Purifying antibodies to citrullinated protein antigens from rheumatoid arthritis patient serum and cross-reactivity with fecal pool bacteria.

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Introduction

- Rheumatoid arthritis (RA) affects millions of people worldwide while the source of autoimmune etiology remains a mystery.
- There is evidence of Rheumatoid factor (RF) antibodies as well as anticitrullinated peptide (ACPA) antibodies bound to antigens at the mucosal surfaces for patients with RA and those at risk for developing RA.[1]
- It has been found that patients diagnosed with RA and patients at high risk for developing RA have elevated IgA antibodies within their circulation.[2]
- This leads us to the question whether there are at the mucosal surface antigens within the at-risk group that act as a catalyst in the creation of RA autoantibodies?
- The microbiome has been an area of focus due to numerous studies that have shown the clinical association of microbial infections and RA.[3]
- Research has shown that the development of an immune response towards citrullinated peptides is initially restricted but expands over time.[4]
- We sought to demonstrate the interaction of RA antibodies with antigens within the feces samples of a broad pool of patients with sero-positive RA as well as those with high risk factors as supporting evidence that the initial cause of disease may be within the intestinal lining microbiome.

Methods

- Pools of banked serum were obtained from subjects previously profiled for high levels of CCP3 antibodies.
- Anti-CCP3 antibodies were purified from the serum using ELISA (Fig 1).
- The anti-CCP3 antibodies were quantified by IgA and IgG via ELISA (Fig 2).
- Purified ACPA were then prepared into a serial dilution by adding 5ul of sample round 1, round 2, round 1+2, and round 3 in incubation buffer.
- Purified anti-CCP3 antibodies were then labeled with PE and mixed with a pool of fecal samples. Fecal samples were obtained from 5 healthy control patients, 5 patients with early RA, 4 patients at risk who were CCP+, and 4 at-risk CPP-.
- Thaw fecal pool samples were added to 3 separate tubes. For a positive control, tube 1 was added with 100ul staining buffer containing *E. coli* primary antibody and SYTO.
- Tube 2 was the negative control and only included unstained cells.
- Tube 3 included the ACPA pool rounds 1 and 2 as well as PE conjugate plus SYTO.
- All samples were then followed by cytometric analysis (Fig 3)

Results

- From measuring the optical density of the ACPA sample showed that our sample was positive for purified ACPA (Fig 1). The high positive control for this assay was 1.627 OD and the negative control was 0.025 OD.
- Our purified ACPA from the serum samples measured significantly more IgG than IgA regardless of ACPA round (Fig 2). The most IgA measured was in the combined ACPA pool (round 1 + 2) of 21.5 whereas the lowest amount measured in rounds 2 and 3 showed 0 measured IgA molecules.
- Flow cytometry confirmed that the sample serum purified ACPA did positively interact with our pooled stool sample (Fig 3).
- The sample ACPA had a PE positive bacteria of 0.043 comparably higher than the SYTO (PE positive bacteria 0.035) and the unstained cells (PE positive bacteria 0.038).
- The E. coli control group had a PE positive bacteria 8.05 for comparison.

Limitation

- The first limitation of the study is size. While the study utilized a broad patient serum and stool sample as sufficient for a proof-of-concept study, ideally the study would have included exponentially higher numbers of patient samples to solidify the results.
- The total IgA collected within our purified ACPA sample was significantly less than the IgG. This is notable as previous research has shown that IgA proliferation at the mucosal surfaces is theorized to be the inciting subtype of antibody responsible for the development of RA.

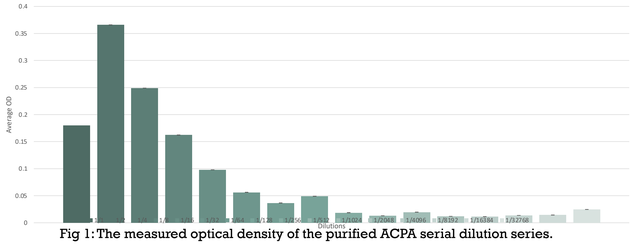

Fig 1: The measured optical density of the purified ACPA serial dilution series.

Source: https://coloradopaincare.com/

Sample	Average IgG/sample
ACPA Round 1	803.3476799
ACPA Round 2	521.7503806
ACPA Round 3	1357.550165
ACPA Pool (1&2)	568.3008565
Sample	**Average IgA/sample**
ACPA Round 1	10.33643165
ACPA Round 2	0
ACPA Round 3	0
ACPA Pool (1&2)	21.50591524

Fig 2: (Top) The average IgA measured within the purified ACPA from rounds 1, 2, 3, and rounds 1 + 2. (Bottom) The measured IgG within the purified ACPA from rounds 1, 2, 3, and rounds 1 + 2.

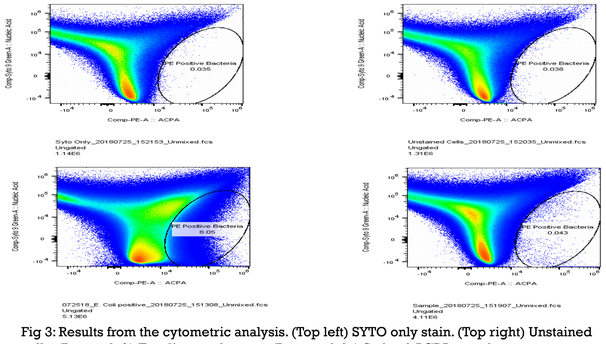

Fig 3: Results from the cytometric analysis. (Top left) SYTO only stain. (Top right) Unstained cells. (Bottom left) E. coli control group. (Bottom right) Stained ACPA sample.

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Discussion

- Our research sought to provide proof of concept for further studies that ACPA did interact with pooled fecal samples and could be useful as a tool for further research to explore.
- ACPA, such as CCP antibodies, are the most specific and sensitive clinical biomarkers for evidence of RA. Evidence that ACPA interacts with antigens within the gastrointestinal tract, in this case within the feces, retroactively provides insight as a potential link to the origin of RA.
- The project was limited by using broad serum and fecal samples to ensure highest possibility of interaction. Further areas of research would aim to specify patient ACPA serum samples as well as the antigen composition which the antibodies target. Nevertheless, flow cytometry analysis illustrated the positive findings of this project which should serve as a basis for the future.
- If the origin of RA is indeed at the mucosal surface due to microbial dysbiosis, then the future endeavors in this field will be in microbial analysis. Identifying the microbial antigen which ACPA interacts is the next frontier.

Acknowledgements

This poster was possible because of the work of Kristi Kuhn, MD/PhD, Meagan Chriswell, Danielle Sansone-Poe, Adam Lefferts, Aaron Vroliik, Michelle Mangette, Cassandra Levens, Eric Norman, American College of Rheumatology.

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Disclaimer: No conflict of interest to disclose