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Rheumatoid arthritis is associated with increased gut permeability and bacterial translocation that are reversed by inflammation control

Rachel Audo^{1,2}, Pauline Sanchez¹, Benjamin Rivière³, Julie Mielle⁴, Jian Tan⁴, Cédric Lukas¹, Laurence Macia⁴, Jacques Morel^{1,2} and Claire Immediato Daïen^{1,2}

Abstract

Objective. To assess how RA and DMARDs affect gut permeability.

Methods. To explore colonic mucosa integrity, tight junction proteins zonula occludens-1, occludin and claudin 2 were quantified by immunohistochemistry on colonic biopsies in 20 RA patients and 20 age- and sex-matched controls. Staining intensity was assessed by two blinded independent readers. To explore intestinal permeability, serum concentrations of lipopolysaccharide binding protein (LBP), soluble CD14 (sCD14) and zonulin-related proteins (ZRP) were evaluated by ELISA in another cohort of 59 RA patients: 21 patients naive for DMARDs [17 before and after introduction of a conventional synthetic DMARD (csDMARD)], 38 patients with severe RA [before and after introduction of a biological DMARD (bDMARD)] and 33 healthy controls.

Results. ZO-1 protein was less expressed in the colon of RA patients than controls [mean score 1.6 (S.E.M. 0.56) vs 2.0 (0.43), $P=0.01$], while no significant difference was detected for occludin and claudin-2. RA patients had higher serum LBP and sCD14 concentrations than controls. LBP and sCD14 levels were significantly correlated with the 28-joint DAS ($r=0.61$, $P=0.005$ and $r=0.57$, $P=0.01$, respectively) while ZRP did not. bDMARD responders had significantly reduced LBP and sCD14 concentrations, unlike bDMARD non-responders and patients treated with csDMARDs.

Conclusion. RA patients have altered colonic tight junction proteins and increased serum biomarkers of intestinal permeability. There was a correlation between serological markers of intestinal permeability and disease activity as well as bDMARD response. These results suggest a link between impaired gut integrity and systemic inflammation in RA.

Key words: RA, microbiota, gut permeability, gut homeostasis, DMARDs

- Patients with RA had altered gut barrier integrity with decreased ZO-1 in the colon.
- Serum markers of gut permeability were increased in RA and positively correlated with disease activity.
- Disruption of gut integrity was reversible, as effective bDMARDs decreased gut permeability biomarkers.

Introduction

RA is the most frequent chronic inflammatory rheumatic disease. Its development is driven by genetic and environmental factors. The gut microbiota, defined by the trillions of bacteria inhabiting the gut, has recently been

highlighted as central in health [1]. Dysbiosis, defined as imbalanced gut microbiota composition, has been reported in RA patients, despite discrepancies about the species involved [2–4].

The gut microbiota is tightly controlled by the host through the gut epithelium and the mucosal immune system. Conversely, changes in gut microbiota composition can impair both the gut epithelial integrity and mucosal

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immune function. Dysbiosis promotes increased gut permeability, which increases exposure of the immune cells to bacterial antigens, leading to a pro-inflammatory response [2].

The epithelial intercellular tight junctions (TJs) provide a physical barrier between the gut lumen and the host. These TJs are mainly composed of claudins and occludins. The intracellular domains of these transmembrane proteins are associated with scaffold molecules such as zonula occludens-1 (ZO-1) that provide a structural scaffold to the TJs. Zonulin is an enterotoxin secreted by intestinal epithelial cells in response to dietary and microbial stimuli that disengages ZO-1 from the TJ complex and therefore increases permeability [5].

