hôpital Fribourgeois), Neuchâtel (Réseau Hospitalier Neuchâtelois), Bâle (Universitätsspital Basel), Zurich (Universitätsspital Zurich), Berne (Inselspital-Hôpital universitaire de Berne), Aarau (Kantonsspital Aarau) and Saint-Gall (Kantonsspital St.Gallen). Figure 3 represents the geographical distribution of enrolled population across the involved Swiss cantons.

Questionnaires

Inclusion questionnaire

At inclusion, participants complete a questionnaire regarding demographic data and environmental factors such as alcohol consumption, nutritional habits, smoking status, infectious diseases, professional exposures, oral health, female hormonal factors and family history of autoimmune disease (table 1).

Longitudinal follow-up questionnaire

Participants receive a yearly follow-up questionnaire assessing articular symptoms, presence of immune disease, current medication and environmental factors such as smoking, nutritional or exercising

Table 1 Summary of questionnaire content for participants, SCREEN-RA cohort, Switzerland, 2009–2020

Questionnaire component	Summary of content
General information	Contact information, age, gender, ethnicity, country of origin, birth weight, years of education
Physical examination	Absence of systemic inflammatory disease, height, weight, blood pressure, number of swollen joints, no of tender joints, presence of rheumatoid nodules.
Family information	Family's country of origin, number of relatives with RA or other autoimmune conditions, number of siblings, no of children, relation to the RA-diagnosed relative, age of beginning of symptoms, age of diagnosis, anti-CCP testing, medication of the RA-relative.
Annual follow-up questionnaire	Joint pain assessment, joint swelling assessment, recent blood test for RA for Lupus, current health issues, current medication, history of infectious disease, history of female hormonal factors, vaccinal status, smoking status, consumption of tea/coffee/soft-drinks, use of vitamin supplementation, alcohol consumption, professional situation, sleeping disorders, physical activity.
Annual follow-up questionnaire (optional)	Dust exposition at workplace, professional health, oral health, consumption of seafoods.
In case of stool sample (optional)	Time since last defecation, stool consistency, recent travel, use of probiotics, use of antibiotics, recent surgery, current periodontitis. A Food Frequency Questionnaire.
In case of oral sample (optional)	Allergies, current medication, smoking status, number of teeth, reason for tooth loss, no of implants, oral hygiene habits, chewing problems, breath problems, periodontal status.

CCP, cyclic citrullinated peptide; RA, rheumatoid arthritis; SCREEN-RA, Evaluation of a SCREENing strategy for Rheumatoid Arthritis.

habits (table 1). Questionnaires are available in three languages (French, English and German). Questionnaires have been established in collaboration with other ongoing studies of at-risk populations, such as the American SERA cohort[64] to allow replication studies in the future.

Clinical visits

At inclusion, a clinical examination is performed by a specialised nurse or a rheumatologist to assess potential tender and swollen joints[65] and rule out the presence of RA or other autoimmune conditions. This examination is repeated yearly for the 'high-risk' participants, in addition to biological sampling.

Biological samples

Blood samples

Full blood samples are collected at inclusion in EDTA collection tubes for genetic testing (HLA; online supplemental file) and additional aliquots for a genomic DNA library. Full blood is further used to collect total RNA using Tempus Blood RNA Tubes (lyses whole blood cells and stabilises RNA). Genomic DNA and total RNA are isolated by standard procedures. Serum samples are collected for the assessment of autoantibodies (ACPA, RF, and anti-Ra-33 in a subset of participants) using commercially as well as non-commercially assays (online supplemental file). Aliquots are stored at −80°C in a serum library. Participants deemed at high risk provide yearly new blood samples, while other participants provide a baseline sample.

Stool samples

A nested case–control study in 2016 was performed, with 133 stool samples. A new collection is ongoing (2019–2020; targeted n=400 stool samples), using collection-devices allowing the creation of several aliquots. Participants receive a stool collection kit and proceed to sampling at home. They temporarily freeze the fresh sample at −20°C, and bring it in a cooler box to the study centre, where the stool samples are stored at −80°C, without any additive according to published methods.[66]

Salivary/dental plaque samples

In a subset of the cohort (n=99), gingival crevicular fluid is collected at one site in each dentition quadrant using membrane strips. The salivary microbiome is sampled collecting unstimulated saliva by spitting in a sterile plastic tube. Finally, the subgingival microbiome is sampled using sterile paper points inserted into the bottom of the pockets, at four different oral sites.

Sample storage and biobank

All biological samples are processed following standard operative procedures and stored at −80°C, in a dedicated biobank. Samples from collaborative centres are regularly shipped on dry ice to the Geneva's main biobank. Table 2 presents the repartition of all available serum, DNA and RNA samples by baseline risk-subgroups. A total of 2301 serum samples were collected during the study period. Each serum sample is divided into 7–9 aliquots (total n=12 390 aliquots). Twenty-eight per cent of participants have at least two sequential samples (mean interval between samples=2.8 years). Moreover, most serum samples are matched with RNA and DNA samples (table 2). Concentration and RNA Integrity Number of RNA samples are available, as well as concentration and 260/280 optical density ratios for DNA samples. A total of 159 participants have at least two sequential RNA samples, allowing future transcriptomic longitudinal studies. In addition, matching of RNA and DNA samples (n=1396, table 2) will be useful for future expression quantitative trait loci analysis. All matched biological samples will also allow studies of predictive associations of biomarkers, combining serological, genomic and transcriptomic information into RA risk-scores.

Data management

Data are collected through a secured online interface. Since late 2019, data are stored and monitored using Research Electronic Data Capture (REDCap) software and hosted on institutional servers, with secure backup. Previously filled-in on paper versions, questionnaires are now sent by email, and reports of physical examination or serological analysis are entered into REDcap. For external data manipulation, each patient is identified

Table 2 Number of available biological samples by baseline risk-subgroups, SCREEN-RA cohort, Switzerland, 2009–2020

Baseline risk-group	Participants (n subjects)	Serum samples (n samples)	At least two sequential serum samples (n subjects)	DNA samples (n samples)	RNA samples (n samples)	Matched RNA and DNA (n samples)
1	1006	1293	171	1060	839	835
2	80	242	59	152	140	140
3	147	379	92	227	213	213
4	143	222	57	149	125	124
5	51	133	32	82	84	84

Baseline risk-groups as defined in figure 2.
SCREEN-RA, Evaluation of a SCREENing strategy for Rheumatoid Arthritis.

Table 3 Baseline sociodemographic characteristics of participants, SCREEN-RA cohort, Switzerland, 2009–2020

Variable		No (%) (total n=1458)
Age group, years	18–25	172 (12)
	25–50	779 (53)
	50–75	494 (34)
	>75	13 (1)
Year of education	0–5	55 (4)
	5–10	83 (6)
	>10	785 (54)
	Not specified	535 (37)
Gender	Female	1086 (74)
Ethnicity	White	1322 (91)
Number of RA cases in participants' family	1	1158 (79)
	2	165 (11)
	>3	51 (4)
	Not specified	84 (6)
Tobacco smoking	Never	727 (50)
	Previous	380 (26)
	Current	283 (19)
	Not specified	67 (5)

RA, Rheumatoid Arthritis; SCREEN-RA, Evaluation of a SCREENing strategy for Rheumatoid Arthritis.

Table 4 Baseline biological and physical characteristics of participants, SCREEN-RA cohort, Switzerland, 2009–2020

Variable		No (%) (total n=1458)
BMI groups	<18	30 (2)
	18 to <25	892 (61)
	25 to <30	385 (27)
	≥30	127 (9)
	Missing	24 (2)
Biology	Provided at least two blood samples	412 (28)
	Total ACPA seropositivity (commercial or non-commercial assays)	78 (5)
	Total RF seropositivity	282 (19)
	IgA RF seropositivity	66 (5)
	IgM RF seropositivity	251 (17)
	Total anti-Ra33 tested	660 (45)
	Anti-Ra33 seropositivity (for any Ig subtype,≥3× ULN)	8 (0.5)
Shared epitope allele no	0 copy	724 (50)
	1 copy	564 (39)
	2 copies	105 (7)
	Not tested	65 (4)

RA-converter subjects.
ACPA, anticitrullinated peptide antibodies; BMI, body mass index; RA, rheumatoid arthritis; RF, rheumatoid factors; SCREEN-RA, Evaluation of a SCREENing strategy for Rheumatoid Arthritis; ULN, upper limit of the norm.

by a numerical code of 2–4 digits, which is also used to label the biological samples. The database is password protected and changes are tracked in logfiles.

Statistical analysis

A descriptive analysis of baseline data was performed (tables 3–5). Continuous variables are expressed as means with standard deviation (SD) whereas categorical variables are described using frequencies (percentage). χ^2 test, or Fisher's exact test for small size samples, were used to compare categorical variables. Continuous variables were compared between groups using Student's t test, or Kruskal-Wallis test if not normally distributed or ANOVA if more than two groups. Two-tailed values of $p<0.05$ were considered significant. Missing data were imputed using value found in the nearest time point in a window of 6 months, when available. Missing data for RF, shared epitope and ACPA status were imputed as last observation carried forward. Incomplete records (ie, participant who never came to inclusion visit or finally refused blood sampling) were excluded. All analyses were conducted using R, V.3.6.2, with package tableone.

Baseline characteristics and evolution of the SCREEN-RA population

Whole study population

On 23 November 2020, SCREEN-RA cohort had enrolled 1458 individuals, 1261 of whom are still actively providing follow-up data. The total follow-up duration equals 7762 patient-years, which represents an average of 5 years of follow-up per participant. The population had a mean age at enrolment of 44 years, was 74% female and 91% from white ethnicity (table 3). The main reason for study discontinuation was loss to follow-up (65%), followed by refusal to participate further (30%). The main enrolment sites were the Geneva centre (35%) and St-Gallen centre (19%). Nineteen per cent of participants were active smokers and the mean baseline body mass index was 24 kg/m^2 (tables 3 and 4).

RA-converter subjects

During the study period, 16 participants developed a classifiable RA, after a mean follow-up of 5.5 years. They provided a total of 48 blood samples, including postdiagnostic samples. At enrolment, compared with other FDRs taken together, RA-converters were significantly more often seropositive for ACPA (38% vs 5%; $p<0.01$) and RF (63% vs 19%; $p<0.01$). Interestingly, frequency of shared epitope alleles among RA-converters was not

Table 5 Baseline characteristics of subjects who provided blood sample, by risk-subgroups, SCREEN-RA cohort, Switzerland, 2009–2020

		Low risk		High risk				
		Mean (SD)		Mean (SD)				P value (ANOVA or χ^2)
Variables (at enrolment)		Otherwise n (%)		Otherwise n (%)				
Risk groups (total n=1458):		NA (n=31)	1 (n=1006)	2 (n=80)	3 (n=147)	4 (n=143)	5 (n=51)	
		Not assigned (serological result awaited)	Asymptomatic without specific autoimmunity	High genetic risk without specific autoimmunity	Asymptomatic with specific autoimmunity	Isolated clinically suspect arthralgias	Clinically suspect arthralgia with specific autoimmunity or high genetic risk	
Variables								
Demographics	Age (years)	42 (12)	43 (14)	44 (12)	45 (15)	50 (14)	48 (13)	<0.001
	Gender (female)	68%	72%	78%	75%	86%	84%	0.004
	White ethnicity	84%	92%	88%	90%	91%	82%	0.044
	BMI	27 (6)	24 (4)	24 (4)	24 (4)	25 (5)	26 (5)	0.02
	Tobacco smoking							0.22
	current	29%	18%	28%	16%	24%	20%	
	previous	26%	25%	33%	31%	26%	24%	
	never	29%	51%	29%	51%	48%	56%	
Biology	ACPA seropositivity (commercial or non-commercial assays)		0%	0%	42%	0%	31%	<0.01
	RF seropositivity (IgA or IgM)							
	at least 1× ULN		12%	9%	66%	17%	53%	<0.01
	at least 3× ULN		0%	0%	61%	0%	43%	
	Anti-RA33 antibodies		0%	0%	3%	1%	2%	0.02
	(3× ULN)							
	HLA-SE							<0.01
	0 copy		55%	0%	52%	54%	35%	
	1 copy		43%	0%	39%	43%	27%	
	2 copies		0%	100%	7%	0%	27%	
	Undifferentiated arthritis	0%	0%	0%	0%	11%	6%	<0.01

'Highgenetic risk' defined as having two copies of the HLA-SE. 'Undifferentiatedarthritis' means : presence of clinically suspect arthralgia + a least oneswollen joint (patient reported or nurse examined). P values computed excludingthe NA group.
ACPA, anticitrullinated peptide antibodies; ANOVA, analysis of variance; BMI, body mass Index; CCP, cyclic citrullinated peptide; HLA-SE, human leucocyte antigen shared epitope allele; SCREEN-RA, Evaluation of a SCREENing strategy for Rheumatoid Arthritis; ULN, upper limit of the norm.

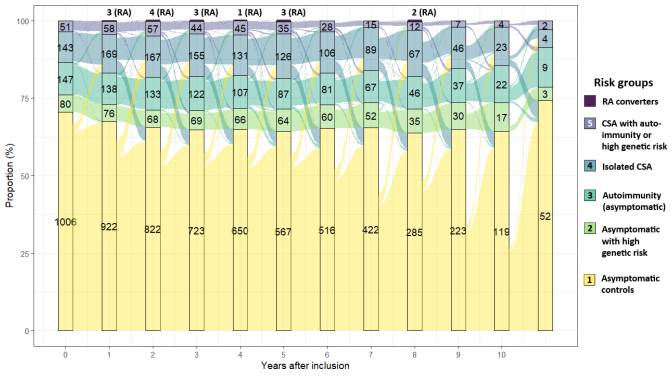

Figure 4 Detailed sequential evolution of risk-group classification of participants, SCREEN-RA cohort, Switzerland, 2009–2020. 'Converter' means 'newly diagnosed'. This figure represents the number of participants by risk-group (ie, columns in table 5) and by years of follow-up. Participants not yet assigned to a group are not represented (hence total N on the figure is 1427 participants). CSA, clinically suspect arthralgia; RA, rheumatoid arthritis. SCREEN-RA :Evaluation of a SCREENing strategy for Rheumatoid Arthritis.

distinguishable from other FDRs, even if the small sample size does not allow definitive conclusions.

Risk-group classification at baseline and evolution over time.

Table 5 presents the baseline classification into 5 subgroups of all recruited subjects. The group '1' comprises asymptomatic subjects without specific autoimmunity associated with RA nor strong genetic risk factors, who are considered to be at 'low risk' for RA development. In contrast, participants in the '2'–'5' subgroups were classified as 'high risk' and followed more closely, with a yearly invitation for blood sampling. Criteria for 'high-risk' classification are based on existing literature and detailed in figure 2. The 'NA' (= 'non-assigned') subgroup in table 5 is comprised of individuals recently enrolled, for whom serological results are still awaited to confirm final classification. The process of both follow-up and sequential blood collection allowed to observe the evolution of 108 subjects from the 'low-risk' group to a 'high-risk' group. Figure 4 represents the detailed sequential evolution of participants, per risk subgroup, across years of follow-up.

FINDINGS TO DATE

Musculoskeletal ultrasound

Musculoskeletal ultrasound was performed by an independent blinded assessor in 273 individuals from the SCREEN-RA cohort, whom 96 (35%) had some signs of inflammatory activity (positive power Doppler) on ultrasound. Power Doppler on ultrasound was associated with the presence of unclassified arthritis (ie, at least one swollen join at physical examination), but not with any of the other preclinical phases of RA, thus not supporting the indiscriminate use of musculoskeletal ultrasound in a screening strategy for RA in a population with a limited risk of developing RA.[67]

Expanded T cell clones

The proportion of highly expanded T cell clones in the peripheral blood of participants in the SCREEN-RA cohort increased the closer the participants were to the onset of RA,[68] which is consistent with the 'mucosal origins hypothesis'.[21] Indeed, antigen-specific T cells are required to build antibody mediated immune responses by activating B-cells. In particular, this cytokine cross-talk takes place at the mucosal level, where B-cells will in turn generate high amounts of secreted IgA,[69] including IgA-ACPA in inflammatory context.[70] An expansion of T-cell clones before RA diagnosis therefore fits in line with the current hypothesis that RA could result from the systemic-spread of an initially local mucosal immune reaction.

Female hormonal factors

The assessment of female hormonal factors among women in the SCREEN-RA cohort suggested that peri-menopausal status was significantly associated with ACPA positivity ($p<0.001$),[71] which underlines the potential role of female hormonal factors in the onset of RA.[7] This is in line with a previous finding that the prevalence of ACPAs increases with age, peaking between 45 and 55 years old for women, but not for men.[72]

Periodontitis

SCREEN-RA participants (n=99) were examined for periodontal status by a blinded periodontist, to assess the link with ACPA seropositivity. This nested case–control study revealed a higher prevalence and severity of periodontitis and poorer periodontal conditions in the ACPA positive subjects, compared with ACPA negative subjects.[73] This finding suggests that periodontitis precedes the development of the disease and may be causally associated with the onset of RA.

Gut microbiota

Bacterial composition of available stool samples was determined by a blinded external research group. 'High-risk' samples were then compared with samples from asymptomatic participants, and revealed an expansion of *Prevotella* species, in particular *Prevotella copri*.[30] This study was the first to confirm intestinal expansion of known RA-associated microbes in the pre-clinical phases of RA, suggesting that the association between gut microbiome and early RA might be causal. A second stool sampling campaign is currently ongoing.

Strengths and limitations

The main strength of the SCREEN-RA study is its longitudinal design, with physical and biological data collected in a controlled environment, using standard operating procedures. The recruitment and long-term follow-up of asymptomatic individuals allows better characterisation of the preclinical stages of RA. The variety of preclinical RA stages enrolled gives the opportunity to realise nested studies, which help to understand the link between environmental factors and specific preclinical stages of the disease, and ultimately apprehend factors driving the onset of RA. The longitudinal

follow-up allows for more accurate causal inferences than typical case–control studies.

