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Elevated serum levels of zonulin-family peptides in ACPA+ at-risk individuals without arthritis

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Abstract

Objective: Recent advances imply that early events triggering rheumatoid arthritis (RA) occur at mucosal surfaces. We aimed to evaluate whether intestinal permeability is altered in patients at increased risk of RA, and/or predicts the development of clinical arthritis, by measuring serum zonulin family peptides (ZFP) levels, which are shown to reflect intestinal barrier integrity. **Methods:** Two independent prospective observational cohorts were studied, including subjects with musculoskeletal symptoms and anti-citrullinated protein antibodies (ACPA), but without clinical arthritis at baseline. In Sweden, 82 such at-risk patients were compared to 100 age-matched healthy blood donors. In the UK, 307 at-risk patients were compared to 100 ACPA-negative symptomatic controls. ZFP was measured in baseline sera by enzyme-linked immunoassays. **Results:** In the Swedish at-risk cohort, ZFP levels were significantly increased in patients compared to controls (mean 41.4 vs 33.6 ng/ml, $p<0.0001$) and Cox regression analysis showed prognostic value of ZFP for arthritis development (Hazard ratio 1.036 per ng/ml ZFP increase, 95% CI 1.005-1.068, $p=0.023$). Elevated ZFP levels among ACPA-positive at-risk patients compared to symptomatic ACPA-negative controls were confirmed in the Leeds at-risk cohort (mean 69.7 vs 36.0 ng/ml, $p=0.0001$) but baseline ZFP were not associated with arthritis development (HR: 1.00 per ng/ml ZFP increase, 95% CI 1-1.01, $p=0.3$). **Conclusion:** Serum ZFP levels are elevated in ACPA-positive at-risk patients when compared to both healthy blood donors and symptomatic ACPA-negative controls. Thus, gut barrier function may be of importance in RA-associated autoimmunity. A possible prognostic value of serum ZFP merits further investigation, preferably in larger prospective cohorts.

Introduction

Rheumatoid arthritis (RA) is an autoimmune, chronic inflammatory disease primarily affecting synovial joints. Autoantibodies against citrullinated proteins (ACPA) are highly specific for RA and may be found in serum several years before the onset of joint inflammation (1), implying that extraarticular locations are inductive sites of immunization in RA (2). In recent years, increasing evidence indicate that mucosal surfaces may be important sites for early triggering events in RA development, in particular concerning autoantibody responses (2). Attention has been drawn to the role of the microbiome and its interaction with mucosal surfaces in the development of chronic inflammatory diseases (3), and gut dysbiosis have been reported both in patients with RA and in patients at increased risk of developing RA (4-7). It remains unclear however, if and how gut dysbiosis specifically promotes synovial inflammation (8).

Zonulin is a human protein which regulates the tight occluding junctions of the digestive tract. The binding of Zonulin to its receptor on the surface of intestinal epithelial cells induces tight junction disassembly and increased intestinal epithelium permeability following a cascade of biochemical events (9), enabling entry of substances that may trigger immune reactions (10). Serum levels of zonulin-family peptides (ZFP) have been shown to associate with intestinal permeability (11), and a dysbiotic microbiota may trigger ZFP release (12, 13), providing a potential link between gut dysbiosis and increased intestinal permeability.

In a study by Tajik et al (14), elevated levels of serum ZFP were found both in patients with RA and in at-risk subjects. Among the latter, elevated ZFP levels associated with an increased risk of developing RA, although cohorts were smaller than in the current study, follow-up only one year, and the results were not adjusted for other risk factors. Interestingly, the study also found that ileal mucosal biopsies from RA patients revealed lower expression of tight

junction proteins in intestinal epithelial cells than healthy controls, and that functional assessment of intestinal permeability in patients showed increased lactulose/mannitol recovery in the urine characteristic for impaired intestinal permeability (14). Finally, in mice, ZFP inhibition restored barrier function and reduced the progression rate to arthritis by 50% (14).