Gut permeability can be assessed through functional assays (i.e. absorption of lactulose/mannitol, chromium-51 ethylenediaminetetraacetic acid or polyethylene glycol) as well as colonic immunohistochemistry to quantify and visualize TJ proteins or by indirect methods of measurement of bacterial product translocation. In the latter method, the lipopolysaccharide (LPS) binding protein (LBP), which is known as a marker of clinical endotoxemia, is also considered as a valid marker of gut permeability [6], and the soluble form of CD14 (sCD14), co-receptor for LPS [7], can be used as a marker of bacterial translocation. Despite some controversy [8], serum zonulin has been correlated with increased intestinal permeability in numerous reports [8–12]. Zonulin belongs to a group of proteins structurally and functionally related to the precursor of haptoglobin 2 [8, 11, 13]. As commercial ELISAs not only detect zonulin, but also other proteins from this family [8, 11, 13], the term zonulin-related proteins (ZRP) is now often used rather than the term zonulin when using ELISA to detect it.

In RA, most of the previous studies failed to conclude about gut permeability [14]. Indeed, early studies explored gut permeability in long-standing RA and were limited in their design, with either a very limited number of patients or no control groups, and methodological issues [14]. Only two recent studies using state-of-the-art methods (i.e. histological analysis and biological markers in blood) found increased gut permeability in early RA [12, 15]. Tajik *et al.* [12] investigated gut epithelial permeability in RA patients focusing on the ileal TJs, but did not evaluate the colonic permeability. Increased gut permeability in RA still needs to be better documented, and whether RA patients also have impaired colonic epithelial integrity is unknown. In addition, whether dysbiosis and subsequent gut permeability, if confirmed, is a cause or a consequence of the disease is still an unresolved question. It has been suggested that the dysbiosis of RA patients might be improved by controlling inflammation with DMARDs [3, 16]. However, whether control of inflammation also improves gut permeability in RA remains poorly explored.

In the present study we investigated whether RA patients had increased gut epithelial permeability through the characterization of TJ proteins in colonic biopsies and the quantification of serum biomarkers of gut integrity at

different stages of disease. We also characterized the influence of different DMARDs on these markers.

Materials and methods

Patients and controls for colonic biopsies

In France, biopsies performed in routine care are conserved for 20 years. We identified RA patients who underwent colonoscopy for cancer screening with normal histology and then identified age- and sex-matched controls (University Hospital of Montpellier, Montpellier, France). As approved by the Institutional Review Board of Montpellier Hospital (2018_IRB-MTP_12-12), all subjects gave their informed consent, in accordance with the 2013 Declaration of Helsinki, to use the remaining tissues for the present research. Control subjects neither suffered from any systemic disease nor received anti-inflammatory or immunomodulatory drugs. They were matched with the RA patients on age (5 years) and sex. To be included, RA patients had to fulfil the ACR/EULAR 2010 criteria. RA patients' biopsies were taken from 2012 to 2017 (median 2015) and control biopsies were from 2014 to 2017 (median 2016). The use of NSAIDs, glucocorticoids and DMARDs was reported, as well as disease characteristics, which are detailed in Table 1. The colonic biopsies were analysed as detailed in Supplementary Table S1 (available at *Rheumatology* online) and the staining intensity of ZO-1, claudin 2 and occludin was assessed by two independent blinded assessors using a score from 0 to 4. The interassessor reproducibility was very good with $\kappa = 0.93$ (s.e. 0.07).

Patients and controls for sera

Healthy subjects were either blood donors or patients seen in the Department of Rheumatology (University Hospital of Montpellier) for mild osteoarthritis or

TABLE 1 Patients' characteristics for colonic biopsy analysis

Characteristics	Controls	RA
N	20	20
Females, % (n)	50 (10)	60 (12)
Age, years, median (IQR)	65 (49–68)	63 (43–69)
Disease duration, years, median (IQR) (/19)	NA	20 (8–28)
RF positive, % (n)	NA	50 (10)
ACPA positive, % (n) (/19)	NA	63 (12)
Erosions, % (n)	NA	70 (14)
Corticosteroids, % (n)	0	20 (4)
NSAIDs, % (n)	0	5 (1)
Ongoing csDMARD, % (n) (/18)	NA	39 (7)
Ongoing bDMARD, % (n)	NA	60 (12: 7 TNFi, 2 TCZ, 3 RTX)
DAS28, median (IQR) (/14)	NA	3.9 (2.2–5.0)