The principal limitation of the SCREEN-RA study is the low incidence and slow rate of RA conversion, which requires the enrolment of numerous asymptomatic participants to ensure the observation of a limited number of individuals developing a definite diagnosis of RA. Moreover, we cannot formally exclude a selection bias since symptomatic individuals could have a higher motivation to participate. However, the observed incidence rate in our cohort (~2.1 case per 1000 person-years) is still compatible with previous studies in the same population.[5 74]

An important part of the collected data is based on self-assessment; hence, we cannot completely exclude the possibility of outcome misclassification and measurement uncertainty on symptom-related items. Notably, our group 4 'Isolated CSA', as presented in table 5, is likely to overestimate the proportion of individuals with true inflammatory arthralgias, because the CSA definition relies principally on self-reported symptomatology and nurse-examination. This overestimation appears on figure 4: some participants classified in group 4 often later regressed to lower-risk and asymptomatic subgroups, probably because of fluctuating aspecific symptomatology. To address this issue of misclassification, we categorised the highest-risk participants (group 5), as those presenting both CSA symptoms and biomarkers. We may further underestimate the proportion of asymptomatic 'high-risk' individuals (group '3', ie. columns 2 and 3 in table 5), because of differential follow-up procedures. Indeed, our risk classification includes biological markers that are not immediately available, hence enrolled individuals are occasionally misclassified as low risk, due to delay in obtaining serological results (ie, non-assigned subgroup in table 5). Second, because of budget limitations, or refusal from the participants, not all individuals have been blood-sampled yearly, since, low-risk participants are not invited to provide additional blood sample unless they develop new symptoms. To address this issue, we consider increasing our blood-sampling capability to include every low-risk individual in the annual serological sampling.

FUTURE PLANS

Currently ongoing, a multicentre collaboration is focusing on characterising antibody production at mucosal site to identify novel biomarkers for the prediction of RA development. Analysis will include immunohistochemistry, 16s RNA sequencing, single cell cloning. A complementary project aims at pinpointing other biomarkers by large 'multiomics' analysis. The collected blood samples will be used to extract genomic DNA (targeted n=500) and total RNA (targeted n=700), which will be compared with DNA and RNA from patients with RA from the SCQM-RA cohort (targeted n=100).[75] Finally, linking periodontitis to ACPA status previously suggested that mucosal inflammation can be an important trigger in the onset of autoimmunity associated with RA.[21] One of the largest mucosal site is the gut, and our initial analysis of intestinal microbiota of SCREEN-RA participants suggested a link between gut dysbiosis and development of RA.[30] Thus, we are currently resampling faecal material of participants at different preclinical stages, using more up-to-date methodology,[66] as well as studying mucosal and serological immune responses against hypothesised 'autoimmunogenic' micro-organisms (such as *P. copri*).[76 77]

CONCLUSION

Started in 2009 in Switzerland, the SCREEN-RA cohort focuses on long-term follow-up of individuals at risk of RA. Both symptoms, signs and biological data have been collected systematically in 1458 FDR of patients with RA. Prospective cohort designs allow more reliable causal inference than case-control experiments, while providing the opportunity to realise nested studies or validation studies.

Despite slow conversion rate toward classifiable RA, the study confirmed the involvement, in early phases of RA, of previously known risk factors, such as female hormonal factors, periodontitis and autoantibodies. Future plans include validation of new RA-associated biomarkers, and assessment of host-microbial immune homeostasis in pre-clinical phases of RA. In the new era of 'personalised medicine', early identification and stratification of at-risk individuals will indeed be key to establish reliable diagnostic approaches. We also expect our future research to demonstrate the efficacy of targeted preventive interventions.

COLLABORATION

Our team welcomes collaborative projects, in particular for biomarker identification and/or replication studies. Contact senior author Pr. FINCKH (ORCID: 0000-0002-1210-4347 - Email: axel.finckh@hcuge.ch).

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content), previously published studies, in particular ref 72. ET: Research coordinator. Involved in updating patient information, various logistics and administrative issues for the cohort, implementation of follow-up questionnaires, coordination with other Swiss centres, communication/recruitment (social media, website, campaigning, etc.), minor corrections in the manuscript. OS: Research nurse. Involved in data acquisition (follow-up visits, sampling), expedition of stool sampling kits, optimisation of questionnaires, minor corrections in the manuscript. DSC: Professor of statistics. Involved in implementation of Redcap database, troubleshooting database, statistical analysis, implementation of the study (in particular, power and sample size calculation), major corrections in the manuscript. AR-R: Professor in a collaborative cenrer (St. Gallen). Involved in substantial sample collection and data acquisition in St. Gallen Canton, corrections in the manuscript. DK: professor in a collaborative centre (Basel). Involved in extension of the study in Switzerland, substantial sample collection and data acquisition in Basel Canton, corrections in the manuscript. BM: professor in a collaborative centre (Bern). Involved in extension of the study in Switzerland, substantial sample collection and data acquisition in Bern Canton, corrections in the manuscript.AF: professor in the Rheumatology division (Geneva) and Principal Investigator of the SCREEN-RA study. Involved in starting the cohort in 2009 (Geneva), development of initial questionnaires, obtention of ethical approval and funding, overall study design, classification of participants in 'risk groups', supervision of most of the previous publications, major corrections in the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Initial approval in 2008 by the 'Comite départemental d'éthique de médecine interne et de médecine communautaire', protocol 08-102, project name : 'Evaluation d'une stratégie de dépistage de la polyarthrite rhumatoïde'. Every modification on the project was then approved by relevant cantonal ethic committees (respectively for each Swiss canton for which the project was extended). The SCREEN-RA cohort has been approved by the relevant ethic Committees (project PB_2016–00889), and participants sign an informed consent before enrolment, in accordance with the Declaration of Helsinki.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. Anonymised data from the SCREEN-RA cohort can be shared on request (contact senior author Pr. Finckh at axel.finckh@hcuge.ch).

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SUPPLEMENTARY MATERIAL

Shared Epitope Genotyping.

DNA was extracted from sampled blood using a modification of the salt-out technique (Nucleaon TM, Scotlab, UK). Reverse polymerase chain reaction was used to determine HLA-DRB1 shared epitope polymorphism (sequence specific oligonucleotide primers), with commercially available reagents validated by the Swiss National Reference Laboratory for Histocompatibility. The method discriminates all the major subtypes in different alleles groups within DRB1*04. HLA-DR1, DR14 and DR4 alleles negative for the SE70-74 motif are also discriminated. Finally, PCR-SSP was used to analyze the SE-positive ambiguities in order to obtain the final 4-digit result.

Assays for assessment of serological ACPA and RF.

Assessment of RF and ACPA serological status was performed using various commercially as well as non-commercially assays. List as follow:

- CCPlus Immunoscan® (anti-CCP2) IgG ELISA (Svar Life Science, Malmö, Sweden)
- QUANTA Lite® CCP3.1 IgG/IgA ELISA (INOVA Diagnostics)
- QUANTA Lite® CCP3 IgG ELISA (INOVA Diagnostics)
- QUANTA Flash® CCP3 IgG CIA (INOVA Diagnostics)
- QUANTA Lite RF IgM ELISA (INOVA Diagnostics)
- QUANTA Lite RF IgA ELISA (INOVA Diagnostics)
- Elia RF IgM (Phadia AB)
- Elia RF IgA (Phadia AB)
- ELIA anti-RA33 (IgA, IgG or IgM isotype), research use only (Phadia AB)

2 – Submitted (RMD Open) - Gut microbiome and intestinal inflammation in preclinical stages of rheumatoid arthritis

Gut Microbiome And Intestinal Inflammation In Preclinical Stages Of Rheumatoid Arthritis

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ABSTRACT

Background:

Fecal Prevotellaceae, and other microbes, have been associated with rheumatoid arthritis (RA) and preclinical RA. We have performed a quantitative microbiome profiling study in preclinical stages of RA.

Methods:

First-degree relatives of RA patients (RA-FDRs) from the SCREEN-RA cohort were categorized in four groups: - Controls: healthy asymptomatic RA-FDRs; - High genetic risk: asymptomatic RA-FDRs with two copies of the shared epitope; - Autoimmunity: asymptomatic RA-FDRs with RA-associated autoimmunity; - Symptomatic: clinically suspect arthralgias or untreated new-onset RA.

Fecal samples were collected and frozen. 16S sequencing was performed, processed with DADA2 pipeline, and Silva database. Cells counts (cytometry) and fecal calprotectin (ELISA) were also obtained. Microbial community analyses were conducted using non-parametric tests, such as PERMANOVA, Wilcoxon and Kruskal-Wallis, or Aldex2.

Results:

A total of 371 individuals were included and categorized according to their preclinical stage of the disease. Groups had similar age, gender, and BMI. We found no significant differences in the quantitative microbiome profiles by preclinical stages (PERMANOVA, $R2 = 0.00798$, $p = 0.56$), and in particular no group-differences in Prevotellaceae abundance. Results were similar when using relative microbiome profiling data (PERMANOVA, $R2 = 0.0073$, $p = 0.81$), or Aldex2 on 16S sequence counts. Regarding fecal calprotectin, we found no differences between groups ($p = 0.3$).

Conclusions:

We could not identify microbiome profiles associated with pre-clinical stages of RA. Only in a subgroup of individuals with the most pronounced phenotypes did we modestly retrieve the previously reported associations.

INTRODUCTION

Recent findings have suggested a mucosal origin of RA.[1] In this context, gut microbiome has been repeatedly analyzed in RA patients, and some potentially relevant microbes have been linked to RA development.[2–12] The mucosal origins hypothesis further entails local mucosal inflammation and increased permeability of the intestinal mucosal barrier for bacterial compounds to trigger autoimmunity.

Mice-derived evidence revealed how these "arthritogenic" bacteria could exacerbate intestinal inflammation and arthritis, and upregulate a number of auto-reactive T-cells.[2,5,6,13–15] Scher et al. demonstrated that colonization with *Prevotella copri* (*P. copri*) by oral gavage of antibiotic-treated mice worsens chemically induced colitis, compared to *Bacteroides thetaiotamicron*.[2] Then, in 2016 Maeda et al. demonstrated that germ-free mice colonized with *Prevotella*-dominated microbiota from RA patients had an increased number of intestinal T_H17 cells and developed severe arthritis when treated with zymosan, compared with "healthy-control-microbiota" colonization.[5] Other microbes have been isolated from RA-patient feces and have proven to aggravate arthritis in mice models; they include *Eggerthella,*[4,6,16] *Collinsella,*[6,9,16] *Subdoligranulum,*[15] or *Fusobacterium nucleatum*.[17] The involved mechanisms may sometimes differ, as the *Subdoligranulum* strain seemed to stimulate T_H17 cells expansion and B-cell activation in gut lymphoid follicles,[15] while *Fosubacterium nucleatum* rather promoted arthritis by secreting antigenic outer-membrane vesicles able to translocate in joints and trigger inflammation.[17]

Regarding *Prevotellaceae*, it has been speculated that they might carry epitopes cross-reactive to arthritis-related autoantigens.[18–20] Alternatively, *Prevotella* species have also been hypothesized to take part in the process of biofilm formation, which in the oral context connects to periodontitis and T_H17 immune responses.[21–25] Also, in the oncologic context, *P. copri* is among the bacteria increasing response to anti-PD1 therapy, suggesting "immunogenic" characteristics.[26] The latter could be a consequence of an immune-stimulant glycolipid that *P. copri* produces, alpha-galactosylceramid, which can activate non-conventional T-cells.[27–29]

We reasoned that, whatever the underlying mechanism, for a causal association between gut microbes and RA to exist, these microbes have to be found before the onset of RA. The present study is an attempt to replicate and expand on previous findings, especially regarding the presence of Prevotellaceae, using a quantitative methodology, in a larger untreated preclinical population at risk for RA.[30]

METHODS

Study population

The SCREEN-RA cohort has been extensively described elsewhere.[31] Briefly, since 2009 the SCREEN-RA cohort has recruited more than 1'500 first-degree relatives (RA-FDRs) of established RA patients, across Switzerland. After having provided a baseline serum sample, participants are followed up yearly using online questionnaires. Individuals at higher risk of developing RA, presenting with autoantibodies associated with RA or clinically suspect arthralgia, are monitored more closely and re-invited for further study visits on a yearly basis, until the development of RA. Participants were excluded if they developed another autoimmune disease or if they initiated an immunosuppressive treatment.

In parallel, untreated new-onset RA patients from the Geneva rheumatology division were also invited to participate in the study as positive controls, before initiating DMARD therapy or glucocorticoids.

Study design

This study is nested within the SCREEN-RA cohort study. We performed a cross-sectional comparison between four distinct at-risk groups, defined based on the current recommendations (details below).[32]

Sample collection

Between September 2019 and October 2021, SCREEN-RA participants have been invited to provide a stool sample paired with a serum sample. Participants were provided with stool collection-devices allowing the creation of several aliquots of stool and proceeded to stool sampling at home. They temporarily froze the fresh stool sample at −20°C, and rapidly brought it to the study centres, to be stored at −80°C without any additive, as previously described.[33] During the study visit, a blood sample was also taken, clotted and centrifuged to store several serum aliquots at -80°C according to SCREEN-RA standard operating procedures.[31] The average time difference between stool sampling and serum sampling was 2.55 days (SD = 13.6 days).

Serum samples processing

Each serum sample was assessed for RF and ACPA serological status. ACPA serology was defined as positive if at least one of the following tests was positive: CCPlus Immunoscan® (anti-CCP2) IgG ELISA (Svar Life Science, Malmö, Sweden), QUANTA Lite® CCP3.1 IgG/IgA ELISA (INOVA Diagnostics), QUANTA Lite® CCP3 IgG ELISA (INOVA Diagnostics), QUANTA Flash® CCP3 IgG CIA (INOVA Diagnostics). Similarly, RF was defined as positive if at least one of the following tests was positive: QUANTA Lite RF IgM ELISA (INOVA Diagnostics), QUANTA Lite RF IgA ELISA (INOVA Diagnostics), Elia RF IgM (Phadia AB), Elia RF IgA (Phadia AB). Results were recorded in the database for each test and interpreted based on the manufacturer's recommended cutoffs.

Stool samples processing

DNA was extracted from a thawed stool aliquot using Qiagen MagAttract PowerMicrobiome DNA/RNA kit bead-beating kit on a robotized platform. DNA samples were then randomized on 96 wells plates, and for bacterial and archaeal characterization, extracted DNA (dilution 1:10) was further amplified in triplicate using 16S rRNA primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'- GGACTACNVGGGTWTCTAAT-3') targeting the V4 region, modified to contain a barcode sequence between each primer and the Illumina adaptor sequences to produce dual-barcoded libraries. Two deep sequencing was performed on a MiSeq platform (2x250 PE reads, Illumina).

Microbial loads of stool samples were measured as described previously.[34] Moisture content was determined as the percentage of mass loss after lyophilization from 0.2 g frozen aliquots of non-homogenized fecal material (−80 °C) as previously described.[34] Finally, fecal calprotectin concentrations were determined using the fCAL ELISA Kit (Bühlmann), on frozen fecal material as described previously.[34]

Exposure of interest

RA-FDRs from the SCREEN-RA cohort [31] were classified into four pre-clinical stages (Figure 1):

1) Control, i.e., healthy asymptomatic RA-FDRs, without clinically significant autoantibody titers (ACPA < the upper limit of the norm (ULN), RF < 3x the ULN, anti-Ra33 < 3x the ULN);

2) High genetic risk, i.e., healthy asymptomatic RA-FDRs with two copies of the shared epitope (SE);

3) Autoimmunity, i.e., RA-FDRs without articular symptoms, but with clinically significant autoimmunity (ACPA titers at least the ULN, or RF or anti-Ra33 at least 3x the ULN);

4) Symptomatic, i.e., RA-FDRs with a clinically suspect arthralgias (CSA) score equal or greater to 4, using the EULAR questionnaire. When one of the CSA items was missing or if concomitant autoimmunity, a CSA score greater to 3 was used to define clinically suspect symptoms for RA (see criteria in Table S1).[35] Finally, this group also includes newly diagnosed RA, i.e. RA-FDRs who developed incident RA and a small number of untreated new-onset RA recruited as positive controls, – as the number of incident RA cases was insufficient to constitute an independent group.

This classification is defined at 1) the time of serum sampling for serum-derived variables (except anti-Ra33 titers), 2) in the 60 days surrounding the date of stool sampling for variables included in the CSA score (maximum score was retained).

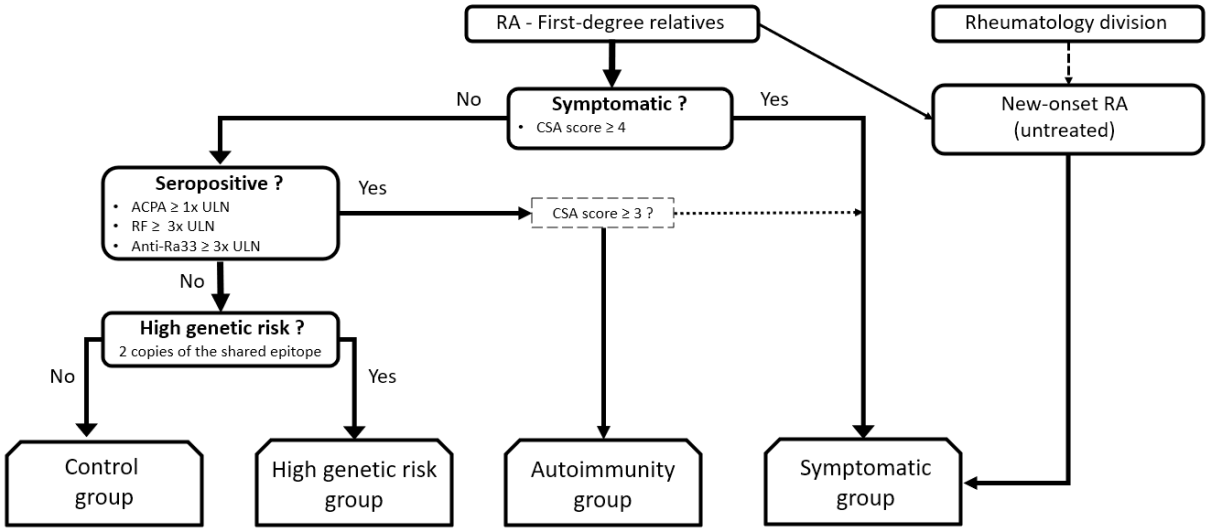

Figure 1: Screen-RA group assignation algorithm. RA = Rheumatoid Arthritis. CSA = Clinically Suspect Arthralgia, defined using EULAR score. ACPA = Anti Citrullinated Peptide Antibodies. RF = Rheumatoid Factor. ULN = Upper Limit of the Norm. New-onset RA recruited from the rheumatology division are not necessarily RA-First-degree relatives. Of note, two cutoffs are possible for CSA score; in case of one or two missing items, or in case of concomitant autoimmunity, the lower cutoff is applied (CSA score of at least 3).

As a secondary exposure of interest, we selected from each group only the 20 most pronounced phenotypes (RA diagnosis, then highest CSA scores and auto-antibodies titers), matching the 20 individuals to controls for sex and age. This subgrouping was pre-planned and used for a parallel project.

Outcomes

The main outcome was the quantitative abundance of Prevotellaceae bacteria in stool samples, expressed as an estimation of the absolute bacterial cell counts per gram of stool (Quantitative Microbiome Profiling, QMP). As a secondary outcome, we examined the percentage of total 16S sequences (Relative Microbiome Profiling, RMP). Other secondary outcomes also included the abundances of other bacterial families and genera of interest, as well as fecal calprotectin concentration.