In addition to an urgent clinical need to identify predictors of arthritis development in patients with ACPA and arthralgia, identification of a link to the intestinal mucosa in RA development has the potential to unravel novel therapeutic targets and strategies. We aimed to evaluate whether intestinal permeability is altered in patients at increased risk of RA, and/or predicts the development of clinical arthritis, by measuring serum zonulin family peptides (ZFP) levels, which are shown to reflect intestinal barrier integrity.

Patients and methods

Patients and controls

This study included two prospective observational cohorts, which both included ACPA-positive patients at-risk of RA. Baseline characteristics of patients and control groups are detailed in Table 1.

At-risk patients

The Swedish TIRx (X-tra timely rheumatology follow-up) at-risk cohort enrolled 82 cases with musculoskeletal complaints of any kind and duration and positive IgG 2nd generation anti-CCP test but no baseline arthritis. Patients were enrolled between 2010-13 at the Rheumatology Unit, Linköping University Hospital, Sweden, and followed up for the

development of clinical arthritis defined by clinical examination by a rheumatologist after 3 and 12 months and thereafter yearly. Importantly, patients were instructed to contact the clinic without delay in case of increased symptoms. The patients were followed for a median time of 72 months (range 1-90). 5 patients were lost to follow-up. The healthy control group comprised 100 age-matched blood donors without arthralgia (15).

The Leeds at-risk cohort consisted of 307 ACPA positive patients with new non-specific musculoskeletal symptoms of any kind and duration but with no clinical evidence of inflammatory arthritis (IA). Participants were enrolled from 2008-2019. The participants were followed every three months for the first year, and annually thereafter, monitoring for the development of clinical arthritis as determined by a rheumatologist. The control group consisted of 100 ACPA negative individuals with new non-specific musculoskeletal symptoms recruited from primary care (16). Of the 307 ACPA positive individuals, full follow data was available for 239 individuals. Participants were followed for a median time of 25 months (range 0 – 120). 9 patients were lost to follow-up. Detailed descriptions of the Leeds study has been previously published (16, 17).

Laboratory analyses

Serum ZFP

Serum samples were drawn at baseline and analyzed at the research laboratory at Linköping University for ZFP by a commercially available competitive Enzyme-linked immunosorbent assay (ELISA) (Immundiagnostik AG, Bensheim, Germany). The samples were stored at -70 degrees until use. Samples diluted 1:20 were together with a tracer added to pre-coated 96-microwell plates. Following incubation at RT and washing the conjugate, peroxidase-labelled streptavidin, diluted 1:101, was added. After incubation the substrate was added followed by

stop solution and optical density was immediately determined at 450nm (TECAN Sunrise, software: Magellan V7.1; Tecan Nordic AB, Mölndal, Sweden). Serum samples were analyzed in duplicates. Positive samples with a coefficient of variation exceeding 20% between duplicates were re-analyzed. A standard curve included in the kit was used to calculate concentrations. Cut-off for positivity was set at the 99th percentile of the 100 healthy blood donor control sera (49.8 ng/ml). The intraassay variation was 4% and the interassay variation 7%.

Autoantibodies

Baseline serum samples from the Swedish at-risk cohort had been previously analyzed for free secretory component (SC), total secretory IgA (TSIgA), and total secretory IgM (TSIgM) by using in-house sandwich ELISAs (18). Serum IgG ACPA and secretory component-containing ACPA (SC ACPA) were both analysed by ELISA using 2nd generation CCP as antigen (CCPlus® Immunoscan, Svar Life Science, Malmö, Sweden), but for SC ACPA the secondary antibody was changed into an antibody directed against secretory component (19). Agglutinating rheumatoid factor (RF) was measured at baseline by nephelometry at the accredited laboratory of clinical chemistry, Linköping University Hospital, Sweden. Cut-off was 30 U/ml.

In the Leeds cohort anti-CCP testing was carried out using 2nd generation CCP as antigen in a Bioplex 2200 machine (Bio-rad), with reference range <2.99 IU/mL. RF was tested using nephelometry, reference range in ≤ 14 IU/mL. Data on free SC, TSIgA, TSIgM and SC ACPA where not available in the Leeds at-risk cohort.