Data are for the 20 RA patients, unless stated (/n). NA: not applicable; RTX: rituximab; TNFi: TNF inhibitor; TCZ: tocilizumab.

mechanical pain with no general pathology or infection and receiving no immunomodulatory drugs. They were gender- and age-matched with the RA patients. To be included, RA patients had to fulfil the ACR/EULAR 2010 criteria and were not treated with a biologic DMARD (bDMARD) or glucocorticoid <10 mg/day. Ongoing and recent infection was an exclusion criterion. Patients were defined as naive RA if they had never received any DMARD [conventional synthetic DMARD (csDMARD) or bDMARD] ($n=21$). Patients were defined as severe RA if they had failed at least one csDMARD ($n=38$) and required the initiation of a TNF inhibitor. A total of 55 of the 59 RA patients were followed after introduction of a csDMARD ($n=17$: methotrexate, $n=13$; leflunomide, $n=3$; sulfasalazine, $n=1$) or introduction of TNF inhibitors [$n=38$: etanercept, $n=33$ (86%); certolizumab, $n=5$ (16%)]. RA patients and controls were recruited from 2008 to 2015 [controls: median 2012 (range 2011–2015); naive RA: median 2010 (range 2008–2015); severe RA: median 2010 (range 2008–2012)]. Disease activity was assessed using the 28-joint DAS (DAS28). Responders were defined using the EULAR response criteria (good or moderate) [17]. An additional group of five patients who were insufficient responders to TNF inhibitors were treated with tocilizumab and were also evaluated for LBP, sCD14 and ZRPs in sera. All subjects signed a written informed consent for the study in accordance with the 2013 Declaration of Helsinki and as approved by the Medical Ethics Committee of Nîmes Hospital, France (CPP_2012-A00592-41). Characteristics of the subjects are detailed in Table 2.

Gut permeability and bacterial translocation assessment in humans

Fasting blood samples were collected, immediately centrifuged and stored at -80°C until analysis. ZRP [8, 11, 13], sCD14 and LBP were assessed in patients' sera using

commercially available ELISA kits according to manufacturer's instructions. LBP (DY870-05) and sCD14 (DY383) were purchased from R&D Systems (Minneapolis, MN, USA) and ZRP were evaluated using a zonulin kit (PK-EL-K5600, from PromoCell GmbH, Heidelberg, Germany).

Cytokines

Cytokines (IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, TNF- α , VEGF) were determined using a proteomic approach on an Evidence Investigator biochip system (Randox, Mauguio, France). The CRP level was determined by an immunoturbidimetric method with the Olympus AU2700 biochemistry analyser (Beckman-Coulter, Villepinte, France) and Randox full-range CRP reagent (Randox Laboratories, Mauguio, France).

Statistical analysis

Continuous variables were presented as median [interquartile range (IQR)] or mean (S.E.M.). The qualitative variables were expressed as numbers and percentages. Mann-Whitney tests were used to compare values of controls, naive RA and severe RA. The Wilcoxon matched-pairs signed-rank test was used to compare values before and after therapy. Correlations with clinical parameters were performed using Spearman's test. P -values <0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA).

Results

RA patients have altered colonic epithelial TJs

The colonic biopsies of 20 patients with RA and 20 matched controls were analysed to assess colonic