Statistical analyses

Population characteristics

Continuous baseline variables were expressed as means with standard deviation (SD). Continuous variables were compared between groups using Kruskal-Wallis test if not normally distributed or ANOVA if more than two groups. Categorical variables were described using percentage, and compared using $\chi 2$ test, or Fisher's exact test for small sample sizes. Two-tailed p values <0.05 were considered significant. Analyses were conducted using R, version 4.3.0, with package *tableone*.

Microbiome

Fastq files obtained from the MiSeq platform were filtered and trimmed using the DADA2 pipeline (v1.16.0) on R (v4.0.3).[36,37] Reads were truncated after 230 (forward) and 150 (reverse) nucleotides. Denoising, merging and chimera removal were performed with default parameters. This generated a set of Amplicon Sequence Variants (ASV), which were subsequently matched to the Silva 16S database (138v) using the DADA2 built-in assigner.[38]

The output of the DADA2 pipeline was visualized on R with packages *phyloseq* (v1.32.0) and *ggplot2* (v3.4.2).[36,39,40] Sample richness was assessed using Shannon Index. For Principal Coordinate Analysis (PCoA), ASV counts were transformed into proportions, and samples were ordinated using a Bray-Curtis dissimilarity matrix (at the ASV level), before PCA plotting. PERMOVA was performed on the Bray-Curtis dissimilarity matrix using function *adonis2()* from R package *vegan*.

The quantitative microbiome profiling (QMP) matrix was built as described previously.[33] In brief, samples were downsized to even sampling depth, defined as the ratio between sampling size (16S rRNA gene copy number-corrected sequencing depth) and microbial load (the average total cell count per gram of frozen fecal material). 16S rRNA genome copies numbers were imputed using RasperGade16S,[41] a new tool that utilizes a heterogeneous pulsed evolution model for predicting 16S rRNA genome copies (also providing confidence estimates for the predictions). A minimum rarefied read counts of <150 was used for QMP analyses. Rarefied ASV counts were converted into numbers of cells per gram.

For enterotyping, observed genus richness was calculated on the genus matrix (downsized to 10,000 reads) using *phyloseq*,[39] as already reported for previous studies. [34] Enterotyping (or community typing) based on the Dirichlet-multinomial mixtures approach was performed

in R as described previously.[34,42,43] It used a combined genus-level abundance RMP matrix including SCREEN-RA samples compiled with 1045 samples originating from the Flemish Gut Flora Project.[44] The optimal number of Dirichlet components based on the Bayesian information criterion was four. The four clusters were named *Bacteroides1* (Bact1), *Bacteroides2* (Bact2), *Prevotella* (Prev) and *Ruminococcaceae* (Rum) as described previously.[33].

Microbial community composition and differential analysis were conducted using non-parametric tests, such as Wilcoxon rank sum and Kruskal-Wallis. To assess other taxa-specific differences between groups, low abundance ASV were removed (i.e., ASV not present at least 10 times in 5% of the samples). Then ASV in this filtered dataset were aggregated at the relevant taxonomical level (Family or Genus level), and sequence counts were compared between groups using R package *Aldex2* accounting for multiple testing and data compositionality (*Aldex2* performs a centered-log-ratio transformation on the count data and applies Benjamini-Hochberg correction on p-values). Other p-values were also corrected for multiple testing using the Benjamini–Hochberg method (reported as *p-adj*) when multiple tests were performed on lists of features.

Fecal calprotectin

Since non-normally distributed, fecal calprotectin values were compared between groups using Wilcoxon signed rank tests (pairwise, with control group as reference, applying Benjamini–Hochberg correction).

Sensitivity analysis

To compare more pronounced phenotypes of the groups, we selected the 20 most persons with the highest autoantibody titers or arthralgia scores. We compared the difference in median abundances of Prevotellaceae using permutation tests with 10’000 permutation samples. For each permutation sample, two groups of 20 individuals were randomly selected from the whole cohort, and difference in median Prevotellaceae abundances (proportions) were compared. The one-tailed p-value was estimated by the proportion of permutation samples with a median difference as extreme or more extreme than the median difference between the two pronounced phenotype groups.

RESULTS

Population description

A total of 371 individuals were included in this study (Figure 2).

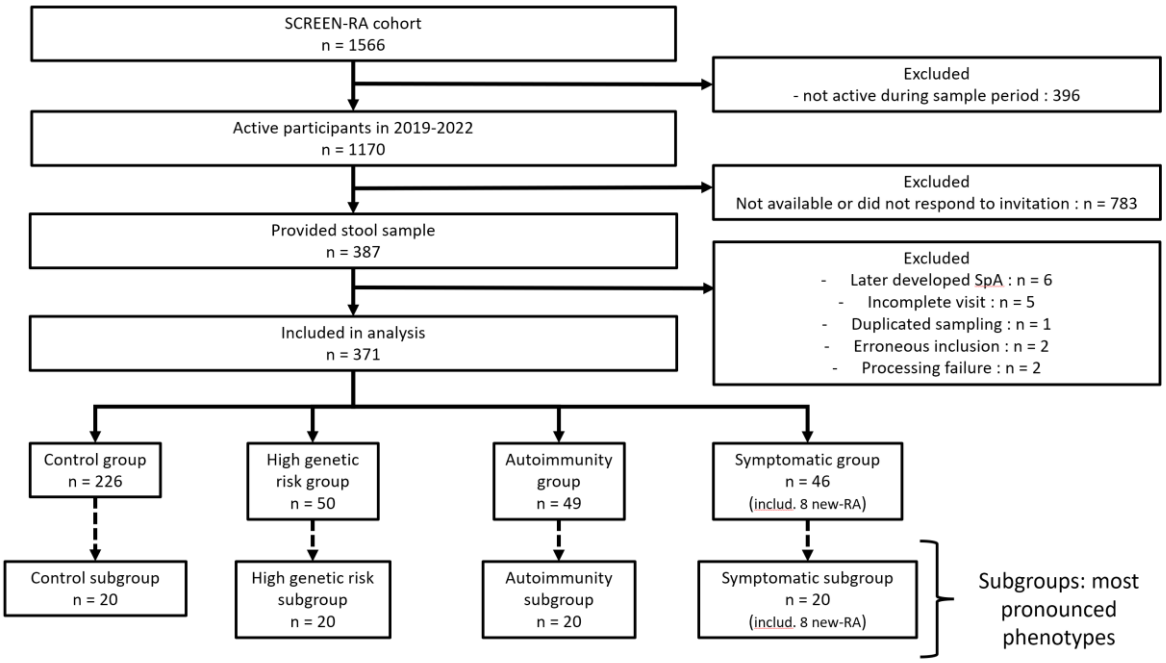

Figure 2: Study recruitment flow-chart. SpA = Spondyloarthritis. RA = Rheumatoid arthritis. New-RA = includes both RA-FDRs from SCREEN-RA who developed a new RA at the time of sampling, and new-onset RA from the Geneva rheumatology division, which are not necessarily RA-FDRs.

We sampled a total of 226 controls, 50 high-genetic risk individuals, 49 individuals with autoimmunity and 46 symptomatic individuals (including 8 new-onset untreated RA). Baseline characteristics per group are presented in Table 1. The subgroups with the most pronounced phenotypes are presented in Table S2.

Table 1: Baseline characteristics of study population, SCREEN-RA

Variable	Control n = 226		High Genetic risk n = 50		Autoimmunity n = 49		Symptomatic n = 46		p value
	n % of total in group Otherwise: Mean (SD)								
		Miss.		Miss.		Miss.		Miss.	
Female	78 %		82 %		73 %		87 %		0.377
Age	52 (14)		53 (12)		55 (16)		54 (12)		0.523
BMI	24 (4)		25 (3)		25 (4)		25 (5)		0.7
Share epitope copies									
0	53 %		0 %		47 %		50 %	2 %	<0.001
1	47 %		0 %		39 %		39 %		
2	0 %		100 %		14 %		9 %		
RA autoimmunity	0 %		0 %		100 %		26 %		<0.001
ACPA									
Negative	100 %		100 %		67 %		83 %		<0.001
Low	0 %		0 %		14 %		4 %		
High	0 %		0 %		18 %		13 %		
RF									
Negative	90 %		94 %		27 %		65 %		<0.001
Low	10 %		6 %		2 %		17 %		
High	0 %		0 %		71 %		17 %		
Anti-Ra33									
Negative	41 %	50 %	72 %	8 %	65 %	10 %	54 %	33 %	<0.150
Low	9 %		20 %		20 %		13 %		
High	0 %		0 %		4 %		0 %		
Clinically Suspect Arthralgia (CSA)									
No	96 %	4 %	100 %		100 %		9 %	2 %	<0.001
Yes	0 %		0 %		0 %		89 %		
CSA score (detail)									
1	64 %		80 %		82 %		4 %		
2	23 %		16 %		18 %		4 %		
3	10 %	4 %	4 %		0 %		9 %	2 %	<0.001
4	0 %		0 %		0 %		61 %		
5	0 %		0 %		0 %		11 %		
6	0 %		0 %		0 %		9 %		
Antibiotics (past 2 months)	6 %		2 %		6 %		6 %		0.710
Probiotics (past month)	10 %		8 %		8 %		9 %		0.965
Surgery (past 2 months)	2 %		6 %		6 %		6 %		0.162
Travel outside Europe (past month)	2 %		2 %		2 %		0 %		0.827

SD = standard Deviation. BMI = Body Mass Index. RA = Rheumatoid Arthritis. ACPA = Anti-citrullinated Peptide Antibodies. RF = Rheumatoid Factors. CSA = Clinically Suspect Arthralgia.

Of note, 4 patients with new-onset RA included in "symptomatic" group due to their diagnosis, however, did not meet threshold for "CSA" because of either missing data in questionnaires or not having obvious symptoms at the study visit (symptoms can fluctuate and regress between flares).

Note: For technical reasons, anti-Ra33 titers were measured on several previous serum samples using kit: ELIA anti-RA33 for IgA, IgG and IgM isotypes (research use only, Phadia AB). Hence, the present study imputes the anti-Ra33 serology based on serological measures obtained months to years before the stool sampling of interest; which also explains the higher missing rate when a recent sample with anti-ra33 dosage was not available.

Microbiome

Shannon index, which reflects the number of different bacterial taxa identified in each stool sample (alpha-diversity), did not differ between the groups (Figure S1). As a gross assessment, each fecal microbiome can be assigned to an enterotype, based on the dominant taxa.[45] Assigning samples in their respective enterotypes did not reveal significant differences between the groups (Fisher's exact test p = 0.64; Figure 3A).

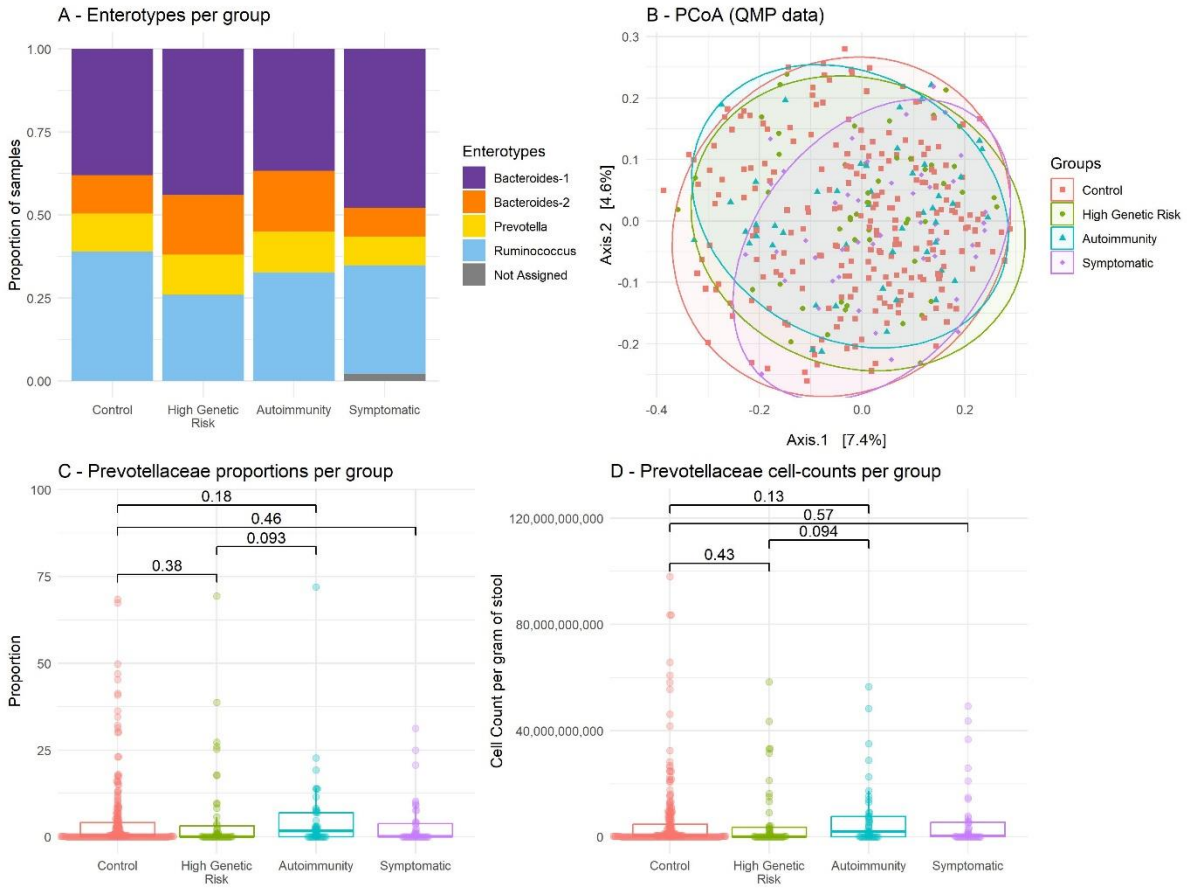

Figure 3: Gut microbiome profiling by group. A – Enterotype distribution by group; Fisher p = 0.64. **B** - Principal Coordinate Analysis performed at the sequence variant level, after quantitative correction (QMP); distance between points reflects their dissimilarity, based on Bray-Curtis's index. PERMANOVA R2 = 0.00798; p = 0.56. **C** – Proportions of Prevotellacae bacteria per group (RMP), boxplots; p-value are Wilcoxon tests. **D** – Prevotellacae estimated cell-counts per group (QMP), boxplots; p-value are Wilcoxon tests.

To assess the main outcome (QMP) at the most granular level, it is possible to compare samples pairwise, using Bray-Curtis distance.[46] This index, ranging from 0 to 1, reflects the ecological difference between two samples, in terms of counts of detected taxa (in our case,

the QMP taxonomic counts per gram of stool). Comparing sample composition to each other using Bray-Curtis index subsequently allows performing a Principal Coordinate Analysis (PCoA); on such a figure, the distance between two points increases when their compositional difference increases, as assessed by Bray-Curtis index. We found no group-wise clustering doing a PCoA on the QMP data at the 16S sequence variant level (PERMANOVA, R2 = 0.00798, p = 0.56; Figure 3B). Also, using the RMP data (uncorrected bacterial proportions) yielded the same results (PERMANOVA, R2 = 0.0073, p = 0.83). Overall, stool profiling was similar between groups, both when assessed as estimated cell counts or as percentages (see Family level, Figure S2).

More specifically, contrary to our previous report,[30] we found no group-differences in Prevotellaceae QMP abundance (Figures 3D; Kruskal-Wallis p = 0.29). Results were similar using the RMP data (Figure S3, Figure 3C, Kruskal-Wallis p = 0.28).

To explore differential abundance of other bacterial taxa, as secondary outcomes, we used *Aldex2* tool. It performs centered log-ratio transformations on crude 16S-count data and applies Benjamini-Hochberg correction on Kruskal-Wallis p-values, to account for multiple testing. *Aldex2* found no significant differences between groups regarding other bacterial families or genera present in the dataset (Figure S4). Also, contradicting previous findings,[47] grouping on shared-epitope genotype, we found no association between shared epitope presence and Prevotellaceae, or *Prevotella* genera (not shown).

Microbiome in subgroups

In the sensitivity analysis, we selected the 20 most pronounced phenotypes in each group (for instance, in the symptomatic group: all 8 RA patients, then highest autoantibody titers or arthralgia scores). We modestly reproduced published results regarding increased Prevotellaceae abundance in autoimmunity and symptomatic groups (Figure S5), both in QMP (Figure 4D) and RMP (Figure 4C). Even though the increase in *Prevotella*-enterotype is visible for the symptomatic subgroup, it was not significant (Figure 4A); also, overall PCoA PERMANOVA remained non-significant (Figure 4B).

As an alternative to Benjamini–Hochberg method, we re-assessed the p-value of these subgroup Prevotellaceae differences, by performing a permutation test (10’000 repetitions). Only 5.541 % of the permutation samples had a median difference of quantitative abundance (QMP) more extreme than observed in the pronounced phenotype subgroups (if comparing control versus autoimmunity), corresponding to a one-sided p-value of 0.054 (0.034 if using RMP data; Figure S6).

