Confirmation of Serological Antibodies Against Novel Citrullinated Proteins in Early Rheumatoid Arthritis

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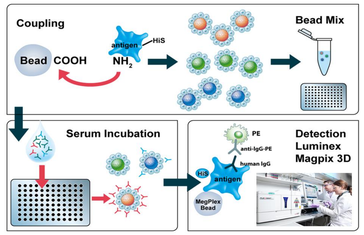
Introduction

Autoantibodies against ACPAs (anti-citrullinated peptide/protein antibodies) show a diagnostic sensitivity of 82% and a specificity of up to 98% in established RA, whereas in early RA sensitivity of ACPA tests may be around 50%.

ACPAs recognize specifically various citrullinated peptides that derive from different antigens. It has been demonstrated that autoantibodies to citrullinated peptides emerge at different stages of RA (Brink et al., 2013). Moreover, some CCP-negative RA-patients can be identified by analyzing additional citrullinated proteins or peptides (Lundberg et al., 2013). We hypothesize that autoantibodies in early RA require distinct protein sequences plus citrulline. In pre/early RA, a rather broad set of different citrullinated protein epitopes is therefore required (fine specificities). To develop a multi-epitope ACPA-test we set out to identify additional fine specificities using SeroTag screens with citrullinated proteins. Here we show validation of such novel ACPAs.



- High-throughput technology with 8,000 human proteins as analytes
- Serum based, minimal sample volume (< 5µl) required
- Stable analytes (IgG - all isoforms, IgM, IgA)
- Luminex systems widely used and accepted by FDA, EMA

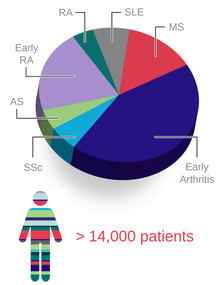
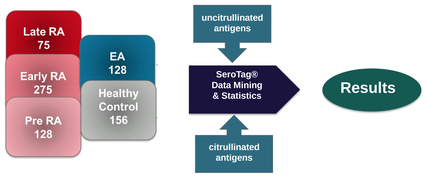


Figure 1:
The SeroTag® autoantibody discovery process is based on bead-based array screening of recombinant human autoantigens in citrullinated and non-modified forms

Methods

SeroTag® autoantibody profiling via the bead based Luminex FlexMAP3D platform in over 14,000 patient samples has revealed numerous novel antigens in autoimmune indications like SSc, SLE, and RA. Whereas normal screens utilize up to 8,000 human proteins expressed in and affinity purified from *E.coli*, in RA we also use enzymatically citrullinated antigens as bait for autoantibodies. In several discovery studies 25 novel ACPAs were selected for further validation in additional early RA cohorts (Figure 2).

Cohorts used:
- CAPEA
- Late RA
- Bavarian Red Cross (BRK) blood donors
- Rheuma Truck



Group	T0	T1
Early Arthritis	Arthritis	Arthritis
pre-RA	Arthritis	Early RA
Early RA	Early RA	Early RA
Late RA	Confirmed RA >> 2 years	Confirmed RA

Figure 2: Patients representing different cohorts of RA patients (pre-RA, early RA, RA, early Arthritis, healthy controls)

Results

Evaluation of the profiling data revealed significant reactivities for 25 novel antigens previously not described in RA. Activity was increased comparing both citrullinated vs. uncitrullinated and RA vs. HC. As expected, serum from patients with later stages of RA recognized a higher number of antigens (Mean +3SD of HC), demonstrating patient specific reactivity patterns. Patients with pre-RA also recognised on average 15 novel antigens, albeit at a higher degree of diversity (fine specificities).

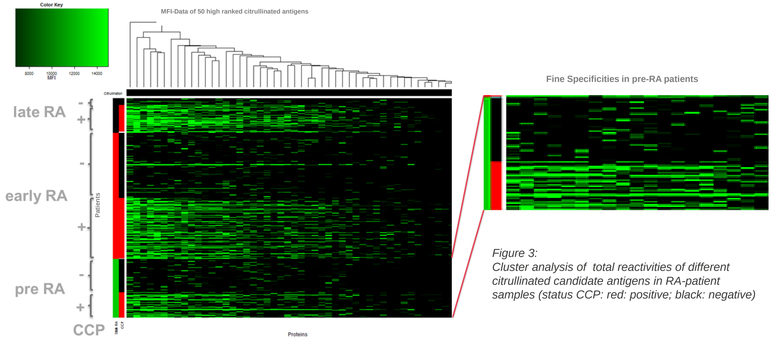


Figure 3:
Cluster analysis of total reactivities of different citrullinated candidate antigens in RA-patient samples (status CCP: red: positive; black: negative)

Disease Stage	Mean Number (± SD) of Reactivities in Total RA Cohorts and HC	Number of Samples (n=)	Mean Number (± SD) of Reactivities in RA Subgroup CCP+	Number of Samples (n=)	Mean Number (± SD) of Reactivities in RA Subgroup CCP-	Number of Samples (n=)
Healthy	11.9 ± **13.3**	156	n.a.	n.a.	n.a.	n.a.
Early Arthritis	15.7 ± 17.0	127	14 ± 1.22	4	15.77 ± 17.65	118
Pre-RA	15.0 ± 17.1	128	15.3 ± **17.3**	55	11.6 ± 10.2	73
Early RA	28.1 ± 31.5	274	29.1 ± 31.7	132	23.6 ± 23.7	142
Late RA	46.5 ± 34.3	73	46.6 ± 34.4	58	31.1 ± 13.2	15

Table 1: Number of positive reactivities (> Mean + 3SD of HC) in samples of RA patients in different disease stages

The graphical display of 25 candidate antigens resulted in prominent differences in reactivity in RA patient samples and healthy controls with a higher reactivity of antibodies against citrullinated antigens while reactivities against uncitrullinated antigens were not significant. In addition to known citrullinated antigens used as benchmarks (e.g. Fibrinogen (FGB) or Vimentin (VIM)) the novel antigens revealed a significant difference in AAB content in sera of RA patients (Table 3, Figure 4).