TABLE 2 Patients' characteristics for serum analysis

Characteristics	Controls	All RA	Naive RA	Severe RA ^a
<i>N</i>	33	59	21	38
Women, %	78	96	95	97
Age, years, median (IQR)	48 (37–60)	57 (46–65)	52 (41–65)	59 (51–64)
Disease duration, years, median (IQR)	NA	3 (1–8)	0 (0–0.1.5)	6 (3–20)
DAS28, median (IQR)	NA	4.1 (3.6–5.3)	4.1 (3.5–5.2)	4.4 (3.3–5.2)
RF positive, %	NA	68	48	81
Erosive disease, %	NA	61	29	84
Glucocorticoids, %	0	66.1	66.7	65.8
Glucocorticoids, mg/day, median (IQR)	0	3 (0–7)	0 (0–5)	5 (0–9)
AINS, %	NA	23.7	42.8	13.2
Number of previous bDMARDs, median (IQR)	0	0 (0–0)	0 (0–0)	0 (0–0)
Number of previous csDMARDs, median (IQR)	0	38	0 (0–0)	38
Drug initiated	0		MTX, $n=17$; LEF, $n=3$; SFZ, $n=1$	TNFi, $n=38$
Combination of bDMARD and csDMARD, %	0	54.2	0	84.2 (MTX, $n=24$; LEF, $n=8$)
Patients with data for EULAR response evaluation, n	NA	41	15	26

^aSevere RA is defined as RA requiring initiation of a bDMARD. NA: not applicable; TNFi: TNF inhibitor; TCZ: tocilizumab.

epithelial integrity by measuring ZO-1, claudin-2 and occludin by immunohistochemistry. RA patients had a significantly lower expression of ZO-1 than healthy controls [mean 1.61 (S.E.M. 0.13) vs 2.00 (0.09) for RA and controls, respectively; Mann-Whitney $P=0.01$], while the expression of claudin-2 [mean 1.65 (S.E.M. 0.14) vs 1.72 (0.16), $P=0.68$] and occludin [2.74 (S.E.M. 0.18) vs 2.54 (0.18), $P=0.26$] were similar (Fig. 1A–C).

We found no correlation between ZO-1 expression and the participants' age (Spearman's $r=-0.24$, $P=0.31$), DAS28 ($r=0.10$, $P=0.70$) or disease duration ($r=0.04$, $P=0.86$) in RA patients. The expression levels of ZO-1 were similar in RA patients regardless of the use of glucocorticoids, csDMARDs, bDMARDs, RF and/or ACPA positivity or the presence of erosions.

RA is characterized by increased gut permeability and bacterial translocation

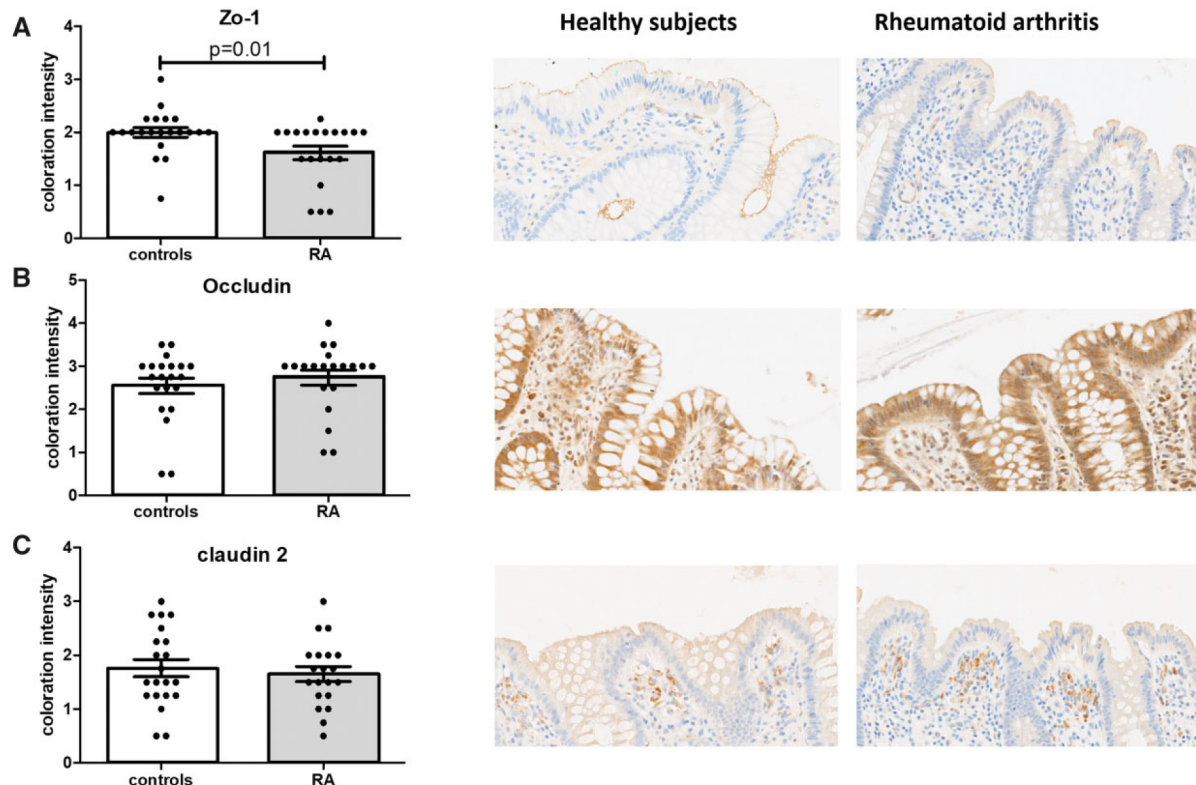
To determine whether impaired colonic epithelial integrity in RA could affect gut permeability, and thus the translocation of bacterial products from the colon to the blood, we quantified the bacterial products in the blood, i.e. LBP and sCD14. We found that RA patients (including patients never treated with a csDMARD and severe patients who did not respond to csDMARD) had significantly higher levels of serum LBP and sCD14 compared with healthy controls (Fig. 2A and B). Serum ZRP levels