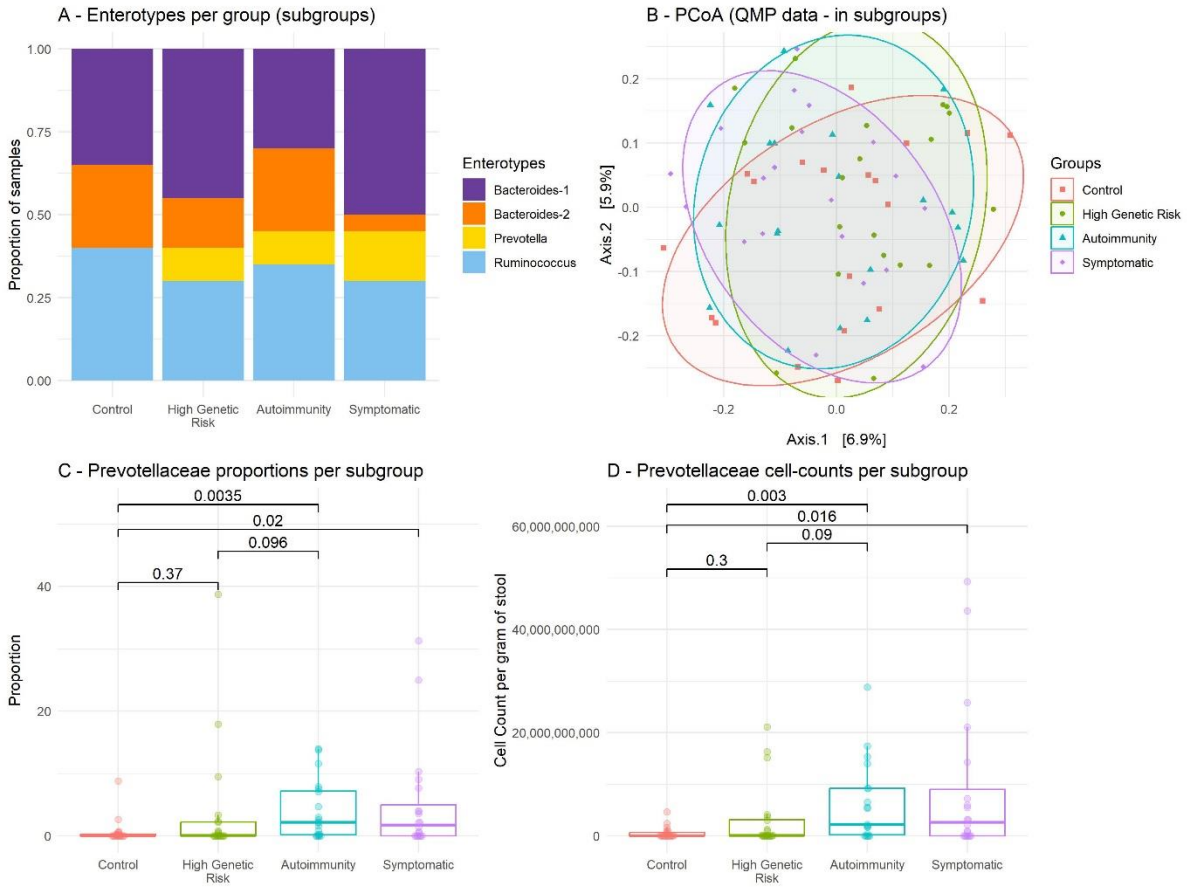

Figure 4: Gut microbiome profiling by subgroup. A – Enterotype distribution by subgroup; Fisher's p = 0.5473. **B** - Principal Coordinate Analysis performed at the sequence variant level with quantitative data; distance between points reflects their dissimilarity, based on Bray-Curtis's index. PERMANOVA R2 = 0.042, p = 0.14. **C** – Proportions of Prevotellacae bacteria per group, boxplot; p-value are Wilcoxon tests (unadjusted). Adjusted p-values are, from bottom to top: 0.37, 0.13, 0.04, 0.014. **D** – Prevotellacae estimated cell-counts per group, boxplots; p-value are Wilcoxon tests (unadjusted). Adjusted p-values are, from bottom to top: 0.34, 0.13, 0.04, 0.014.

Fecal calprotectin

Examining a biomarker of mucosal inflammation, we found no overall difference in fecal calprotectin between groups (Kruskal-Wallis p = 0.3; Figure 5A). When restricting the analysis to the most pronounced subgroups, a trend was noticeable, with a modest increase in the autoimmunity group compared to control group, which disappeared after correction for multiple testing (p = 0.076; p-adj = 0.23; Figure 5B). Also, *Prevotella* genera were not among the bacteria associated with mildly elevated (>100 ug/g) calprotectin in this dataset, as assessed using *Aldex2* (associated microbes were *Streptococcus* and an unclassified *Clostridia UCG-014*) (data not shown).

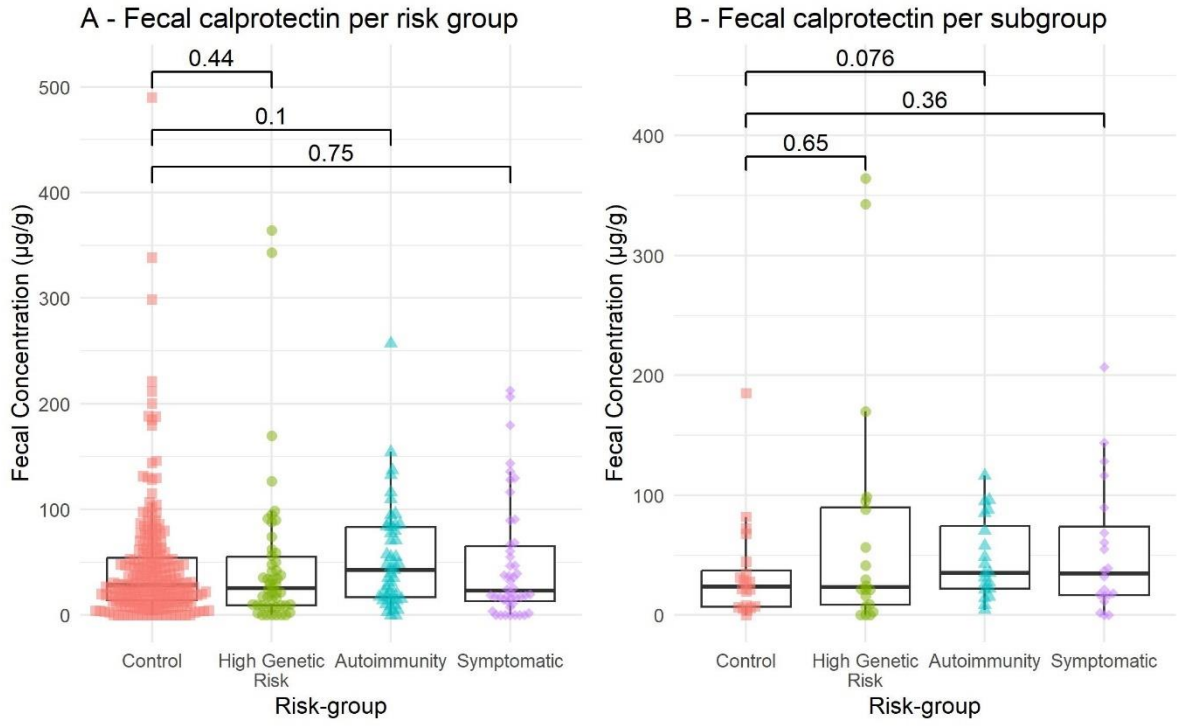

Figure 5: Fecal calprotectin by risk group. Measured with ELISA in fresh frozen stool. **A** – In all included stool samples. P-values are Wilcoxon tests. **B** – Only in the most pronounced phenotypes subgroups. Displayed p-values are Wilcoxon test, non-adjusted. P-adj values for subgroups are respectively (from bottom to top) : 0.65, 0.55, 0.23 .

DISCUSSION

In this study, we investigated the association between fecal bacterial composition, fecal calprotectin, and different "preclinical" stages of RA. We found no association between "dysbiosis", or specific bacterial taxa, and the pre-clinical RA grouping. However, when analyzing a subgroup of individuals with the most pronounced phenotypes, we retrieved some modest associations in line with the previously reported findings, namely an increased prevalence of Prevotellacae in later pre-clinical stages. Also, fecal calprotectin levels did not differ significantly between the groups, which confirms our previous finding on serum biomarkers of intestinal damage.[48] Though a trend for calprotectin elevation in the most pronounced autoimmunity subgroup became non-significant after p-value correction for multiple testing.

To our knowledge, the only comparable study is a previous work from our group, in the same cohort.[30] However, Alpizar et al. used a simpler exposure (merging autoimmunity and symptomatic groups, without including new onset RA cases), a slightly more stringent control group, a different stool sampling procedure together with a different bioinformatical pipeline (though also 16S-based, providing RMP), and an earlier version of the SCREEN-RA database. These technicalities might partly explain the differences in results. However, as the design is conceptually identical, not being able to reproduce the results while using very similar techniques underlines the tenuousness of such associative findings.

Scher et al. were the first to report increased proportions of Prevotellaceae in untreated new-onset RA patients.[2] We only recruited 8 new-onset RA, precluding any reliable conclusion in this subpopulation; still, at first sight RA patients in our study did not have extreme values of Prevotellaceae abundances (whether QMP or RMP, not shown). Other microbiome studies in RA have mostly compared treated chronic RA cases with unrelated healthy controls,[3–12,17,49–51] which do not make them exactly comparable to our study setting due to the impact of antirheumatic therapies on microbiome and intestinal health.

Limitations

The main limitation of this study is related to misclassification of the exposure, in that the classification of at risk population used is based on expert opinion and usual terminology used

in the field.[35] Because our cohort enrolls RA-FDRs before they develop RA, we cannot ensure that our "higher-risk" groups are actually comprised of individuals who will develop RA in the future. Of note, since study completion, two individuals newly developed RA, but at the time of stool sampling (~2 year before), they were assigned to the control group (seronegative, with no clinically significant symptoms – they later developed ACPA-negative RA). Figure S7 illustrates the definitional overlap between groups (Principal Component Analysis, using grouping variables as input).

Our cases and controls are all derived from the same source population of RA-FDRs. By comparing fecal sample from this unique population, we aimed at neutralizing confounding by genetic background, and maybe overall lifestyle, as well as ensuring clinical applicability of potential findings. However, a drawback of this approach is a more phenotypically homogenous population, making any statistical signal even less prominent. Many "mild-phenotypes" did not reaching the thresholds for CSA or for autoantibody seropositivity, and were attributed into our large "control" group (Table 1; Figure S7). Also, the CSA score involves self-reported items and/or nurse's clinical assessment, which could also lead to exposure misclassification because of limited specificity. Overall, imprecise exposure assessment and non-differential misclassification generates a bias towards the null, which could explain the absence of a clear signal. To address the possibility of a dilution of the effect, we had defined *a priori* a subgroup of participants with more pronounced phenotypes, which did confirm, even though modestly, some of the findings previously reported.[2,30]

Last but not least, fecal samples are only a proxy of the gut microbiome, and it is unclear to what extent microbes in feces are informative about the mucosal barrier micro-environment. Microbiome and inflammation on other mucosal sites have also been hypothesized to favor the development RA-autoimmunity, which have not been studied in this analysis. Finally, we have not been able to account for the possible confounding effect of diet, antibiotic treatments, or the use of probiotics; however, given the prevalence of these potential confounding factors was balanced across groups (Table 1), we think it is unlikely that they biased our findings.

Strengths

The main strength of this study is a larger sample size. Our methodology also included for the first time in this population an estimation of fecal bacterial loads, which might be more meaningful than a simple proportion of bacterial taxa, given the high inter-individual variability in total fecal biomass.

To avoid confounding by immunosuppressants and antirheumatic treatments, we only enrolled participants without DMARD therapy. The multimodal assessment of serum autoantibodies, fecal inflammatory biomarker and microbiome composition may also provide precious insights into how these parameters covary. In addition, given the long-term follow-up in the SCREEN-RA cohort, the data we generated will be usable retrospectively, if more individuals develop incident RA.

Perspectives

Even though we confirm the detection of the RA-associated bacterial genera in our cohort of at-risk individuals (Figure S8), we could not find significant group-wise differential abundances. Also, trying to reproduce Pianta et al. findings regarding anti-*Prevotella* serum Ig reactivity, Seifert et al. only retrieved modest results.[52] Similarly, in a recent work we were not able to demonstrate significant increases of serum anti-P *copri* IgG in the context on RA, but we noticed a high variability in reactivity depending on the *P copri* strain tested.[53]

The latter underlines how using only one bacterium as a biomarker may be too simplistic. Future research should rather explore what these different RA-associated microbes have in common (in terms of gene function, surface antigens, mucus-invading capabilities, etc.), while the strain-level variability of *P. copri* should be better accounted for. Alternatively, obtaining gut biopsies from diseased and at-risk individuals would certainly help unravelling host-microbe interactions in the context of RA; but given the inconvenience and ethical issues, this will remain rarely possible. Repeated longitudinal sampling should be considered, to monitor time-variation in the parameters. Finally, one could argue that fecal microbiome transfer trial, as recently done in psoriatic arthritis,[54] might be more a pragmatic way to assess if the gut microbiome impacts RA development.

Conclusion

Most microbes previously associated with RA development could be identified in a FDR-RA population. However, the presence of these microbes did not appear to correlate with the known preclinical stages of RA. Yet, in a subgroup analysis of only the most pronounced phenotypes, we noticed a modest signal for increased fecal Prevotellaceae abundance, mirroring previous reports. Fecal calprotectin levels did not significantly associate with RA autoimmunity or clinically suspect arthralgia, being normal in most of the enrolled individuals.

OTHERS

Conflicts of interest

Authors have no conflicts of interest to declare.

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Ethical Review and Regulatory Considerations

This observational study has been approved by the Geneva ethical review boards (ERBs) as required by local law (protocol n° PB_2016-00889). Every participant has signed an information and consent form at inclusion in the SCQM registry. Hence, this study has been conducted in accordance with the ethical principles of the Declaration of Helsinki and is consistent with Good Pharmacoepidemiology Practices.

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Data availability

The dataset generated for this study will be made publicly available on YARETA.

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3 – Frontiers – Brief report: assessment of mucosal barrier integrity using serological biomarkers in preclinical stages of rheumatoid arthritis

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Brief report: Assessment of mucosal barrier integrity using serological biomarkers in preclinical stages of rheumatoid arthritis

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Background: The pathogenesis of rheumatoid arthritis (RA) is believed to initiate at mucosal sites. The so-called 'mucosal origin hypothesis of RA' postulates an increased intestinal permeability before disease onset. Several biomarkers, including lipopolysaccharide binding protein (LBP) and intestinal fatty acid binding protein (I-FABP), have been proposed to reflect gut mucosa permeability and integrity, while serum calprotectin is a new inflammation marker proposed in RA.

Methods: We analyzed serum samples of individuals genetically at increased risk of RA in a nested-case-control study. Participants from a longitudinal cohort of first-degree relatives of RA patients (SCREEN-RA cohort) were divided into three pre-clinical stages of RA, based on the presence of risk factors for subsequent RA onset: 1) low-risk healthy asymptomatic controls; 2) intermediate-risk individuals without symptoms, but with RA-associated auto-immunity; 3) high-risk individuals with clinically suspect arthralgias. Five patients with newly diagnosed RA were also sampled. Serum LBP, I-FABP and calprotectin were measured using commercially available ELISA kits.

Results: We included 180 individuals genetically at increased risk for RA: 84 asymptomatic controls, 53 individuals with RA-associated autoimmunity and 38 high risk individuals. Serum LBP, I-FAPB or calprotectin concentrations did not differ between individuals in different pre-clinical stages of RA.

Conclusion: Based on the serum biomarkers LBP, I-FABP and calprotectin, we could not detect any evidence for intestinal injury in pre-clinical stages of RA.

KEYWORDS

rheumatoid arthritis, gut permeability, intestinal inflammation, autoimmunity, biomarker

1 Introduction

Rheumatoid arthritis (RA) is an auto-immune disease leading to joint destruction and extra-articular manifestations. Researchers have hypothesized that RA autoimmunity is initially triggered at the mucosal level, for instance in the oral cavity or gastro-intestinal tract (1, 2). In particular, the breakdown of intestinal mucosal barrier integrity and translocation of bacterial products to the circulation and lymphoid organs could constitute a key step (3).

Tajik et al. have first demonstrated in a collagen induced arthritis mouse model how intestinal inflammation and loss of permeability surprisingly precedes the onset of arthritis. Targeting intestinal permeability, using butyrate or zonulin antagonist, reduced the severity of the observed arthritis (4). Using two different mouse models, Matei et al. have confirmed such findings, showing a loss of intestinal integrity before arthritis development. This included epithelial erosion, crypt elongation, reduced expression of tight junction protein 1, translocation of bacterial products to serum and lymphoid organs, and increased serum lipopolysaccharide (LPS) and lipopolysaccharide binding protein (LBP) levels. These observations depended on the presence of gut microbiota, and modification of the intestinal permeability also affected arthritis severity (5). Still, the exact molecular mechanisms linking translocated bacterial products to arthritis remain unclear.

Assessing gut barrier function in humans is challenging. Standard measures of gut mucosal barrier permeability are indirect and rely on the ingestion of passively absorbed probes, most commonly lactulose and mannitol, for which the excreted quantity can subsequently be measured in the urine. A higher urinary lactulose/mannitol ratio (LMR) is believed to indicate a higher small intestine permeability (6). Such functional tests of gut permeability are logistically complicated, time-consuming, and can be compromised by concomitant intestinal disease or NSAID intake (6–8).

To simplify the assessment of gut mucosal barrier integrity, several circulating biomarkers, such as lipopolysaccharide binding protein (LBP), intestinal fatty acid binding protein (I-FABP) and zonulin, have been proposed, even though their reliability in this context is still debated (9). LBP is mostly secreted by the liver and can opsonize gram negative bacteria (10). It also binds circulating LPS, thereby allowing the formation of a ternary complex with CD14 (11) and signaling through TLR4 to induce antibacterial responses (12). Given the technical limitations that prevent direct serum LPS assessment (13), elevated serum LBP levels are sometimes considered to reflect chronic LPS translocation from the intestinal lumen to the circulation (14–18). Serum LBP has also been studied as a marker of inflammation and disease activity in RA patients (19), was reported to modestly correlate with RF titers (20, 21), but has never been assessed during pre-clinical stages of the disease. I-FABP, also known as fatty acid binding protein 2 (FABP-2), is a tissue specific intracellular protein only expressed in enterocytes (22). It is released into the peripheral circulation after epithelial cell injury and is thus used as a marker of intestinal damage, for instance during small bowel ischemia (23), in obesity (24), or in the context of active RA (5).

In the 90', several authors have evidenced that established RA patients have an increased intestinal absorption of orally administered probes, such as polyethylene glycol, or milk beta-lactoglobulin (7, 25, 26). However, if compromised intestinal mucosal integrity plays a role in the human RA pathogenesis, one would expect mucosal permeability to be altered prior to disease onset, during pre-clinical or early stages of RA (27). Pre-clinical stages of RA are defined by an increased risk for RA development based on genetic or environmental risk factors, the presence of circulating auto-antibodies, or inaugural articular symptoms (27). Only very few studies have assessed intestinal integrity during pre-clinical stages of RA. In their research, Tajik et al. showed elevated serum zonulin in 32 individuals positive for anti-cyclic citrullinated peptide (CCP) auto-antibodies compared to healthy seronegative individuals (5), but using a Cusabio ELISA kit with low reliability (28). Matei et al. assessed serum biomarkers for intestinal integrity in only 7 asymptomatic individuals with RA-related autoimmunity and 7 patients with early stage undifferentiated arthritis. They found no difference in serum I-FABP concentration compared to healthy controls, but slightly elevated serum LPS and LBP concentrations (5).

In this study, we assessed intestinal mucosal barrier integrity in individuals at risk for RA, using LBP and I-FABP as serological surrogate markers. We reasoned that improved feasibility compared to complex oral functional tests would increase participation, and compensate the loss of precision.

