Development and Validation of a Diagnostic Bead-Based Multiplex Autoantibody Assay: Screening for Autoantibodies to Detect "Seronegative" Rheumatoid Arthritis

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Introduction

Autoantibodies (auto-ab) against citrullinated peptides (ACPA) and rheumatoid factor (RF) are important biomarkers for the diagnosis of rheumatoid arthritis (RA). However, RF or ACPA are undetectable in up to 30% of RA patients who can be referred to as "seronegative". In these cases, the diagonsis is often challenging. Early diagnosis in turn is important for improved outcome. In search for an optimized serologic diagnostic strategy, a comprehensive auto-ab screening was performed in RA patients.

Methods

5,892 recombinant human antigens were covalently coupled to fluorescent beads, incubated with patients' sera and detected by a secondary, fluorescent antibody using Luminex xMAP technology (Fig.1). Initially, auto-ab profiles from sera of 72 established RA patients according to ACR/EULAR criteria were compared to 71 matched healthy controls, and 129 systemic lupus erythematosus (SLE) patients in an age- and gender-adjusted manner (exploration phase). Predefined multi- and univariate analyses were employed to generate a diagnostic panel of auto-ab for further testing. The diagnostic potential of this panel was assessed in 116 RA patients from a randomized controlled trial (HIT-HARD) against 116 matched healthy controls (validation phase). Logistic regression modelling was employed to calculate sensitivity and specificity at maximal area under the curve (AUC).

Fig. 1
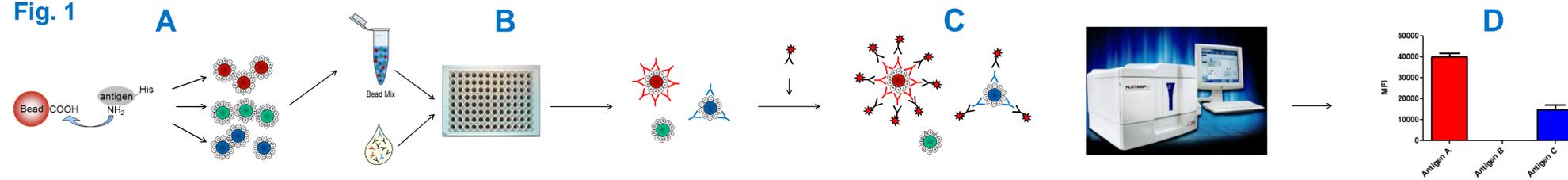
Schematic overview of antibody profiling:
A. Covalent coupling of recombinant antigens to fluorescent beads.
B. incubation with patients' sera.
C. Incubation with secondary fluorescent antibody.
D. Anaylsis of fluorescence intensity.

Results

1) **Panel-Identification:** A panel consisting of 11 auto-ab including ACPA was selected for further testing based on a combination of uni- and multivariate analyses (Tab. 1, Fig. 2)
2) **Exploration:** In the exploration phase, the discriminatory capacity of the selected panel was excellent for RA patients and controls, or SLE patients, respectively (Tab. 2). Omitting ACPA only slightly reduced the discriminatory capacity between RA patients and healthy controls to sensitivity 0.9, specificity 0.86, AUC 0.95.
3) **Validation:** In the validation phase, a reduced discriminatory capacity of the panel was noted with respect to the exploration phase (Tab. 3). Disregarding clinical criteria, the combination of RF and CCP within the patient cohort would have identified 72% of RA patients (18% were seronegative), whereas the tested auto-ab panel identified 71%. In seronegative patients, the auto-ab panel resulted in a high specificity, albeit very low sensitivity (Tab. 3).

Tab. 1 – Functions of proteins selected for the auto-ab Panel

1. citrullinated filaggrin (ACPA)
2. RNA-binding/second messenger
3. second messenger NFkB pathway
4. amino acid synthetase
5. cytoskeleton
6. cytoskeleton
7. unknown (protein kinase?)
8. protein-protein/nucleic acid interaction
9. second messenger PKC pathway
10. nucleolar protein (snRNP associated)
11. growth factor receptor

Fig. 2 - antigens: healthy vs. RA
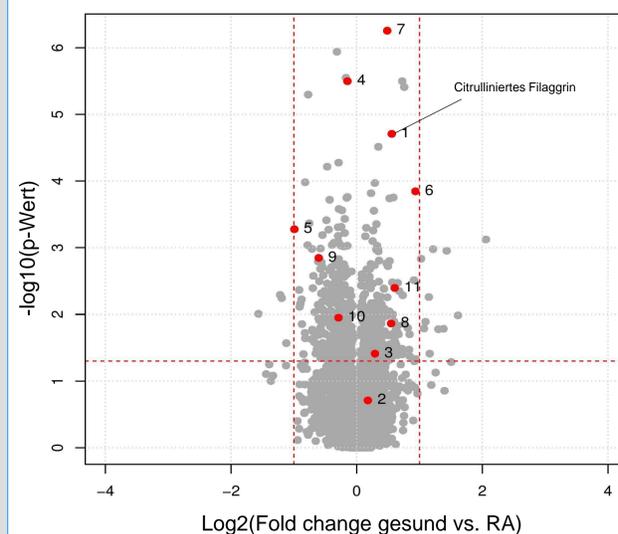
Tab. 2 – exploration phase

Test	sens.	spec.	AUC
RA vs. healthy	0.9	0.9	0.97
seroneg RA vs. healthy	1.0	1.0	1.0
RA vs. SLE	0.79	0.92	0.93

Tab. 3 – validation phase

Test	sens.	spec.	AUC
RA vs. healthy	0.71	0.53	0.68
seroneg RA vs. healthy	0.09	0.96	0.78

Conclusions

The auto-ab panel showed a diagnostic quality for RA independent of ACPA. Reductions in the discriminatory capacity of the auto-ab panel in the valiadation phase underlines the potential for over-fitting in explorative studies using multiplex approaches. Improved models with increased sensitivity for seronegative RA patients could potentially enable serologic diagnosis of „seronegative" RA in the future.

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