were higher, yet not significantly, in patients with severe RA compared with controls (Fig. 2C). It is known that NSAIDs promote damage to the epithelial lining of the gut [14, 18] and the use of NSAIDs was reported in ~43% of naive RA patients (Table 2). However, when naive RA patients with NSAIDs were excluded from the analysis, LBP and sCD14 were still increased compared with controls ($P=0.0018$ and $P=0.0017$, respectively; Supplementary Fig. S1, available at *Rheumatology* online). Impaired gut permeability in RA patients naive for DMARDs correlated with disease activity, as shown by the positive correlations between DAS28 and serum LBP and sCD14 levels ($r=0.61$, $P=0.005$ and $r=0.57$, $P=0.01$, respectively; Fig. 2D–F). LBP also correlated with CRP ($r=0.45$, $P=0.045$), ESR ($r=0.72$, $P=0.001$) and TNF- α ($r=0.68$, $P=0.002$), but not with the other cytokines tested. To confirm that the increase in LBP and sCD14 was not related to infection, we measured the activity of phospholipid transfer protein (PLTP), a protein involved in LPS clearance found to be increased in plasma during infection [19], and no correlation was found with DAS28 or CRP ($r=-0.174$, $P=0.50$ and $r=-0.16$, $P=0.5037$, respectively; $n=17$).