Finally, we also assessed serum calprotectin. Calprotectin is a heterodimer of zinc and calcium binding proteins S100 A8 and A9, released by activated macrophages, granulocytes and monocytes. It has a bactericidal effect and promotes inflammatory responses. Serological calprotectin is currently being studied as a promising biomarker for RA disease activity (29–31). Baseline levels at RA diagnosis can predict erosive damage (32, 33) and response to methotrexate (33), while better reflecting disease activity than acute phase proteins (34). Recently, Bettner et al. have demonstrated that serum calprotectin was also elevated in a subset of individuals prior the onset of RA. Combined with RF and ACPA serologies, it improved predictive positive value for future RA diagnosis (35).

2 Materials and methods

2.1 Study design and population

We analyzed a set of serum samples obtained from the SCREEN-RA cohort, a cohort that follows individuals genetically at risk for RA, namely first-degree relatives (FDR) of established RA patients. This cohort has been extensively described elsewhere (36). Briefly, blood samples are collected at inclusion for genetic testing of the human-leucocyte antigen (HLA), in particular to detect "shared epitope" alleles. The "shared epitope" refers to a group of alleles of the HLA, which strongly increases the risk of RA in case of homozygosity. Serum samples are divided into aliquots, some of which are used for assessment of RA-associated autoantibodies (anti-citrullinated protein antibodies, ACPA; rheumatoid factor, RF; and anti-Ra-33 in a subset of participants) with previously proposed cutoffs (36). The remaining serum aliquots are stored at −80°C. High risk participants for RA, namely participants with auto-immunity associated with RA (i.e. ACPA, RF or anti-Ra33), high genetic risk based on HLA alleles or articular symptoms are followed-up closely and provide new blood samples yearly. All participants also undergo an articular examination at each visit, and complete yearly online questionnaires about lifestyle habits and medical history.

We performed a nested case-control study in a subset of the SCREEN-RA participants (Figure S1). Participants were divided into three groups, at sampling timepoint, using combinations of the EULAR proposed terminology for pre-clinical stages of RA (27), which are believed to reflect increasing risk of future RA development (Figure 1):

1. Low risk: asymptomatic individuals without RA-specific autoimmunity and without high genetic risk.
2. Intermediate risk: asymptomatic individuals, but with RA-specific auto-immunity, defined as the presence of either RF or anti-Ra-33 antibodies at ≥ 3 times the upper limit of the norm, or the presence of ACPAs at least at the upper limit of the norm.
3. High risk: symptomatic individuals, defined as fulfilling at least 3 of the EULAR criteria for clinically suspect arthralgia (37), regardless of serological or genetic status.

We invited available intermediate and high-risk participants to provide a new blood sample between 2019 and 2021. In parallel, low-risk participants, who are in excess in the cohort, were selected so that the age and sex ratio were comparable to the two other groups, and invited likewise for sampling. Consecutive patients with untreated new-onset RA were also recruited from the rheumatology division of Geneva University Hospital, during the study period.

We performed a sensitivity analysis taking into account the longitudinal evolution of these individuals during an average follow-up of 1.76 years. Depending on the individual's change in symptoms or signs of the disease, we categorized participants as 'progressors', 'regressors, or 'stable' and analyzed the correlations with the biomarkers of interest.

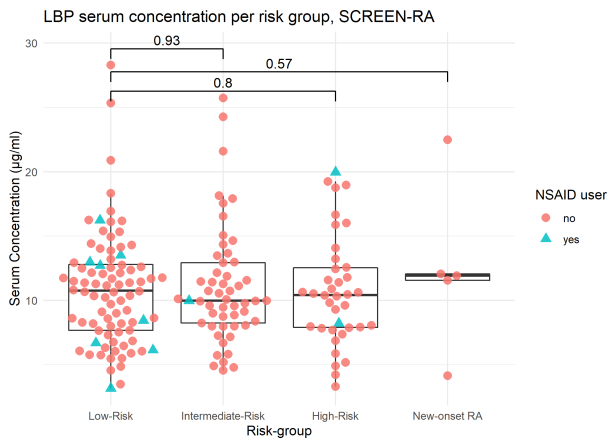

FIGURE 1
LBP serum concentration per risk subgroup, SCREEN-RA. Serum concentrations of LBP, in: – Low-risk: asymptomatic seronegative FDR of RA patients. – Intermediate-risk asymptomatic FDR with autoimmunity (ACPA, RF, or Ra33). – High-risk FDR with clinically suspect arthralgia, based on a combination of EULAR criteria. – New-onset untreated RA patients. RA, Rheumatoid Arthritis. Outliers are included in the analysis. p-values are displayed (Wilcoxon test). NSAID, NonSteroidal Anti-Inflammatory Drug.

2.2 Sample analysis

We used commercially available sandwich DuoSet ELISA kits from R&D Systems (Minneapolis, MN), for LBP (DY870, range 0.78 – 50 ng/ml), calprotectin (DY1820, range 93 - 6000 pg/ml) and I-FABP (DY3078, range 31 - 2000 pg/ml). Samples were randomized and divided into three batches. Each batch was aliquoted in several 96-wells plates, at the appropriate dilution. Then, for a given marker to be tested, ELISA tests were run in duplicate, during 3 consecutive days, according to the manufacturer's instructions (Figure S2). For the LBP and calprotectin assays, samples were diluted 1/1000, while for the I-FABP assay, samples were diluted 1/10, in reagent diluent. Due to the preparation procedure, all samples were thawed twice before measurement (i.e. initial freezing, thawing, dilution and aliquoting, re-freezing, final thawing and testing).

Optical density was determined using a LEDETECT 96 automatic reader, set to 450 nm with a correction filter at 570 nm. Finally, for each plate, the standard curve was constructed with R code using the *drm* function from the *drc* package v.3.0-1 to convert optical densities into concentration values. For each duplicated measurement, the inter-assay coefficient of variation of the two optical densities was computed as (standard deviation)/(mean). Only samples with <10% CV were included in the final analysis. The marker concentration was obtained by averaging the two measured concentrations, and multiplying by the dilution factor.

2.3 Statistical analysis

For baseline characteristics, continuous variables are expressed as means and standard deviations (SD), while categorical variables are expressed using percentages. ANOVA, $\chi 2$ test or Fisher's exact test for small size samples were used to compare baseline characteristics between groups.

The biomarker concentrations were compared to the low-risk group using two-sided Wilcoxon rank tests. Correlations between the biomarkers were calculated using Spearman coefficient, with the related p-value. All statistics were performed using R 2022.02.3 with package *tableone* and *stats*.

3 Results

Out of the 1539 participants of the SCREEN-RA study, we selected 180 individuals, matching low-risk with intermediate- and high-risk participants for sex and age. This resulted in: 84 low-risk individuals, 53 intermediate-risk individuals, and 38 high-risk individuals. Five untreated new-onset RA patients were also recruited, and sampled at the time of RA diagnosis, prior to antirheumatic treatment initiation. There were no significant differences between the groups in terms of age, gender and BMI (Table 1).

LBP, I-FABP and calprotectin concentrations were assessed in the serum of all participants. The mean inter-assay coefficient of variation (CV), computed on optical densities, was 1.7% for LBP, 2.2% for I-FABP, and 3% for calprotectin. One sample was excluded from the I-FABP analysis, and 7 samples were excluded from the calprotectin

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TABLE 1 Baseline characteristics.

Variable	Groups			
	Low-risk n = 84	Intermediate- risk n = 53	High-risk n = 38	New-Onset RA n = 5
Female gender	81%	75%	87%	100%
Age mean (SD)	54 (13)	54 (16)	55 (11)	53 (17)
BMI mean (SD)	25 (5)	24 (4)	24 (5)	26 (6)
ACPA positivity (>1x norm)	0%	32%	8%	60%
RF positivity				
1 to 3x the norm >3x the norm	11% 0%	8% 66%	16% 10%	0% 60%
Anti-Ra33 positivity				
1 to 3x the norm >3x the norm	13% 0%	21% 4%	13% 0%	NA
With detectable RA auto-immunity (low threshold >1x norm)	20%	100%	42%	60%
Shared epitope alleles				
0 allele 1 allele 2 alleles	48% 52% 0%	49% 38% 13%	55% 39% 7%	40% 40% 20%

ACPA, Anti-Citrullinated Protein Antibodies. RF, Rheumatoid Factor. Anti-Ra33, anti-Ra33 autoantibodies. CSA, Clinical Suspect Arthralgia according to the EULAR definition.
Low risk = asymptomatic RA-FDR without specific RA- autoimmunity. Intermediate risk = asymptomatic RA-FDR with specific RA-autoimmunity. High risk = symptomatic RA-FDR. New-onset RA are untreated at sampling time. NA, Not Assigned, i.e. the new onset RA were not tested for anti-Ra33 antibodies.

analysis, because the difference between the two replicates was too large (CV >10%). Overall, the mean values of the three biomarkers did not differ between the groups (Figures 1, 2 and S3; Table 2). Outliers were kept in the analysis.

We found no correlation between LBP and I-FABP levels (Spearman rho -0.06; p = 0.40), nor between I-FABP and calprotectin serum concentrations (Spearman rho -0.07; p = 0. 36; Figure S4). LBP modestly correlated with systemic inflammation, as reflected by serum calprotectin levels (Spearman rho = 0.32; p < 0.001; Figure S3), but not with RF status (data not shown). In complementary experiments, we noticed that additional thawing cycles reduced detectable protein concentrations for LBP, but not for I-FABP and calprotectin (Figure S4).

Finally, follow-up data was assessed in January 2023. Average time-difference between serum sampling and last news date was 1.76 years (SD = 0.74). In terms of risk group attribution, 16 individuals progressed, 32 individuals regressed, and 132 remained in the same risk group. Of note, one patient, enrolled in the low-risk group, developed RA 25 months after serum sampling (measured LBP = 25.34 µg/ml; I-FABP = 1025 pg/ml). We found no evidence of higher serological concentration of I-FABP or LBP associated with progression toward auto-immunity or clinically suspect arthralgia during this time-frame (Figure S6).

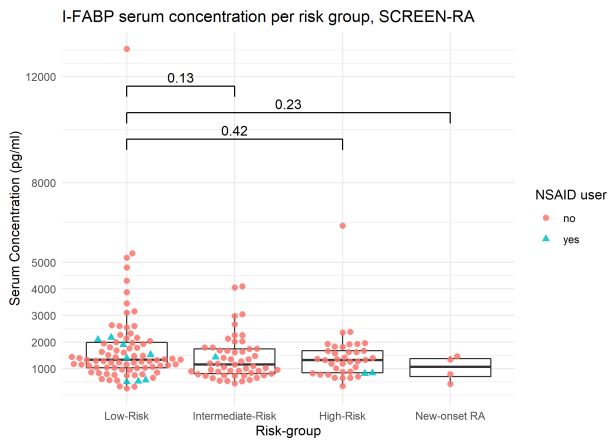

FIGURE 2
I-FABP serum concentration per risk subgroup, SCREEN-RA. Serum concentrations of I-FABP, in: – Low-risk: asymptomatic seronegative FDR of RA patients. – Intermediate-risk asymptomatic FDR with autoimmunity (ACPA, RF, or Ra33). – High-risk FDR with clinically suspect arthralgia, based on EULAR criteria. – New-onset untreated RA patients. RA, Rheumatoid Arthritis. Outliers are included in the analysis. p-values are displayed (Wilcoxon test). NSAID, NonSteroidal Anti-Inflammatory Drug.

4 Discussion

It has been evidenced in several mouse models that compromised intestinal integrity and increased translocation of bacterial compounds precede and affect the onset of arthritis (4, 5). In this work, we measured a panel of three biomarkers, which have been proposed to reflect respectively exposure to LPS translocation (LBP), intestinal integrity (I-FABP) and systemic inflammation (serum calprotectin), in the serum of individuals in preclinical stages of RA. Given the uncertainty concerning commercially available zonulin ELISA tests, we did not assess zonulin. Indeed, zonulin ELISA kits also target related (38) and unrelated peptides, such as properdin, which belongs to the zonulin family, and complement C3 (28). It is consequently still unclear what the commercially available zonulin tests actually detect (39).

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TABLE 2 Biomarker concentrations.

Variable	Groups			
	Low-risk n = 84	Intermediate-risk n = 53	High-risk n = 38	New-onset RA n = 5
Number of samples analyzed, n LBP, n I-FABP, n Calprotectin, n	84 84 82	53 53 50	38 38 36	5 4 5
LBP (µg/ml), mean (SD)	10.83 (4.39)	11.07 (4.55)	11.75 (4.27)	12.44 (6.53)
I-FABP (pg/ml), mean (SD)	1746 (1617)	1393 (823)	1438 (965)	1009 (487)
Calprotectin (ng/ml), mean (SD)	2043 (1396)	1860 (1163)	1629 (1114)	1897 (649)

LBP, Lipopolysaccharide Binding Protein; I-FABP, Intestinal Fatty Acid Binding Protein; SD, Standard Deviation.

Unexpectedly, we observed no differences in LBP and I-FABP between our groups of interest. One potential hypothesis explaining the negative findings is that our assessments were performed too late, or too early, in the timeframe of disease development. Indeed, our intermediate- and high-risk groups were already displaying auto-immunity or symptoms for several months or years, while still being a long time before possible arthritis onset.

Several considerations should also be discussed regarding the use of biomarkers to assess intestinal integrity.

First, we could not find a consensual definition of normal serum LBP levels. Mean reported values in healthy control groups range between 5 and 19 ug/ml (5, 40–47). In addition to natural inter-subject variability, the observed variations in these control concentrations might reflect different handling procedures, different dilutions, and different numbers of thawing cycles before measurement. The latter is rarely reported, and we observed that one additional sample freezing step reduces the measured concentration of LBP by approximately twofold (Figure S5), which could partly explain conflicting results. Also, it has to be kept in mind that RF can sometimes interfere with immunoassays, inducing falsely positive results (48).

After carefully re-considering the literature, we feel that previous findings regarding LBP should be interpreted with caution. It has been known since the 90' that LBP induction in the liver depends on IL-1β and IL-6, which makes it an acute-phase protein (49), although extra hepatic secretion by adipocytes has also been documented (50). In the context of RA, this could explain why LBP correlates with disease activity markers, such as erythrocyte sedimentation rate and C-reactive protein (CRP) (5, 19, 51). On the other hand, LPS is by itself a strong pro-inflammatory agent, which might contribute to low-grade systemic inflammation, adding even more confusion to the matter (13). Thus, it is not clear whether increased LBP is to be seen as the result of systemic inflammation, LPS-induced endotoxemia, or both. In our study, we noticed a modest correlation between LBP and serum calprotectin, while LBP serum concentrations did not differ between the three studied groups. Similarly, Matei et al. were not able to distinguish healthy controls from individuals in pre-clinical stages of RA using LBP (5).

Reported mean I-FABP values in healthy individuals range from ~300 pg/ml to ~1300 pg/ml, depending on the ELISA methodologies and suppliers (5, 52–54). Factors potentially confounding I-FABP serum level include intensive exercise (52, 55) and NSAID intake (56). Matei et al. have shown I-FABP to be significantly elevated in active RA patients, compared to healthy controls (5). This difference was not observed when comparing controls to individuals in the pre-clinical stages of RA, even though for the latter comparison sample size was limited (7 pre-clinical RA versus 34 controls) (5). The latter finding was independent of 2NSAID, NonSteroidal Anti-Inflammatory Drug intake or disease activity. Similarly, we did not observe any differences in serum I-FABP levels between high-, intermediate- and low-risk probands. Only a minority of participants in our study reported NSAID treatment, which did not appear to interfere with our conclusions. Noteworthily, we found no correlation between I-FABP and LBP levels, which was also noticed by Amarrudin et al. in a population of children treated for helminth infection (15).

Serum calprotectin reflects granulocyte activation, and usually does not exceed 1000 – 1500 ng/ml in healthy state (34, 57–59). In the context of acute disease, such as severe COVID-19, or active Crohn's disease, serum calprotectin can reach 10'000 to 20'000 ng/ml (58, 59). In the present study, we found a modest correlation between LBP and calprotectin serum levels. However, asymptomatic auto-immunity associated with RA or clinically suspect arthralgia did not appear to significantly influence serum calprotectin levels.

4.1 Limitations and strengths

The ELISA testing procedure used has several limitations: first, serum samples were thawed twice, which could lead to an underestimation of high marker concentrations, in particular for LBP (Figure S5). Also, for technical reasons, samples were divided into 3 different batches, for which ELISAs were run on different days. Even though these three batches were analyzed on three consecutive days with the same procedure, by the same operator, we cannot *a priori* exclude batch effects. We tried to minimize the impact of potential batch effects by randomizing samples across the three batches.

The major limitation of this study is that we did not perform oral-sugar intestinal permeability tests, because of practical considerations. Hence, we cannot formally exclude that the three groups may differ in terms of LMR. Overall, there is only limited evidence that serum biomarkers reflect mucosal barrier permeability or LMR ratios (Table

S1). Also, recent studies have underlined that plasma should be preferred to serum for calprotectin measurement – thus we may have overestimated calprotectin concentrations due to monocyte and granulocyte release during blood coagulation (60). Finally, NSAID usage was self-reported by online questionnaire. We can thus not exclude that some NSAID users have not documented their treatment. The main strengths of this study are its large sample size, duplicate measurements, and multiple marker dosage in a single serum sample.

Including only FDR individuals ensured comparable genetic background between groups. A drawback is the lack of healthy asymptomatic controls, without family history of RA. However, it is important to underscore that the incidence of RA remains low in FDR of RA patients, with life-time risk of developing RA between 1-2% (61), so that we believe that the risk of RA in asymptomatic first degree relatives is a good proxy for a healthy control population. To our knowledge, it is unclear if genetic risk for RA correlates with baseline levels of serum I-FABP and LBP. Finally, the few untreated new-onset RA that we managed to sample did not allow to constitute a group with sufficient size. Future research would certainly benefit from studying such new-onset RA patients, given that they exhibit a clear phenotype, without the interference of immunosuppressive medications.

4.2 Conclusion

We found no association between putative serum biomarkers of intestinal integrity (LBP and I-FABP) and preclinical stages of RA development. Also, serum LBP did not correlate with I-FABP, but correlated with serum calprotectin, which further questions the relevance of LBP as a marker of gut epithelial health. Future research needs to clarify if LBP truly signals changes in the intestinal integrity or instead merely reflects systemic inflammation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Cantonal Commission on Research Ethics (CCER) of Geneva. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BG: sampling, sample analysis, data interpretation, article writing. CL: biobanking, data interpretation, article correction. LA: study design, data interpretation, article writing. TS: study design, data interpretation, article correction. ER: protocol optimization, sample analysis, data interpretation. GP: study design, protocol optimization, data interpretation, article correction. AF: study design, supervision, data interpretation, article correction. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1117742/full#supplementary-material

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V. APPENDIX (EXTENDED BACKGROUND)

AUTO-ANTIBODIES IN RHEUMATOID ARTHRITIS

Rheumatoid Factor (RF)

It is called likewise for having been first noticed in sera of RA patients.(460,461) Rheumatoid factor is a group of IgA and IgM antibodies that target the Fc region of IgG antibodies. Consequently, they are called auto-antibodies because they target other antibodies (even though not a self-tissue per say).