Statistical analysis

ZFP levels were compared between groups of patients and controls with the Mann-Whitney U test. Cox regression analyses were applied to test whether ZFP levels (continuous) or status (categorical) were prognostic of arthritis development. In the TIRx cohort, we also performed multivariable Cox regression analyses for ZFP (levels or status) and adjusted for age, sex, ACPA levels, RF levels, symptom duration, smoking habits, sedimentation rate and C-reactive protein levels as these markers were acknowledged to potentially influence ZFP, or showed significant difference in the univariable analysis. Spearman´s test was used to test for correlation between levels of autoantibodies and ZFP. We used graphpad prism 9 to calculate receiver operator characteristic (ROC) curves for ZFP levels versus progression to arthritis. We also used graphpad prism 9 to calculate sensitivity (i.e.% positive samples among risk patients), specificity (i.e.% negative samples among controls), and positive predictive value (i.e.% of risk patients among all positive samples), at a cutoff level set at the 99th percentile among blood donors (49.8 ng/ml).

Ethical considerations

All patients and controls gave their written informed consent to participate, and the ethics Review board in Linköping, Sweden approved the study protocol (decision number M220-09 and 2015/236-32). For the Leeds cohort ethical approval was granted by Leeds, West Yorkshire research ethics committee (reference number 06/Q1205/169).

Results

In the Swedish at-risk cohort arthritis development occurred in 39 out of 82 patients (48%, hereafter referred to as ´progressors`), after median 6 months (IQR: 3-24). Serum levels of ZFP were, in the Swedish at-risk cohort, significantly increased in patients compared to healthy controls (mean 41.4 vs 33.6 ng/ml, $p<0.0001$), Figure 1A). At a cutoff set at the 99th percentile among blood donors (49.8 ng/ml), the sensitivity of a positive test was 28% (95%

CI 20-39%), the positive predictive value 96% (95% CI 80-100%), and the negative predictive value was 63% (95% CI 55-70%).

Progressors showed a trend towards higher baseline ZFP levels compared to non-progressors (mean 44.3 vs 38.7ng/ml, p=0.081). In a ROC curve with ZFP levels versus progression to arthritis, the AUC was 0.61 (95% CI 0.49-0.74). Univariable Cox regression analysis showed an association between ZFP levels at baseline and progression to arthritis (Hazard ratio (HR) 1.038, 95% CI 1.008-1.068, p = 0.012) and a trend for ZFP status at baseline and progression to arthritis (HR 1.760, 95% CI 0.914-3.391, p = 0.091). In a multivariable Cox regression analysis including multiple possible confounders, baseline ZFP levels and status were prognostic for arthritis development (levels: HR 1.036 per ng/ml ZFP increase, 95% CI 1.005-1.068, p=0.023) and status: HR 2.64, (95% CI 1.04-6.6, p=0.040, Figure 2A). Mann Whitney´s test showed no association between NSAID use at inclusion (yes/no) and ZFP levels (p=0.52). Markers associated with mucosal immunity where only analysed in the Swedish at-risk cohort. We found no correlation between levels of IgG ACPA and ZFP (rho 0.156, p = 0.162), but there were weak correlations between ZFP and levels of SC ACPA (rho 0.221, p = 0.046) and for TsIgA (rho 0.289, p = 0.009), respectively. No significant correlations were found with TsIgM, free SC or RF levels.

In the Leeds ACPA positive at-risk cohort, 101 individuals developed arthritis (42%, hereafter referred to as ´progressors`), with median time to arthritis of 12 months (IQR: 6-29). ZFP levels were significantly raised in the Leeds at-risk cohort compared to ACPA negative symptomatic controls (mean 69.7 vs 36.0 ng/ml, p=0.0001), Figure 1 B). The sensitivity of a positive test was 44% (95% CI 40-48%), the positive predictive value 99% (95% CI 98-100%), and the negative predictive value was 24% (95% CI 21-29%). In the Leeds ACPA positive at risk cohort a ROC curve with ZFP levels versus progression to arthritis yielded an

AUC of 0.56 (95% CI 0.49-0.64). ZFP was not associated with progression in Cox regression analysis (status: HR 0.98, 95% CI 0.65-1.47, p = >0.9; levels: HR 1.00 per ng/ml ZFP increase, 95% CI 1-1.01, p=0.3, Figure 2 B). Furthermore, there were no associations with NSAID use (p= 0.35) or IgG ACPA levels (rho = 0.077, p = 0.199), respectively.