bDMARDs restored gut integrity in EULAR responders

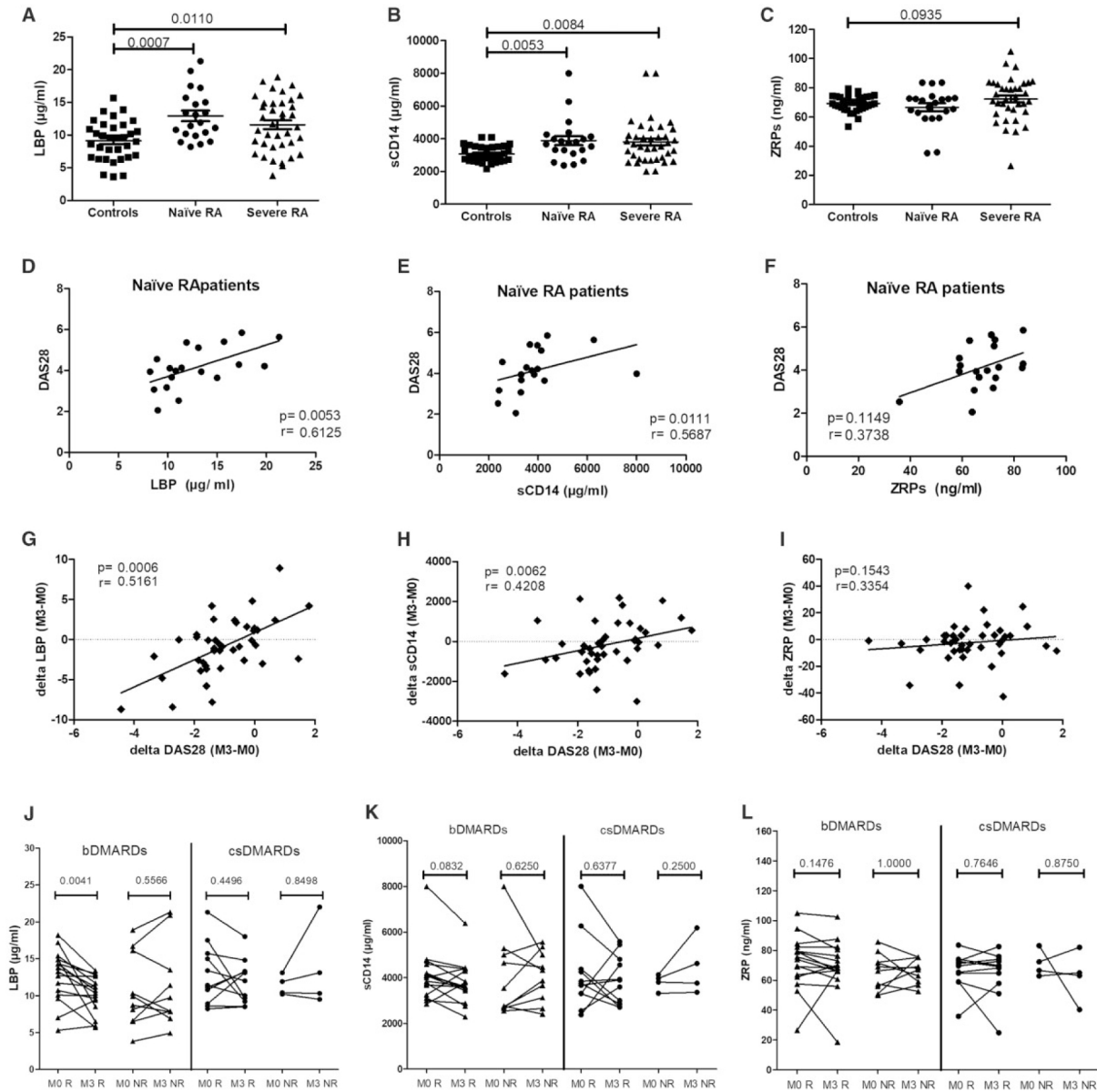
To determine whether RA disease activity could modulate gut permeability, we assessed levels of LBP, sCD14

Fig. 1 TJ markers in the colon of long-standing RA patients compared with age- and gender-matched controls



Quantitative scoring of intensity was performed by two independent readers blinded to the biopsy status. Mean (S.D.) scores and representative pictures are presented for (A) ZO-1, (B) occludin and (C) claudin-2.

Fig. 2 Disease control normalized intestinal permeability and bacterial translocation biomarkers in sera from RA patients



Gut permeability was assessed in sera using commercial ELISAs for (A) LBP, (B) sCD14 and (C) ZRP in patients with RA naïve to any DMARD ($n = 21$), severe RA ($n = 43$) and controls ($n = 33$). Values are presented as mean (S.E.M.). The correlation is shown between disease activity measured by the DAS28 and gut permeability markers (D) LBP, (E) sCD14 and (F) ZRP at baseline in naïve RA patients. The correlation is shown between variations of disease activity measured by Δ DAS28 (M3-M0) and variations in gut permeability markers (G) LBP, (H) sCD14 and (I) ZRP (M3-M0). (J) LBP, (K) sCD14 and (L) ZRP were assessed before and 3 months after introduction of bDMARDs or csDMARDs. Responders (R; good or moderate response) and non-responders (NR) were defined using EULAR response criteria. The Wilcoxon matched-pairs signed-rank test was used.