IgG are secreted in a monomeric form, so they shall not be able to cross-link surface receptors and activate B-cells in a thymus-independent manner – whereas low-affinity IgM (most RF) are the usual result of T-independent B-cell activation. Which is why Janeway et al. (462) suggest that in some cases, for instance after a significant infection, newly created IgG immune complexes could activate B-cells in a thymus-independent manner to produce low-affinity anti-IgG IgM or IgA (RF). The latter secretion would only be transient, not lasting too long after immune complexes have been cleared.

Recent reviews further add that some rheumatoid factors are actually of high affinity.(463) Which means, they must result from macrophage or antigen-presenting-cell uptake of IgG-antigen complexes, MHC-II presentation and T-cell mediated B-cell activation. Looking closer it appears that the Fc-IgG-epitope targeted by RF is only accessible after IgG-antigen binding, explaining how RF can circulate next to IgG antibodies without constantly creating immune-complexes.(464) Consequently, instead of seeing RF as an mistakenly produced auto-antibody, some authors rather support the view that RF can have a physiological regulatory role: if produced in a low-affinity fashion, these anti-IgG IgM pentamers would help clearing IgG antibodies and immune complexes from the circulation, after infection has ceased. But if produced in a high affinity fashion (for instance during sustained infection), after further affinity maturation of the B-cell, RF would stabilize (concentrated) IgG to make them more effective against a pathogen.(463)

Anti-citrullinated-Peptides Antibodies (ACPA)

ACPA were discovered in 1964 as “antiperinuclear factor”,(465) because marking cytoplasmic regions around nucleus of cultured buccal mucosal cells (on their keratohyalin granules), and also modestly binding the nucleus. This “antiperinuclear factor” was later shown to be directed against filaggrin, mostly expressed in the human epidermis.(466) In 1998, it was further demonstrated that this “antiperinuclear factor” was actually binding to antigens containing citrulline residues, located on filaggrin and filaggrin-related proteins.(467) Like RF, ACPA can be of several isotypes: IgM, IgA or IgG.(468,469)

Citrullination is the posttranslational conversion, within a peptide, of arginine to citrulline (**Figure 54**). The process is catalyzed by a family of calcium-dependent peptidyl-arginine deiminases (PAD). It irreversibly modifies protein charges, and thus hydrophobicity and function of the protein.

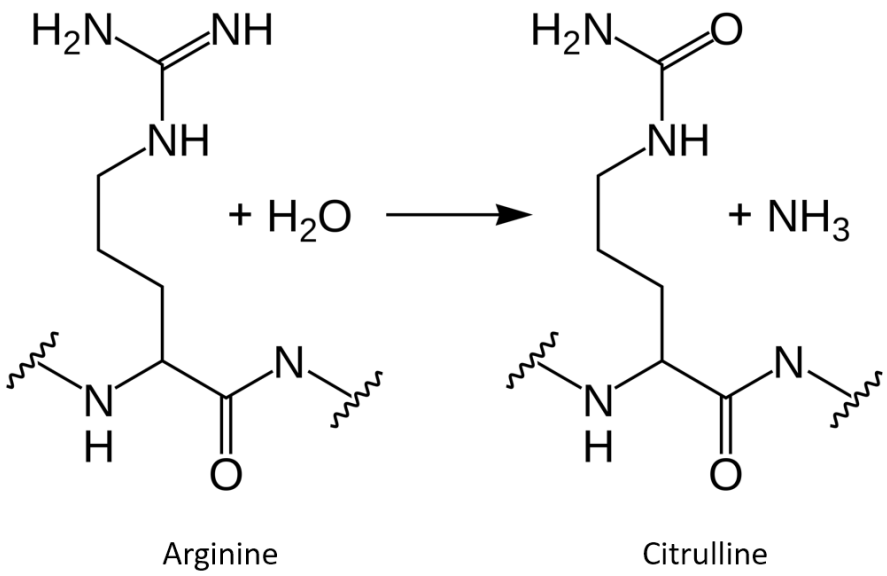

Figure 54: Citrullination. It is the chemical conversion of arginine to citrulline, performed, among others, by PAD enzymes (Peptidyl Arginine Deiminases). At neutral pH, arginine is positively charged, whereas citrulline has no charge. Thus, citrullination increases the hydrophobicity of the protein. Image adapted from public domain content (https://en.wikipedia.org/wiki/Citrullination).

Citrullination is linked to cell apoptosis and terminal differentiation, which increase intra-cellular calcium concentration and activate PAD enzymes, so citrullination itself is not specific for RA.(470) The histone citrullination is common in neutrophil death, for instance during NETosis.(157) However, citrullination is markedly increased in tissues during two scenario : perforin-mediated cell death (triggered by neutrophils) and membrane attack complex (complement system activation, forming a lytic pore), probably because pore-formation results in a strong calcium influx inside the cells.(471) Only the later “hyper-citrullination” resembles convincingly the citrullination pattern observed in joints of RA patients.

The first generation of serum test detecting ACPA were designed using linear citrullinated filaggrin peptides as target antigen. The process was later refined by giving a cyclic structure to the peptides, which increased the assay’s sensitivity. The second generation of anti-cyclic-citrullinated peptides used

a larger set of synthetic peptides.(472) Nowadays a third generation of anti-CCP test is proposed, which allegedly has a better specificity.(473)

The confusing point is that artificial peptides in CCP2 and CCP3 tests do not correspond to any human protein sequence, but simply mimic possible "*in vivo–generated*" citrullinated auto-antigens. It is still unclear what is the physiological target of ACPA, or if this target is the same in every patient (probably not). So far, have been best described (citrullinated) (155) : fibrin and fibrinogen,(474) vimentin,(475) collagen type II,(476) and α-enolase;(477) – these four are all expressed in the joints. Notice that these citrullinated auto-antigens are often targeted also in their native form (non-citrullinated) by patient's sera.(155) Last but not least, many other citrullinated self-proteins have been reported as targets of ACPA, such as : actin, aldolase, calreticulin, collagen type I, histone H4, histone H2A, phosphoglycerate kinase 1, protein disulfide-isomerase, tenacin.(471,478)

It is still debated if ACPA are pathogenic or not.(479) On one hand, since present before the onset of symptoms, they do not look sufficient to induce joint inflammation and bone destruction. On the other hand, plausible mechanisms of pathogenicity are proposed, such as ACPA acting as agonists for a receptor-mediated response, complement system activation, immune complex formation, Fc-receptor activation, cross-reactivity to joint cartilage and neutrophil extracellular trap-related mechanisms.(480) Furthermore, contrary to the other auto-antibodies discussed here, ACPA often bear signs of intense adaptative immune response : they are mostly IgG or IgA isotypes, have undergone hypermutations (thus the producing B-cells must have been activated by T-cells), and can be post-translationally modified, further increasing their affinity (in particular by N-glycosylation).(480–482) Such glycosylation of the ACPA-IgG V-domain thus suggest an affinity-maturation-process preceding the onset of the disease.(483) IL-23-activated T_H17 cells are able to instruct B-cells via IL-22- and IL-21 to regulate IgG glycosylation profile and to induce a pro-inflammatory state.(484)

However, recombinantly produced patient-derived ACPA fail to induce arthritis in mouse models – and some ACPA even exert a protective effect in antibody-induced arthritis models!(485) One possible explanation is that ACPA could also help clearing the citrullinated byproducts of inflammation; for instance it has been reported that ACPA directed against citrullinated histones can suppress neutrophil extracellular traps.(486)

IgM ACPA can still be found repeatedly after RA onset, reflecting recruitment of new B-cells, thus suggesting a continuous (re)activation of ACPA response – i.e. something keeps on "fueling" autoimmunity during RA course.(468) Also, recent works have suggested that citrullination could also render fibrinogen structurally similar to the usual IgG epitope targeted by RF, hence making the latter reactive to other modified-self-proteins.(487)

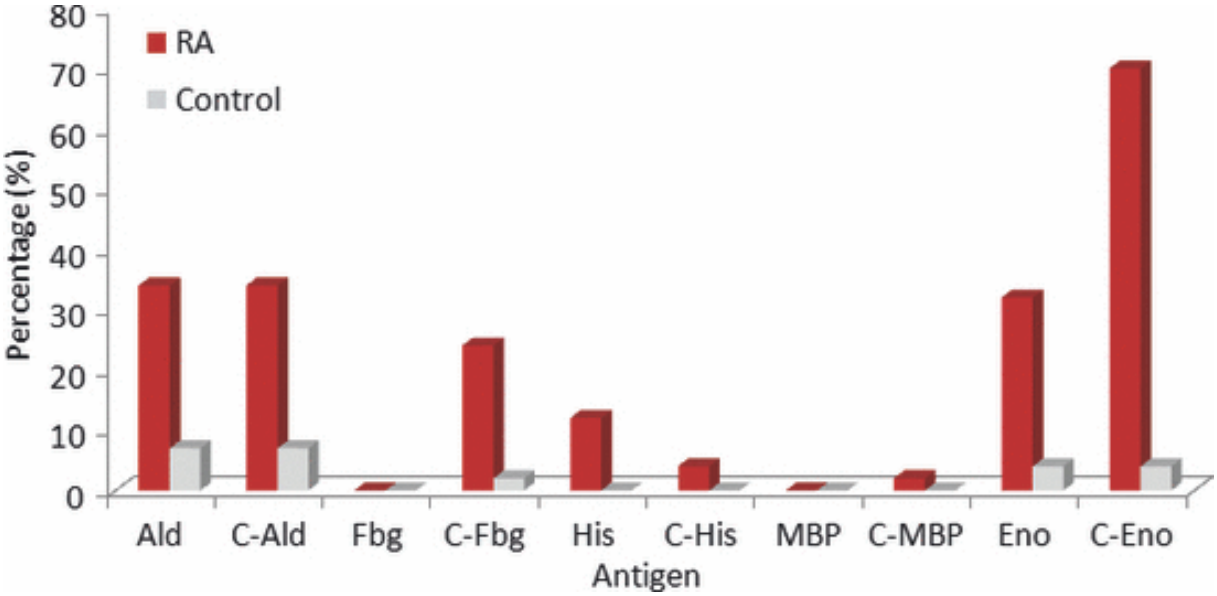

Figure 55: Percentage of serum samples from RA patients (red bars) and healthy controls (grey bars) that are seropositive for the respective native or citrullinated antigens. Serum samples from 50 patients with RA and 45 healthy controls were screened by immunoblotting for IgG reactivity to aldolase-A (Ald), fibrinogen (Fbg), total histones (His), myelin basic protein (MBP), and α-enolase (Eno), either in their native or in vitro citrullinated form (C-). Significant reactivity in RA serum was observed with aldolase-A, fibrinogen, histones, and α-enolase, though increased reactivity with the citrullinated form of the proteins was observed only for fibrinogen and α-enolase. Reproduced from Wegner et al., Immunological Reviews, 233: 34-54. https://doi.org/10.1111/j.0105-2896.2009.00850.x , under license n° 5601350045568.

Anti-Modified Protein Antibodies (AMPA)

As other post-translationally modified proteins are targeted by auto-antibodies in the context of RA, and sometimes in a cross-reactive manner with citrullinated antigens, this category has been proposed.(488) It is not clear if the initial target is the modified or non-modified peptide.

Anti-modified protein antibodies include:

Anti-hinge antibodies, which specifically bind only to pepsin-cleaved IgG (on the "hinge" region), and not the intact parent IgG antibody.(489)

Anti-acetylated protein antibodies. Similarly to citrullination, acetylation is another post-translational modification of lysine, which can be recognized by auto-antibodies.(490) About 40% of RA have anti-acetylated vimentin antibodies (490) (but the structure is quite close to homo-citrulline, so maybe ACPA are cross reacting ?).

Anti-malondialdehyde-acetaldehyde (MAA) and anti-malondialdehyde (MDA). MDA results from lipid peroxidation (for instance in the context of smoking),(491) which can form a more stable compound after reaction with acetaldehyde : MAA. Anti-MAA antibodies are strongly associated with RA, and APCA, but their specificity remains to be clarified.(492)

Anti-carbamylated protein antibodies (anti-CarP). Carbamylation is a non-enzymatic post-translational modification, during which a cyanate (metabolite of urea) molecule binds to a protein, usually on the lysine residue.(493) Notably, carbamylation is increased during inflammation, and smoking, probably relating to neutrophil's myeloperoxidase enzyme activity.(494) It is rather a toxic process which negatively affects protein synthesis and function.(493) About 45% of RA patients have antibodies against carbamylated proteins.(89,495) Such antibodies can be of various Ig classes (IgM, IgG, IgA) and are cross-reactive for multiple targets, including self- and non-self-proteins.(496,497)

Anti-Acetylated Peptides Antibodies (AAPA) recognize epitopes in which lysine, often within vimentin (a protein from cytoskeleton), has been modified enzymatically to carry an acetyl group. This happens frequently in the mitochondria.(498) Even though present in other diseases, AAPA also seem quite specific for RA.(102)

Anti-Ra33

Heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 is a ribonucleoprotein (i.e. proteins + RNA) of ~33kDa, discovered because targeted by auto-antibodies in the context of RA (hence the name).(499) Ribonucleoprotein hnRNP-A2 is involved in mRNA splicing and transport and is ubiquitously expressed.(500) Anti-Ra33 are quite specific for RA, but only ~30% of patients present them.(501) It

seems the antigen is initially targeted in its native form, while in chronic RA the citrullinated version of hnRNP-A2 is additionally targeted (this begs the question if citrullination is really responsible for the loss of tolerance, since in that case, citrullination apparently comes after the loss of tolerance).(502)

Anti-PAD4

Peptidyl-arginine deiminases (PAD) are a group of enzymes that catalyze citrullination, and which comprises five isoenzymes (PAD1-4 and PAD6 – "isoenzyme" means they have different amino-acid sequences but catalyze the same reaction).(503) PAD4 is the one responsible for histone citrullination, which is a key step of neutrophil extracellular traps formation (citrullination decondenses histones).(503) Intriguingly, about 30% of RA patients have antibodies directed against this enzyme, usually of IgG class, and anti-PAD4 titers do not always correlate with ACPA serology.(504,505) Also, PAD2, 3 and 4 are expressed in the synovial tissue of RA patients, particularly in zones with necrotizing cells.(506) The apoptotic neutrophils can apparently release active PAD4 in the synovial fluid.(507) Furthermore, PAD4 is able to auto-citrullinate (i.e. individual PAD4 molecules can citrullinate one another),(508) which could be a reason why thy enzyme is preferentially detected by T-cells as a neo-autoantigen. Finally, anti-PAD4 IgG are strongly correlated with radiographic progression (bone erosion) in RA patients.(509)

Others auto-antibodies

Screening approaches identified more auto-antibodies related to RA, even though less established.(510) They comprise:

- Anti-PTX3. Pentraxin-3, produced by many cell types during inflammation, is the prototype of pentarexins, which are soluble pattern-recognition molecules. They opsonize microbes, but are also involved in the clearance of cell debris.(511)

- Auto-antibodies against various intracellular enzymes such as: dual specificity phosphatase 11 (DUSP11), P antigen family member 5 (PAGE5), deoxyhypusine hydroxylase (DOHH), methyltransferase 21C (METTL21C), fibroblast growth factor 12 (FGF12), UBX domain protein 10 (UBXN10), TBC1 domain family member 19 (TBC1D19) and serine/threonine-protein kinase 3 (STK3). I cannot comment more since little is known about their physiological role and/or involvement un RA. Reviewing *Uniprot.org* I notice some of them are involved either in protein modification (DOHH, METTL21) or apoptosis (STK3), or simply localized in the nucleus (DUSP11, STK3) – some of them are linked to NETosis.(510) MCM2 (minichromosome maintenance complex component 2 or mitotin), ribosomal protein S6 (RPS6) and selenoprotein P, have also been suggested.(512)

- Further anti-nuclear-antibodies, which are common in many immune diseases, can also be found in RA. However, contrary to ACPA, RF, and other arthritis-related auto-antibodies, they seem to appear only after RA diagnosis, hence there are not thought to drive the disease development.(513) They most probably constitute immune epiphenomenon of self-tissue damage and accidental exposition of intracellular content.

Other biomarkers

Besides auto-antibodies, a few biomarkers have been proposed for RA. An example would be "14-3-3η", an intracellular chaperone protein that is present in the synovial fluid of RA patients.(514) It is also detectable in the serum and may be useful to complement auto-antibody serologies.(515) But so far it is unclear how relevant 14-3-3η is in pre-clinical RA.

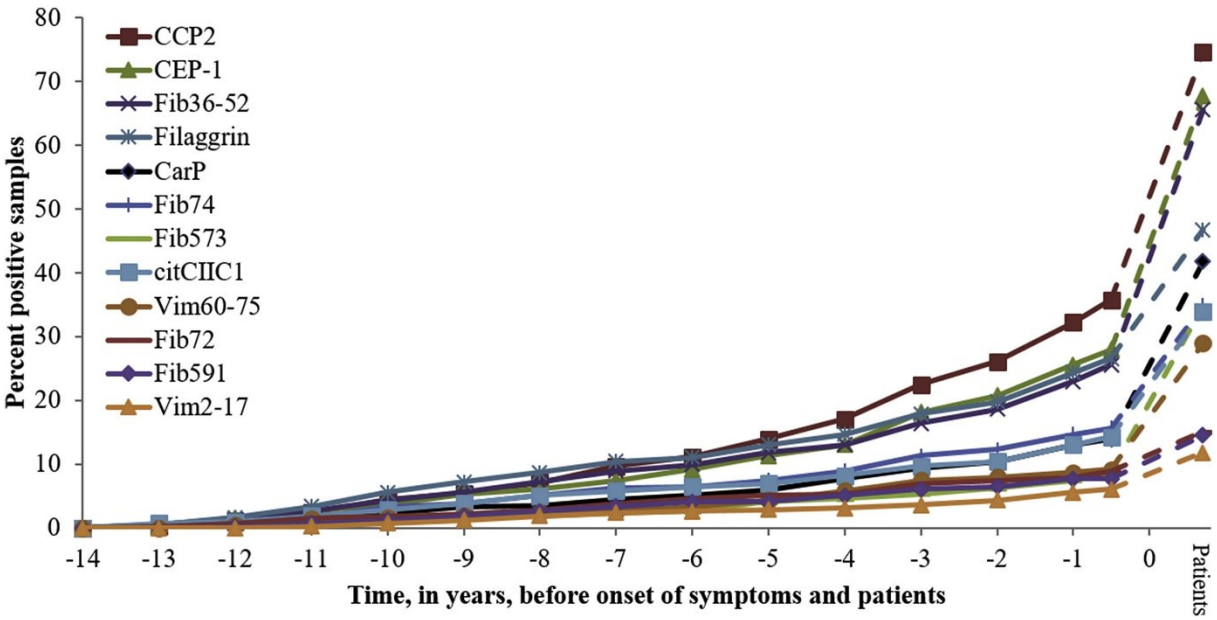

Figure 56: autoantibodies in rheumatoid arthritis before symptom onset. ACPA, anti-citrullinated protein antibodies; anti-CarP, antibodies against carbamylated proteins; anti-CCP2, anti-cyclic citrullinated peptide 2. Measured on 252 RA patients (423 serum samples) and 197 controls. Reproduced from Brink et al., Arthritis Res Ther 17, 25 (2015), https://doi.org/10.1186/s13075-015-0536-2 , under license n° 5601360308811.