ProteinID	p-value	p-value adjusted	AUC [CI]	Sensitivity [CI]	Specificity [CI]
citFGB	< 0.0001	0.0042	0.619 [0.583, 0.656]	0.482 [0.446, 0.519]	0.67 [0.608, 0.732]
citVIM	< 0.0001	<0.0001	0.649 [0.599, 0.7]	0.530 [0.479, 0.581]	0.725 [0.61, 0.84]
Cit-Antigen 1	< 0.0001	< 0.0001	0.742 [0.688, 0.797]	0.563 [0.527, 0.6]	0.804 [0.696,0.913]
Cit-Antigen 2	< 0.0001	< 0.0001	0.728 [0.683,0.774]	0.566 [0.526,0.607]	0.782 [0.687,0.878]
Cit-Antigen 3	< 0.0001	< 0.0001	0.732 [0.68, 0.785]	0.519 [0.453, 0.586]	0.864 [0.776, 0.954]
Cit-Antigen 4	< 0.0001	< 0.0001	0.715 [0.66,0.772]	0.541 [0.466,0.617]	0.850 [0.773,0.927]
Cit-Antigen 5	< 0.0001	< 0.0001	0.721 [0.668,0.775]	0.548 [0.497,0.601]	0.780 [0.702,0.86]

Table 3:
Results of explorative testing for MFI values
RA (CCPneg and CCP pos) vs. HC (Wilcoxon-Rank Sum test)

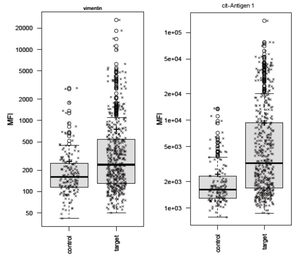


Figure 4:
Boxplot of reactivities of antibodies against citrullinated vimentin and cit-antigen 1 in RA (target) and healthy controls (control)

Frequency of the novel citrullinated antigen 1 was >25% in RA cohorts (69% late RA, 36% early RA, 27% pre RA) compared to 3% in healthy controls or 2% in early Arthritis patients. In contrast to that, antibodies against the respective uncitrullinated antigen could not be identified in a higher frequency in RA patient cohorts (Figure 5).

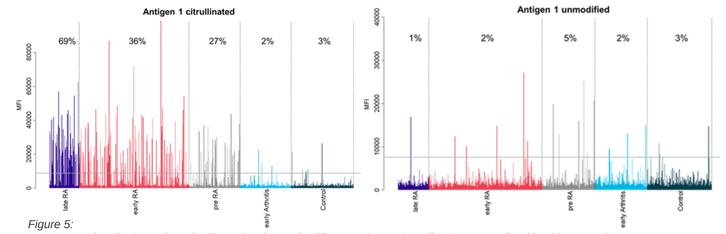
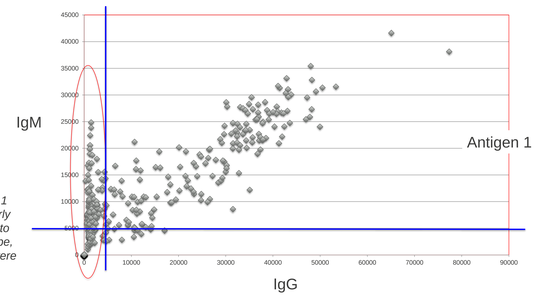


Figure 5:
Frequency of antibody against citrullinated antigen 1 in different cohorts. Cut-off: 97% percentile of healthy controls

Three of the novel ACPAs were assessed in additional 300 early RA samples from a European cohort study. In these patients, the frequencies of the respective antigens was found to be from 25 to 45%, and in combination over 55%. In addition, to autoantibodies of IgG-type, also AABs of IgM-type were measured (Figure 6). A significant proportion of patients exhibited reactivity against IgM, only. Combined assessment of IgG and IgM leads to increased sensitivity and might also outline class switch status of B-cells.

Figure 6:
The citrullinated antigen 1 was assessed in 300 early RA samples. In addition to autoantibodies of IgG type, also AABs of IgM type were measured.



Conclusion

SeroTag®-screens using citrullinated human antigens demonstrated 25 novel ACPA-candidates of which 3 were successfully validated in a large European Early RA cohort. Using those novel ACPA an identification of early- or pre RA patients might be possible based on increased presentation of diverse citrullinated peptide epitopes with better sensitivity and specificity and by assessing both IgG and IgM. Furthermore, improved identification of ACPA in CCP-negative patients may reveal an additional diagnostic potential and might help to improve disease management for those patients.

Lundberg K, Bengtsson C, Kharlamova N, *et al. Ann Rheum Dis* (2013). doi: 10.1136/annrheumdis-2012-201484
Brink M, Hansson M, Mathsson L, *et al ARTHRITIS & RHEUMATISM (2013)* Vol. 65, No. 4, pp 899–910