and ZRP before and after DMARDs. We observed a significant correlation between changes in disease activity (DAS28) and improved gut epithelial integrity as shown by the correlation between disease severity and LBP or sCD14 levels ($r = 0.52$, $P = 0.0006$ and $r = 0.42$, $P = 0.006$, respectively) (Fig. 2G and H). Changes in CRP

levels also correlated with LBP and sCD14 variations ($r = 0.56$, $P = 0.0002$ and $r = 0.35$, $P = 0.026$, respectively; Supplementary Fig. S2, available at *Rheumatology* online). We then assessed the specific effect of bDMARDs (TNF inhibitors) compared with csDMARDs on gut permeability. Interestingly, only patients who responded to

TNF inhibitor treatment had significantly reduced gut permeability, as shown by a reduction in LBP ($P=0.0041$) and a decrease in sCD14 ($P=0.08$), while non-responder had no changes (Fig. 2J and K). On the other hand, ZRP levels were not affected by anti-rheumatic therapy (Fig. 2I, L). An additional group of five patients treated with tocilizumab, another bDMARD targeting IL-6, was included to assess whether the effects observed with TNF inhibitors were class dependent or not. All tocilizumab-treated patients achieved a good ($n=3$) or moderate ($n=2$) clinical response and had decreased LBP and sCD14 at 3 months (Supplementary Fig. S3, available at *Rheumatology* online), suggesting that both anti-TNF and anti-IL-6R drugs improved gut permeability. Altogether, these results suggest that both cytokine blockade and efficient control of inflammation are required to improve gut permeability.

Gut permeability in RA patients is not associated with subsequent response to DMARDs

As gut permeability correlates with inflammation and drug response, we explored whether we could identify responder vs non-responder based on their basal gut integrity status. We found no correlation between EULAR response at 3 months and baseline markers of gut permeability (Supplementary Fig. S4, available at *Rheumatology* online).

Discussion

In the present study we establish that patients with established RA had an altered gut barrier integrity in the colon, with a decrease of colonic ZO-1; patients with RA had increased serological gut permeability markers, which positively correlated with disease activity; and a clinical response to bDMARDs correlated with a decrease in these gut permeability markers. To our knowledge, this is the first study to investigate TJs in the colon of patients with RA and changes in gut permeability induced by DMARDs.

Early studies that explored gut permeability in long-standing RA were limited in their design, with either a very small number of patients or no control groups, making it difficult to conclude about gut leakage in RA. The only possible conclusion from these previous studies was a detrimental effect of NSAIDs on gut integrity, which is well known [14, 18]. Two recent studies described increased gut permeability in early RA [12, 15]. Tajik *et al.* [12] analysed using a lactulose/mannitol absorption test and TJ on ileal mucosal biopsies in nine new-onset and five established RA patients. They found a significant decrease of both occludin and claudin-1 in new-onset and established RA patients compared with controls. In addition, they showed an increase of zonulin in patients with early RA and also before development of the disease. Here we described for the first time an alteration of epithelial TJs in the colon of RA patients, with a

decrease of the key component of TJ protein ZO-1. The other study, from Matei *et al.* [15] found an increase of LPS in pre-RA subjects—defined as patients with serological evidence of arthritis and pain, but no synovitis, and an increase of LBP in early and established RA. In the present study, we confirmed the increase of the gut permeability markers LBP and sCD14 in the sera of RA patients, including early RA. In addition, we demonstrated a link between systemic inflammation and these markers of gut permeability, with a significant correlation of LBP and sCD14 with DAS28. Many studies have associated zonulin/ZRPs in sera to a leaky gut, including in RA [8–12]. The commercial kits used to detect zonulin in sera are controversial [20, 21] and are suspected of detecting a larger family of proteins that are structurally related, named ZRPs [8, 11, 13]. This may explain why we did not detect significant changes in RA patients. Alternatively, other mechanisms independent of zonulin might participate in leaky gut in established RA [22, 23].