MORE ON GUT MUCOSA IN RHEUMATOID ARTHRITIS

Factors influencing intestinal permeability

Table 10: Known factors influencing intestinal barrier function.

Impairing the intestinal barrier	Restoring function (normal impermeability)
- NSAID (human data).(211,212)	- High dose probiotics (mice data).(537)
- Hyperglycemia (mice data) (516)	- Anti-TNF medication (human data).(538)
- Gliadin (mice data), increases intestinal permeability and exacerbates NSAID-induced small-intestinal damage, but did not increase the mRNA expression levels of IL-1β and TNF-α and did not induce visible small-intestinal damage when given alone).(517)	- Divertin (drug which blocks MLCK1 recruitment); mice data, is more effective than TNFi in colitis models.(539)
- Acute stress (mice data).(518)	- Omega-3 derived resolvin (mice).(540)
- NAFLD and NASH (human data) have increased endotoxin levels.(519)	- Butyrate (mice data) (235,541) and other short-chain fatty acids.(542)
- High-fructose diet (Monkey and pig data), with notably high increase in serological LBP-1,(520,521) which can be prevented by antibiotics.(522)	- Cannabinoid CB1 receptor agonist (mice data).(235,543)
- High fat diet (HFD) (mice data) increases gut permeability and LPS levels. Can be reversed by antibiotic treatment.(523) In human, endotoxins increase during HFD, however, permeability test with oral sugars do not change between before and after.(524)	- Larazotide acetate (zonulin receptor antagonist) (mice data).(235)
- High fat (cream) intake challenge (contrary to water or orange juice) increased serological biomarkers TLR4 and LPS (human data).(525)	- Lubiprostone (derived from prostaglandin E) which counteracts NSAID effect (human data).(544,545)
- High dose (cancerology) MTX (rat (526) and human (527) data).	- Dietary fibers in RA patients reduced serum zonulin calprotectin (human data).(433)
- Vitamin D deficiency (mice data – deficient mice had 50x higher bacterial infiltration in colon tissue) (528)	- *Bifidobaterium* strains, reduce sucralose/lactulose ratios in obese volunteers (human data).(546)
- MTX, low dose (in children) (527)	- *Akkermansia muciniphila* supplementation (mice data).(547)
- Acute psychological stress and corticotropin (human data).(529)	- Other probiotics.(548–552)
- Intensive physical activity (human data).(530)	- L-citrulline, 10g intake (human data).(553)
- Emulsifiers, such as polysorbate-80, carboxymethylcellulose, carrageenan (in vitro and mice data).(436)	- Glutamin, in enteral nutrition (human data).(554)
- Other surfactants such as such as monoglycerides, lecithins (rat and in vitro data).(531,532)	- Resveratrol (hypothesized by authors, at end of their review).(555)
- alcohol (human and mice data).(533–535)	- Sucralose, taken before intense physical exercise (human data).(556)
- *Candida albicans* (which can cleave e-cadherins) (in vitro data).(536)	- Intestinal alkaline phosphatase, which reduces LPS formation and for instances prevents alcohol-induced gut barrier dysfunction in mice.(557)
- Wheat, yeast, milk, and soy when applied on the duodenal mucosa of IBD patients (assessed with endoscopic confocal microscopy).(240)	- Zinc carnosine helps normalizing LMR after indomethacin treatment (human data).(558)
	- Other compounds mostly used in combination (arginine, glutamate, glutamine, glutathione, glycine, vitamin A, zinc, and specific lipids) Reviewed elsewhere.(559)
	- Berberine (Asian alkaloid plant extract) (mice data).(560,561)
	- Fermented fibers such as oligofructose or inulin (mice data) (562,563) (human data).(406)
	- Curcumin (polyphenolic yellow pigment of curcuma) (mice data).(564)

Mice data overview

Rheumatoid arthritis does not exist *per say* in mice, and researchers are merely trying to "mimic" such an arthritis by various artificial means – each model partly recapitulates certain aspects of the human disease.(565)

HLA-transgenic mouse or rats

We know since the early 90' that rats genetically modified with human HLA-B27 and human β2-microglobulin are susceptible to arthritis.(566) These rats spontaneously develop a disease involving gastro intestinal tract, skin, nail, genital tract, and joints (which resembles more spondylarthritis than RA – but we can find similitudes as well).

Surprisingly, germ free state was noticed to abrogate the disease.(567)

IL-1RA knockout mouse

These mice are deficient for the IL-1 receptor antagonist, which is an endogenous inhibitor of IL-1. It results in a spontaneous destructive arthritis, dependent on IL-17 and T-cells.(568)

In 2008, Abdollahi-Roodsaz et al. showed that the spontaneous arthritis was abrogated in the absence of gut microbiota (and triggered again after colonization with Lactobacillus, **Figure 57**).(569)

Also, additional TLR4 knockout (Tlr4–/–), in contrast to TLR2 knockout, protected against severe arthritis (lower numbers of T_H17 cells, and reduced capacity to produce IL-17).(569) TLR4 is involved in sensing bacterial LPS, which suggested an effect of the gut microbiome in the development of arthritis, as well as the involvement, at least in this model, of T_H17 cells and TLR4 pathway.

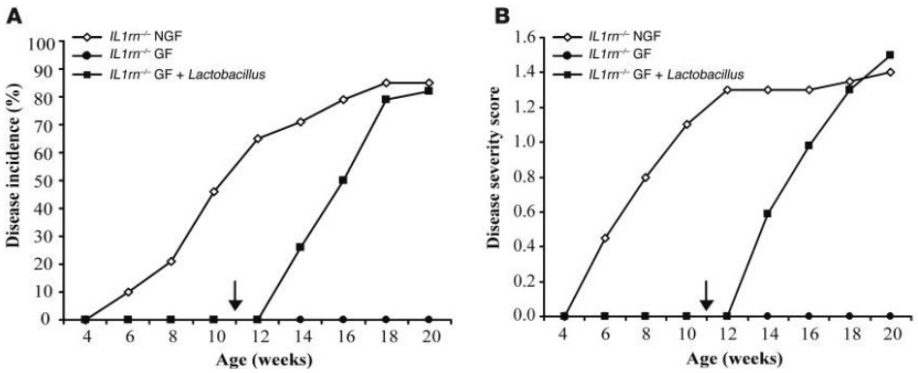

Figure 57: IL-1RA-ko mice do not develop arthritis when germ-free. The incidence (**A**) and severity (**B**) of arthritis are completely abolished in germ-free (GF) IL1rn–/– mice, and infection of GF mice with Lactobacillus (arrows) leads to the same disease expression as in non–germ-free (NGF) animals. Arthritis severity was scored on a scale from 0 to 2 for each paw; n ⩾ 6 per group. Reproduced from Abdollahi-Roodsaz et al., 2008, J. Clin. Invest, https://doi.org/10.1172/JCI32639 under a CC BY 4.0 license.

Collagen-induced arthritis (CIA)

Here the idea is to "vaccinate a mouse against itself". This model works by inducing a first immunization with an intradermal injection of type II collagen (murine or bovine), mixed with complete Freund's adjuvant (a suspension of *Mycobacterium tuberculosis* fragments in mineral oil). The subsequent incidence of arthritis depends on mouse (or rat) strain susceptibility. In general, a later intraperitoneal or intradermal injection of the same collagen is required to trigger the arthritis, which involves both autoreactive B- and T-cells.

Germ-free DBA/1J do not develop arthritis after completing the procedure, but when conventionalized with the microbiota from CIA-susceptible mice, they show a higher frequency of arthritis induction than those conventionalized with the microbiota from CIA-resistant mice.(570)

Moreover, Jubair et al. showed that the induction of the arthritis is also associated with microbiome, intestinal inflammation and increased gut permeability, implying a T_H17 inflammation pattern, before arthritis becomes apparent.(571) Then, treating mice with antibiotics (broad spectrum cocktail, given just after the second immunization) reduced the severity of the collagen induced arthritis (**Figure 58**).(571)

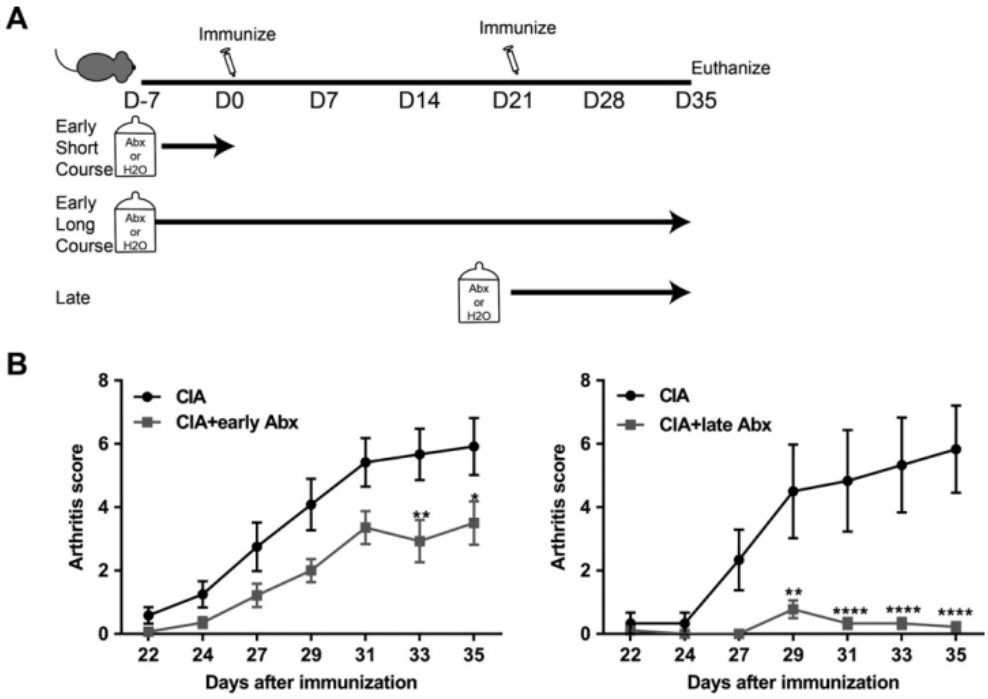

Figure 58: Antibiotic treatment reduces collagen induced arthritis. A – Study design. **B** - Arthritis scores, assessed after day 21 (n = 6–14 mice per group). Values are the mean ± SEM. Abx = Antibiotics. Reproduced from Jubair et al., 2018, Arthritis Rheumatol, https://doi.org/10.1002/art.40490 , under license n° 5602410441234.

But the mice treated with antibiotics only had mildly reduced anti-collagen antibodies. However, strikingly, anti-collagen antibodies from the serum of mice treated with antibiotics late in the course of arthritis failed to activate C3 compared to those purified from serum of control-treated or early antibiotic–treated mice. These "less active" antibodies were actually less glycosylated.(571)

Also in this model, oral inoculation of periodontal pathogens *Porphyromonas gingivalis* and *Prevotella nigrescens* induced periodontitis, and aggravated arthritis severity, skewing T-cells in lymph nodes draining inflamed joint towards a T_H17 phenotype.(572) Let's however notice that orally administered *P. gingivalis* in these mice changed the gut microbiota, elevated serum endotoxins and inflammatory markers, together with an impairment of the gut barrier function (and little trend to more anti *P. gingivalis* IgG).(573) In similar contexts (gavage with *P. gingivalis*), orally applied antimicrobial therapy (metronidazole or chlorhexidine) reduced the incidence and severity of the collagen-induced-arthritis, comparably to methotrexate.(574)

Last but not least, Tajik et al. demonstrated how intestinal inflammation preceded the onset of arthritis in these mice.(235) Interestingly, fecal transplantation between mice also transferred the leaky barrier and mucosal inflammation. Finally, butyrate or zonulin-inhibitors were able to attenuate development of arthritis by targeting intestinal barrier dysfunction.

Using the same model but modified with an humanized RA-associated MHC-II (HLA-DQ8 gene), Balakrishnan et al. demonstrated that gavage with RA-associated bacteria (*Eggerthella lenta* or *Collinsella aerofaciens*) also increased the gut permeability in a sex and age-dependent manner, compared to gavage with non-associated bacterial species (*Prevotella histicola* or *Bifidobacterium sp*.).(575) Since they based their experiment on data from Chen et al. (276)**,** they did not test *P. copri*. However, their results suggest that RA-associated bacteria are not just "inflammation-associated" taxa, but rather active contributors to the chronic inflammatory state. Recently, Nii et al. focused on *P. copri* gavage and they demonstrated that the aggravation of arthritis depended on the considered *P. copri* strain (one of which had a virulence factor in a conjugative transposon).(314) In this case, it induced T_H1 and T_H17 cells in popliteal lymph nodes, and even though milder, findings were transposable to SKG- model.(314)

Fusobacterium nucleatum (compared to *Escherichia coli*) was lately shown to also worsen arthritis in this model, due to secretion of antigenic outer membrane vesicles which translocated and accumulated in mice joints.(282)

Probiotics such as *Lactobacillus casei* reduced the incidence and development of collagen-induced arthritis, and downregulated the cellular and humoral immune responses to collagen in a dose-dependent manner.(576) Similarly, rats with collagen-induced arthritis had reduced signs of arthritis

after 12 weeks of *L. casei* oral administration. Lymphocyte infiltration and cartilage degradation were also reduced compared with control animals.(577) In addition, rats receiving *L. casei* had lower levels of proinflammatory cytokines and reduced T-cell proliferation, as well as increased production of the anti-inflammatory cytokine IL-10.(577) *Lactobacillus delbrueckii* or *Parabacteroides distasonis* also displayed these beneficial effects.(283,578) Last but not least, *Prevotella histicola* reduced arthritis severity and intestinal permeability (by increasing expression of tight junction proteins), when compared to colonization with *Prevotella melanogenica*.(307) Furthermore, butyrate, a beneficial short-chain-fatty-acid produced by some fibers-fermenting bacteria was also shown to suppress or attenuate arthritis in this model (and upregulate T_{reg} cells);(288,579) so did the potent butyrate producer *Faecalibacterium prausnitzii* when administrated to the diseased mice.(580) Also, other metabolites, such as lithocholic acid derived from *Parabacteroides distasonis,* had beneficial effects*,* by pushing macrophages towards M2 differentiation.(581)

Finally, Chriswell et al. demonstrated that gavage of germ-free mice (with the genetic background normally used in collagen-induced arthritis) with a strain of *Subdoligranulum* was sufficient to trigger a mild form of arthritis in these mice (with autoantibodies, T_H17 activation, mucosal invasion and increased intestinal lymphoid follicles), <u>without</u> proceeding to the collagen immunization.(582) Arthritis was not observable when gavage was done with other control bacteria (including *P. copri)*.

Antigen-induced-arthritis (bovine serum albumin)

Antigen-induced arthritis works by immunizing mice against bovine serum albumin. Later, the same albumin in injected in a joint, where it triggers a self-resolving inflammation. Only the challenged joints are inflamed.

Supplementation with *Lactobacillus GG* allegedly reduces histological inflammation in these rats compared with rats fed plain yogurt or milk.(583) Similar results were reported with *Lactobacillus fermentum*.(584)

Matei et al. also used this model to validate their main finds (on SKG mice).(239) Mice in the acute phase of arthritis also displayed morphological gut alterations, decreased zonulin epithelial expression, and increased intestinal permeability (assess by FITC-dextran oral administration before serum dosage). Authors also mentioned an increase in fecal Prevotellaceae abundance.(239) The phenotype was aggravated by claudin-8 knock-out; while treatment with zonulin antagonist reduced arthritis severity.

Spontaneous arthritis (K/BxN mouse)

This mouse model was generated fortuitously by crossing a TCR-transgenic line (specific for a bovine pancreatic ribonuclease) with the non-obese-diabetic (NOD) strain (a known model of spontaneous type-1 diabetes). All offspring develop a joint disease evoking RA, due to a recognition of a NOD-derived

MHC-II molecule by the transgenic TCR. The progression to arthritis involves CD4+ T-cells, B-cells, and probably myeloid cells, which are targeting a self-peptide derived from glucose-6-phosphate isomerase.

K/BxN transgenic mouse do not develop arthritis when raised in germ-free conditions,(585) while re-introduction of segmented-filamentous-bacteria is enough to drive arthritis (**Figure 59**).(585) Indeed, segmented filamentous bacteria are able to promote T_H17 function and differentiation in mice gut (586).

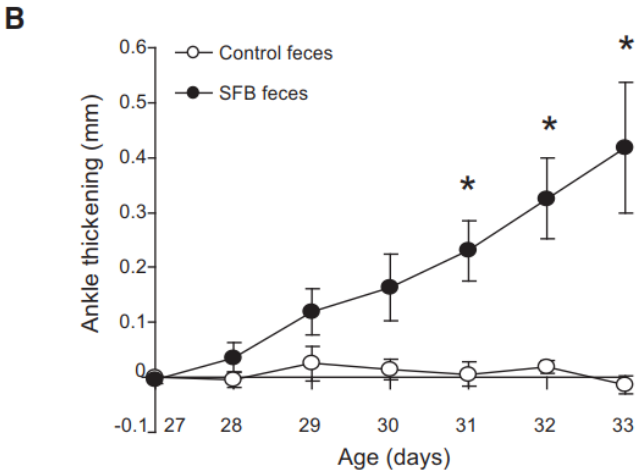

Figure 59: Segmented filamentous bacteria triggers arthritis in susceptible mice. (B) Measurement of ankle thickness beginning on day 27. n = 9 for SFB-treated and n = 5 for controls from four independent experiments. Asterisks indicate statistical significance via the Student's t test, $*p < 0.05$. Reproduced from Wu et al., Immunity, 2010, https://doi.org/10.1016/j.immuni.2010.06.001 , under license n°5602410757707.