Discussion

This study shows increased ZFP levels in serum in two independent ACPA positive at-risk cohorts with musculoskeletal (MSK) symtoms. This finding supports the hypothesis that increased intestinal permeability may be involved in early events of RA development. It is also in line with the only previous study concerning serum ZFP in RA at-risk populations (14). To our knowledge, the current study is the first to show increased ZFP levels among ACPA positive at-risk patients in comparison to ACPA negative individuals with MSK symtoms, implying that increased intestinal permeability may be connected to the development of autoantibodies rather than MSK symtoms.

There was a statistically significant association between serum ZFP and progression to arthritis in the Swedish at-risk cohort, also after adjusting for known risk factors. This agrees well with the previous study by Tajik et al (14) and suggests that serum ZFP may hold promise as a prognostic marker of progression into clinical arthritis in ACPA-positive at-risk populations. However, we could not confirm the prognostic value of ZFP in the independent Leeds at-risk patient cohort despite similar recruitment, patient characteristics, and follow-up procedures. The rather striking differences in baseline ZFP levels between the countries suggest that environmental factors are important and genetic differences may also be involved. However, the median time to arthritis development was shorter in the Swedish at-

risk cohort compared to the Leeds at-risk cohort, implying that ZFP, as a marker of increased intestinal permeability, may have a specific period during disease development where it is readily detected and/or important for arthritis development. These differences between the cohorts, including divergent mean baseline levels, may partly explain the lack of association with arthritis progression in the Leeds cohort. Nevertheless, ZFP levels were markedly increased among at-risk patients compared to controls also in the Leeds setting, and in both cohorts a positive test showed high specificity and positive predictive value for identifying ACPA-positive at-risk patients versus controls. Thus, we conclude that ACPA-positive at-risk patients have elevated ZFP levels, and that the prognostic value of serum ZFP needs to be further evaluated.

Although correlations between antibody levels and ZFP were generally weak, we noted with interest that the only significant findings were made concerning the mucosa-related markers SC ACPA and TsIgA, which are also elevated prior to arthritis development (18, 20).

Zonulin can be detected in multiple tissues (21) and therefore one cannot completely disentangle the origin of serum ZFP, although the previously shown correlation between ZFP and intestinal permeability (22, 23) imply that intestinal mucosa is of substantial importance.

It is known that NSAIDs can influence intestinal permeability (24), but in our study we found no relationship between ZFP levels and NSAID use, suggesting that iatrogenic damage to mucosal membranes is not a major cause of the raised ZFP levels in the circulation.

Concerns have been raised about the specificity of the ZFP assay (25) (26). Indeed, the kit is not zonulin-specific, but rather recognizes a wider range of less characterized zonulin-family peptides (27). However, regardless of the exact variants of ZFP detected by the kit, levels have been convincingly shown to correlate with established readouts of intestinal permeability, including the gold standard lactulose-mannitol ratio test (22, 23, 28).

Nevertheless, a drawback of the current study is the lack of comparison between serum ZFP and a dual sugar test. We also lack data on diet and microbiome, but the otherwise well-characterized patients from “real-world” contexts increase the generalizability of the results.

Conclusion

Serum ZFP is increased in ACPA positive at-risk patients without arthritis, both compared to age-matched healthy blood donors and ACPA negative symptomatic controls. This suggests that increased intestinal permeability may be of importance in the development of RA-related autoimmunity. The prognostic value of ZFP concerning progression to arthritis merits further evaluation, preferably in larger prospective cohorts.

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Figure Legends

Figure 1

Zonulin-family peptide (ZFP) levels in serum from at-risk patients and controls from Sweden (A) and Leeds (B). P-values were generated using Mann-Whitney U test.

Figure 2

Survival plot illustrating baseline ZFP status versus progression to arthritis in Sweden (A) and Leeds (B). The survival plot was generated using Kaplan-Meier and the hazard ratio using Cox regression.