Dysbiosis and subsequent gut leakage have been observed in numerous disease models, with the unanswered question of whether it is a cause or a consequence of the disease. In mice, restoration of the intestinal barrier, either by directly targeting intestinal epithelial barrier function or using zonulin antagonist, was found to attenuate arthritis [12, 15]. Zonulin/ZRPs was found to be increased in patients with RA and in individuals at risk who may develop RA in the future [12], and LPS was increased in pre-RA [15], also suggesting that gut barrier disruption precedes disease development. It is interesting to note that in both studies only half of the at-risk subjects presented markers of gut disruption, suggesting that in other patients, disease may be driven by other environmental factors such as smoking or periodontitis [24]. Identifying gut leakage in patients at risk of RA could guide the evaluation of prevention strategies targeting the intestinal barrier, e.g. fibre or short-chain fatty acid supplementation, specifically in this population.

On the other hand, systemic inflammation also induces dysbiosis and gut barrier disruption. In this study, inflammation and disease activity correlated with the gut permeability biomarkers and their decrease after DMARD treatment correlated with a decrease of these biomarkers, confirming a dynamic link between the gut integrity and systemic inflammation. In line with this, we found that patients who responded to DMARDs had significantly reduced gut permeability biomarkers while non-responders did not. Our data highlight the need to explore gut permeability in at-risk subjects before any symptoms occur (i.e. first degree relatives and/or ACPA-positive asymptomatic subjects), as the presence of systemic inflammation by itself induces increased gut permeability. Furthermore, the presence of arthralgia is often associated with the use of NSAIDs, which also induces gut leakage. Interestingly, the effect of the inflammation's control on gut permeability was only found in patients treated with bDMARDs (in combination with csDMARDs), suggesting the implication of specific

cytokines as previously proposed [15, 22, 23]. In line with this, we found a significant correlation between LBP and TNF- α concentrations. To our knowledge, this is the first study comparing the effect of different DMARDs on gut permeability in RA. The clinical relevance of using early bDMARDs targeting TNF- α or IL-6 rather than csDMARDs alone in patients with increased gut permeability deserves to be explored in prospective study.

Our study suffers from some limitations. Due to the retrospective design, we were unable to obtain sera and colon biopsies from the same patients. For future studies, it would be ideal to have both to confirm the validity of those biomarkers in the context of inflammation. However, the invasive nature of such a procedure, especially if it has to be repeated over time, limits the feasibility. Another limitation is the use of NSAIDs, especially in patients naive to DMARDs and constituting the csDMARD-treated group during follow-up. However, we confirmed the increase of LBP and sCD14 in the subgroup of naive RA patients without NSAIDs. The use of NSAIDs was only reported in one of the two recent studies on RA gut permeability [15]. One could argue that the use of NSAIDs in the csDMARDs group could hide the impact of csDMARDs on gut permeability. However, NSAIDs use was on demand and decreased as patients improved. We would thus expect permeability markers to decrease in naive RA patients who respond to csDMARD treatment, in contrast to what we observed. Therefore the impact of csDMARDs on gut barrier dysfunction in RA seems limited.

In conclusion, we confirmed that RA is associated with increased gut permeability, as TJs were altered in colonic tissue of RA patients and serum biomarkers of gut permeability were significantly increased in both early and established RA patients. Serum biomarkers of intestinal permeability appear to be related to disease activity and effective control of disease activity by bDMARDs targeting TNF- α or IL-6 normalized gut permeability, suggesting a dynamic crosstalk between the gut and systemic inflammation. The effect was not observed in patients treated with csDMARDs, highlighting the potential implication of these cytokines in gut permeability.

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Data availability statement

Data available from the authors upon reasonable request.

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