In this susceptible model, segmented-filamentous-bacteria are also involved in the lung autoimmune complications accompanying arthritis.(186) Even though segmented-filamentous-bacteria are specific to the mice microbiota it supports the relevance of gut microbiome in RA.

Using such arthritic K/BxN mice, Matei et al. histologically further demonstrated a progressive loss of intestinal epithelial integrity during disease development, with reduced expression of tight junction protein 1, and increased serum LBP.(239) This depended on the presence of gut microbiota, involved bacterial translocation and was associated with a shift toward higher proportion of *Prevotella* genus in mice feces. Treatment of mice with zonulin antagonist ameliorated arthritis and prevented gut permeability development.(239)

Moreover, in K/BxN mice, arthritis induced by SFB-containing feces appeared to be driven by the migration of T_{fh} cells from the Peyer's patches to the peripheral lymphoid tissues, where auto-

antibodies are produced.(199) Teng et al. evidenced this cell trafficking using KikGR.K/BxN transgenic mice (KikGR background expresses a green-to-red photoconvertible fluorescent protein in all cells), which allowed, through a surgical procedure, to specifically photoconvert intestinal cells before tracking them.(199)

Spontaneous arthritis (SKG mice)

SKG mice carry a mutation of ZAP70 gene (signaling pathway for development and function of T-cells).(587) They develop chronic autoimmune arthritis due to perturbation in TCR signaling and thymic selection. These mice require an environmental triggers for disease onset, such as fungal beta-glucan (zymosan ; or curdlan, which is a proinflammatory bacterial glucose polymer, binding to pathogen–associated molecular pattern receptors).(588) SKG mice fail to develop disease in sterile conditions.(127,589)

Serum transfer arthritis

This model works by transferring, in wild mice, the serum from a collagen-induced-arthritis mice. It results in immune complexes formation, and activation of complement system, during an acute self-resolving disease. The recipient mice do not develop endogenous T-cell or B-cell autoimmune responses. The operation can also be made using serum from the K/BxN mouse model. Recently, an "enhanced severity" version has been proposed, using a larger cocktail of anti-cartilage antibodies, as well as intraperitoneal injection of LPS or mannan as adjuvants.(590)

Transfer of purified IgG from K/BxN mice to other mice (here BALB/c strain), induced robust arthritis, and the inoculation of *P. gingivalis* (mutated, not to be able to citrullinate) aggravated arthritis and intestinal inflammation as much as the normal *P. gingivalis* (control was saline gavage. **Not** germ-free conditions).(591) This suggests that protein citrullination might NOT be the (only) way *P. gingivalis* can interfere with murine arthritis.(591) K/BxN serum-transfer-induced arthritis also resulted in downregulation of tight-junction-protein 1 and lysozyme 1 (antimicrobial peptide), with increased plasma concentration of endotoxins, and a reduced intestinal concentration of omega-3-derived resolvins.(540) Subsequent inoculation with *P. gingivalis* again exacerbated these changes, while administration of the resolvin restored the barrier function and reduced joint inflammation.(540) The latter could partly explain why omega-3 supplementation is effective as an adjuvant therapy for RA.(592)

Adjuvant-induced arthritis

Even though it is more a model of spondyloarthitis, I shall mention it for its similarities with RA mouse models. A variety of agents can trigger immune arthritis in Lewis rats; for instance extracts from mycobacteria, *staphylococcus*, *streptococcus*, or muramyl dipeptide (similar to the experiments of

Nanna Svartz (147)).(593) In this case, one intradermal injection is sufficient to activate a variety of autoreactive lymphocytes.

Gut microbiota also modulates this model, but germ free rats are more susceptible (100%) to develop the arthritis, as if gut microbiota was necessary to ensure proper "maturity" of the tolerogenic systems.(594)

In this model, Hecquet et al. reported increased gut permeability before the onset of arthritis (assessed by serum zonulin and I-FABP, and ileal zonulin expression).(595) But in this case, mRNA expression of occludin and tight-junction protein 1 were not altered. However, the ELISA kit used to measure zonulin is not mentioned, and overall, this study mostly relies on biomarkers, which have to be interpreted cautiously.

Pan et al. have shown that gavage with the probiotic *Lactobacillus casei* could alleviate adjuvant-induced-arthritis, similar to methotrexate treatment.(596)

Gut microbiome studies in rheumatoid arthritis

See Table below, which is extensive and up to date for mid-2023 (human data only).

Table 11: Human analysis of gut microbiome in rheumatoid arthritis

Author / year / ref.	Patients	Controls	Method	Results
Olhagen et al. 1968 (269)	Classical or definite RA according to Rope's et al. Criteria. n = 186	Healthy volunteers belonging to the hospital personnel and arthrosis patients. n = (46 + 24)	Dilutions, anaerobic/aerobic cultures. "Manual" cell count and colony characterization. Focusing on *Clostridium perfringens* (n = 186). Serum samples tested against dilutions of alpha-toxin (n tested: 160).	125/186 (67%) quantitatively abnormal ($\geqslant 10^5$ *Clostridium perfringens* per g of feces). 125/160 (78%) had raised alpha-anti-toxin serum titer compared to controls.
Shinebaum et al. 1987 (270)	Seropositive RA patients (latex titer >1 in 40). No antibiotics or long-term anti-rheumatoid agents. N = 25	age-and sex-matched controls n = 25	Aerobic and anaerobic culture of fresh samples.	Only difference identified: increased number of *Clostridium perfringens* 22/25 (88%) patients, vs 12/25 (48%) controls ($p < 0,01$).
	RA patients, regardless of stage and treatment n = 113	age-and sex-matched controls n = 38	Aerobic and anaerobic culture of fresh samples, focusing on *C. perfringens.*	79 of the additional patients (70%) and 17 of the controls (45%) carried *C. perfringens* ($p < 0.01$). Overall carriage rate of 73% amongst RA patients and 46% amongst controls ($p < 0.001$).
Eerola et al. 1994 (271)	RA diagnosed according to the ACR criteria, < 12-months duration, without intestinal disease. N = 74	Hospital patients with diseases where no alteration of intestinal flora is expected to occur and without any medication affecting the intestinal flora n = 91	Computerized gas-liquid chromatography for bacterial cellular fatty acids present in the stool.	Spectra in stool samples of RA patients were significantly different from those of non-RA controls. Erosive RA patients most clearly different from controls. Anaerobic bacteria are primarily responsible for the differences observed.
Toivanen et al. 2002 (597)	Early, disease modifying anti-rheumatic drugs, naive RA patients. n = 25	Control patients suffering from non-inflammatory pain. n = 23	Whole cell hybridization with seven fluorescently labelled 16S rRNA-targeted oligonucleotide probes (details not available).	Patients with early RA had significantly less bacteria belonging to the *Bacteroides*, **Prevotella** and *Porphyromonas* genera than the controls (4.7% vs. 9.5%, $p = 0.00005$).
Vaahtovuo et al. 2008 (598)	Non-hospitalized early RA diagnosed according to ARC < than 6 months ago. n = 51	fibromyalgia patients n = 50	Flow cytometry, 16S rRNA hybridization, and DNA-staining (8 oligonucleotides probes) (Detail not available)	RA patients had significantly less bifidobacteria and bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *Bacteroides fragilis* subgroup, and *Eubacterium rectale – Clostridium coccoides* group. Results from the 8 probes showed a significant overall difference between the two groups.

Scher et al. 2013 (273)	New-Onset Rheumatoid Arthritis (NORA): diagnosis according to EULAR or ACR, with seropositive RA, duration between 6 weeks and 6 months. n = 44	Chronic RA: at least 6 months after diagnosis n = 26 Healthy: age-, sex-, and ethnicity-matched with no personal history of inflammatory arthritis n = 28 (Psoriatic arthritis) (n = 16)	16S rDNA amplification and sequencing. Shotgun sequencing on a subset of 44 samples. Colonization of antibiotic treated C57BL/6 mice (not germ-free) with *P. copri* by oral gavage. Control mice: media-only-gavage.	Threshold for presence of >5% relative abundance of *P. copri:* NORA: 33/44 (75%) Chronic RA: 3/26 (11,5%) healthy: 6/28 (21.4%) Psoriatic arthritis: 6/16 (37.5%) Increases in *Prevotella* abundance correlated with a reduction in Bacteroides and a loss of reportedly beneficial microbes in NORA subjects. Relative abundance of *P. copri* in NORA inversely correlates with presence of shared epitope. Shotgun sequencing reveals *P.* copri strain-level differences between NORA and control patients. Colonization of mice revealed the ability of *P. copri* to dominate the intestinal microbiota and resulted in an increased sensitivity to chemically induced colitis compared to control mice.
Liu et al. 2013 (599)	RA according to ACR/EULAR 2010, onset in the previous 6 months n = 15	Healthy volunteers n = 15	qPCR of Lactobacillus 16SrRNA-specific primers. Nb: this methodology focuses on *Lactobacillus* species. No data on other taxa.	RA patients exhibit increased production and community structure changes of fecal lactobacilli, where *L. salivarius*, *L. iners*, and *L. ruminis* become the predominant bacteria, and the presence of L. mucosae is observed.
Zhang et al. 2015 (286)	RA according to ACR/EULAR 2010, disease duration at least 6 weeks. (n = 77 + 17 treatment naive) (n = 21 DMARD treated)	Healthy controls (n = 80 + 17).	Metagenomic shotgun sequencing and metagenome-wide association study of fecal, dental and salivary samples.	Concordance observed between gut and oral microbiomes. Dysbiosis in gut and oral microbiomes of RA patients, partially resolved after RA treatment, correlated with clinical measures and could be used to stratify individuals based on their response to therapy. *Haemophilus* spp. depleted in RA, at all three sites, and negatively correlated with serum auto-antibodies. *Lactobacillus salivarius* over-represented in RA at all three sites and present in increased amounts in very active RA. No significant difference among relative abundance of *P. copri*.
Maeda et al. 2016 (275)	RA diagnosed according to ACR/EULAR, duration < 2 years. n = 17	Healthy controls n = 14	16S rRNA amplification and sequencing + qPCR of bacterial 16S rRNA targeting *Prevotella* (+ inoculation of human feces into SKG germ-free mice)	Clustering analysis at genus level showed 4 clusters, with one dominated by *Prevotella* species (80% *P. copri*) and composed of only RA patients (n = 6, all RF and ACPA seropositive), whereas the other clusters included both RA patients and healthy controls. Quantitative PCR analysis showed markedly increased *Prevotella* bacterial counts in RA patients in this "*Prevotella*-dominated" cluster vs other RA patients and healthy controls. Germ-free SKG mice colonized with microbiota from "*Prevotella*-dominated" RA patients had an increased number of intestinal T_H17 cells and developed severe arthritis when treated with zymosan (vs healthy-control-microbiota SKG mice).
Chen et al. 2016 (276)	Treated RA patients, diagnosed according to ACR. Median duration = 1,5 years. n = 40	Asymptomatic household first-degree relatives n = 15 Healthy unrelated sex and age matched controls	V3-V5 16S DNA amplification and sequencing. The blood levels of 44 metabolites were measured in both RA patients and their FDRs.	Patients with RA have decreased gut microbial diversity compared with controls, with expansion of rare taxa, *Actinobacteria (Nb : !! does NOT contain Aggregatibacter actinomycetemcomitans)*. *Eggerthella* and *Collinsella* genus demonstrated the most significant association with RA (relative abundance expanded.). Decrease of the genera Faecalibacterium.

		n = 17 Total = 32	Testing of RA-Associated microbes on human epithelial cell line and a humanized mouse model of collagen-induced-arthritis.	Association of *Prevotella copri* as with new-onset untreated RA was **not** observed. *Collinsella* genus correlated with high levels of three metabolites (beta-alanine, alpha-aminoadipic acid, and asparagine), while exhibiting an inverse relationship with allo-isoleucine. CIA mice given *Collinsella aerofaciens* (n = 10) developed arthritis with increased incidence and severity compared *with* non-treated mice (n = 8). Epithelial cells culture: a significant decrease in the expression of the tight junction protein ZO-1, and increase in the expression of interleukin (IL)-17A, in the presence of *Collinsella* vs *E.coli.* *Collinsella* administration led to a significant increase in gut permeability in mice compared with *E. coli.*
Alpizar-Rodriguez et al. 2019 (274)	Preclinical-RA: ACPA+ / RF+ and/or 'symptoms and signs associated with possible RA' as defined by the Connective Tissue Disease Screening Questionnaire with or without undifferentiated arthritis. n = 83	First degree relatives of RA-patient without any autoantibodies or symptoms associated with possible RA. n = 50	16S rDNA amplification and sequencing.	Significant enrichment of *Prevotellaceae*, particularly *Prevotella copri*, in the 'preclinical-RA' group (p=0.04). However, *Prevotellaceae* were not present in all preclinical-RA patients. Decreased relative abundance of *Oxalobacteraceae* preclinical-RA stool. No difference in alpha or beta diversity.
Jeong et al. 2019 (277)	Female pre-clinical RA and <u>untreated</u> early RA n = 29	Healthy female control n 25	16S rRNA gene amplification and sequencing.	*Prevotella* genus slightly enriched in RA and preclinical-RA subjects (not really significant). *Collinsella* was <u>decreased</u> in RA.
Kishikawa et al., 2020 (287)	RA patients according to the ACR or EULAR 2010 criteria. 73% with disease duration < 1 year and 71% untreated. Exclusion: - extreme diets - treatment with antibiotics for at least 3 months prior to sampling - history of malignancy or serious diseases n = 85	Age and sex matched individuals n = 42	Metagenomic shotgun sequencing and metagenome-wide association study of fecal microbiota.	Enrichment of *Prevotella* species in the RA group (*P denticola, marshii, disiens, corporis amnii*), giving the impression of an intestinal overgrowth of oral microbes. Diminution of R6FCZ7 gene, which plays a role in electron transfer, redox catalysis and gene regulation. No difference in alpha or beta diversity between controls and cases.

Mena-Vazquez et al. 2020 (278)	Patients with RA, under stable treatment n = 40	Sex-age matched healthy controls n = 40	16S sequencing	No difference in richness or alpha-diverstiy. At genus level: *Enterococcus*, *Sedimentibacter*, and *Collinsella* were significantly more frequent in RA than in controls. Conversely, decrease in *Sarcina* and *Porphyromonas*. In particular, *Collinsella aerofaciens* increased in RA patients. Associated with high levels of ACPA.
Y. Tong et al., 2020 (abstract) (279)	High-risk preclinical-RA ACPA positive. n = 42 Confirmed RA patients n = 31	Healthy controls n = 38	16S sequencing of V3-V4 regions. FMT on collagen-induced-arthritis mouse model.	*Bacteroidaceae* abundance decreases in preclinical-RA and RA. Collagen Induced arthritis occurred earlier and was more severe in mice receiving stool from preclinical-RA. This associates with reduces gut barrier function and microvilli epithelial damage in small intestine.
Artacho et al., 2021 (600)	Untreated new-onset RA n = 26	Idem (Validation group) n = 21	16S sequencing To predict later response to methotrexate	Responders had lower baseline microbial diversity, with increased proportions of taxa from *Bacteroides* and *Prevotella* genera. Suggestion that these bacteria are the one capable of metabolizing methotrexate. But their predictive model did not work very well on the validation group (AUC ~ 0.6)
He et al., 2022 (288)	Cohort I : Untreated new-onset RA n = 40 Cohort II : Treated established RA patients n = 37	Cohort I : Healthy controls n = 29 Cohort II : Healthy controls n = 31	Shotgun sequencing (HiSeq 2000-2500 platform, Trimmomatic, human DNA filter, Kraken) Samples in cohort were actually selected to be pairs of similar microbiome profiles.	RA patients had significantly more butyrate consumer bacteria. These detrimental species also associated with ACPA (also in the validation cohort). Finally, they show that butyrate suppresses arthritis in collagen-induced-arthritis model (increasing the number of T_{reg} cells).
Jung Hee Koh et al., 2023 (280)	Patients with chronic treated RA (EULAR), in Seoul. n = 94	Healthy adult controls n = 30	16S V3-V4, DADA2 pipeline with SILVA. V138.	*Streptococcus*, *Lachnospiraceae*, and *Weisselia* were relatively more abundant in patients with RA, whereas genera *Romboutsia*, *Collinsella*, *Bifidobacterium*, *Clostridium sensu stricto 1*, and *Lactobacillus* were enriched in healthy participants. Too many subgroups' analyses and some risky statistical stuff. (i.e. they try to predict response to MTX based on microbiota...).
Chen et al., 2023 (281)	Hospitalized RA patients stratified in 3 subgroups according to DAS-28 n = 131	Healthy controls n = 50 + 93 individuals as validation cohort	16S rRNA and Internal Transcribed Spacer (ITS) Sequencing (for fungi assessment)	When increased disease activity, proportion of *Firmicutes* decreased, while *Proteobacteria* increased. Lactobacillus, and *Escherichia-Shigella*, were more abundant is disease group. Fungi were mainly composed of *Ascomycota* and *Basidiomycota* - *Ascomycota* and *Candida* was enriched in the disease groups.

Hong et al., 2023 (282)	RA patients n = 49	Healthy controls n = 25	16S rRNA amplicon sequencing (DADA2, Silva v132)	Genera *Ruminococcus*, *Fusobacterium*, *Erysipelatoclostridium*, and *Mitochondria* (?) are significantly more abundant in RA patients. abundance of *Fusobacterium* is positively correlated with DAS-28. *Fusobacterium nucleatum* aggravate CIA arthritis compared to E coli.
Sun et al., 2023 (283)	Established RA patients n = 37 (+25)	Healthy subjects n = 13 (+22)	16S Sequencing + (shotgun metagenomics)	*Collinsella, Erysipelothichaceae, Roseburia, Dorea, Erysipelatoclostridium, Faecalibacterium, Bacteroides, Subdoligranulum, Alistipes, Coprococcus, Parabacteroides* and *Blautia* were decreased in RA patients. *Parabacteroides distasonis* was associated with DAS-28 score (decreased in RA patients) When given to collagen-induce-arthritis mice, it had a beneficial effect comparable to TNFi. *Parabacteroides distasonis* -derived lithocholic acid, deoxycholic acid, isolithocholic acid and 3-oxolithocholic acid (3-oxoLCA) had similar result (promoted M2 macrophage polarization).
Thompson et al. 2023 (289)	Individuals with rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis, in UK n = 221	Healthy controls (age-matched) 219	Shotgun sequencing	Several taxa were associated with diseases, paralleling paralleled previously studies in IBD, including the clades *Streptococcus* sp., *Escherichia coli*, and *R. gnavus*. No *P. copri* change. Also, changes in folic acid metabolism, iron sequestration, metabolism of broad classes of B vitamins, and production of isoprenoids. Folic acid metabolism in particular (microbial processing of folate to downstream compounds) was more abundant in patients with arthritis and higher circulating CRP.

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