Autoantibody Profiling of Late and Early RA Patients Against Citrullinated Proteins

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Introduction

The discovery of autoantibodies (AAB) against citrullinated proteins (ACPA) and the development of serological tests using cyclic citrullinated peptide (CCP) has improved diagnosis of rheumatoid arthritis (RA). ACPAs recognize specifically various citrullinated peptides that derive from different antigens. CCP-positive RA patients show autoantibody specificities against different citrullinated antigens. It has also 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 follow the hypothesis that discovery of additional citrullinated antigens might improve early diagnosis of RA based on epitope-determined heterogeneity of AAB-reactivity.

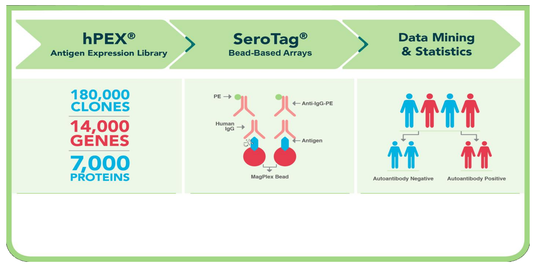

Figure 1:
The SeroTag® autoantibody discovery process is based on bead-based array screening of recombinant human autoantigens in citrullinated and non-modified forms

Methods

The SeroTag® technology utilizes the bead-based Luminex xMAP technology to profile the reactivity of autoantibodies in patient sera against 7000 human recombinant antigens (Figure 1). For the identification of novel RA-biomarkers, a set of 417 antigens was selected from earlier studies in several autoimmune diseases like RA, SLE or SSc. The set of antigens was enzymatically citrullinated and also used in the unmodified form as control. In this study, the AAB-profiles of 763 serum samples of CCP+ and CCP- RA patients with late-, early - or pre-RA were tested and compared to profiles of healthy volunteers and patients with early Arthritis (EA) (Table 1).

Group	n	Mean Age	Gender f/m	Diag T0	Diag T1	CCP positive	CCP negative
Early Arthritis	127	56.5 ± 14.6	86 / 41	Arthritis	Arthritis	4	118
Pre-RA	128	56.1 ± 14.1	75 / 53	suspected RA	Confirmed RA*	55	73
Early RA	275	54.6 ± 14.6	185 / 90	Confirmed RA	Confirmed RA	132	142
Late RA	75	57 ± 13	54 / 21	Confirmed RA >> 2 Years	Confirmed RA	58	15
Healthy	158	51.3 ± 13.3	109 / 49	n.a.	n.a.		

* according to ACR-1987 criteria

Table 1:
Data of patients included in the analysis. The samples have been selected to represent different cohorts of RA patients depending on the diagnostic status)

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 + 3 SD of HC), demonstrating patient specific reactivity patterns. Patients with pre-RA also recognised on average 15 novel antigens.

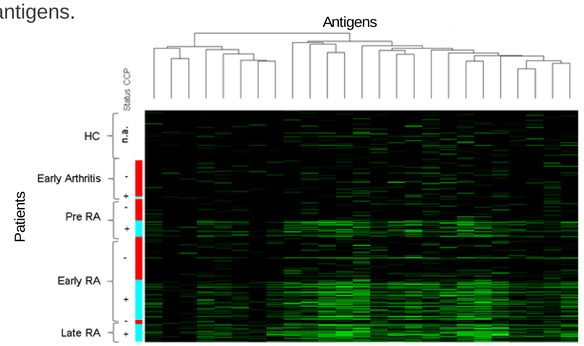

Figure 2:
Cluster analysis of total reactivities of 25 different citrullinated candidate antigens in RA-patient samples (status CCP: red: negative; blue: positive)

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 2:
Number of positive reactivities (> Mean + 3SD of HC) in samples of RA patients in different disease stages

The analysis 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 (Figure 4) 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 3).

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)

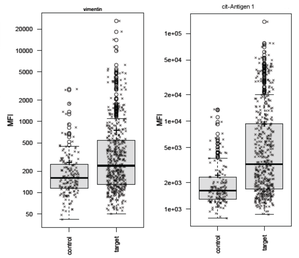

Figure 3:
Boxplot of reactivities of antibodies against citrullinated Vimentin and cit-Antigen 1 in RA (target) and healthy controls (control)

Frequency of the novel citrullinated antigen 1 was >25% in RA cohorts (69% lateRA, 36% earlyRA, 27% preRA) 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 4).

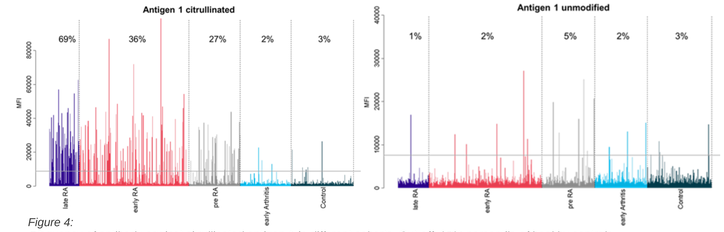

Figure 4:
Frequency of antibody against citrullinated antigen 1 in different cohorts. Cut-off: 97% percentile of healthy controls

In order to investigate the potential of novel citrullinated antigens to identify CCP-negative RA-patients the marker candidates have been analyzed in the subgroup of CCP-negative RA-patients (Table 4, Figure 5). AAB against citrullinated antigen 11 could be identified in 8 - 27% of CCP-negative samples of RA patients.

ProteinID	p-value	p-value adjusted	AUC [CI]	Sensitivity [CI]	Specificity [CI]
Cit-Antigen 11	<0.00001	0.0078	0.634 [0.57,0.699]	0.472 [0.425,0.52]	0.754 [0.699,0.809]
Cit-Antigen 12	0.0001	0.1180	0.618 [0.547,0.691]	0.445 [0.406,0.484]	0.711 [0.6,0.823]
Cit-Antigen 13	0.0014	1.0000	0.601 [0.524,0.678]	0.455 [0.393,0.519]	0.699 [0.595,0.805]
Cit-Antigen 14	0.0018	1.0000	0.604 [0.519,0.691]	0.404 [0.291,0.519]	0.714 [0.624,0.806]
Cit-Antigen 15	0.0019	1.0000	0.592 [0.535,0.649]	0.440 [0.381, 0.5]	0.678 [0.612,0.746]

Table 4:
Results of explorative testing for MFI values RA (CCPneg) vs. HC

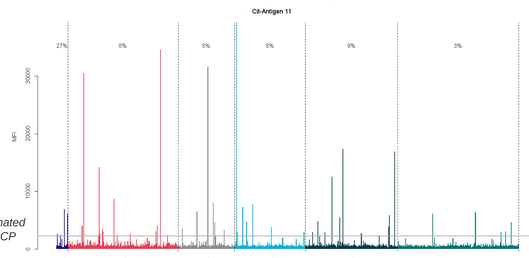

Figure 5:
Frequency of AABs against citrullinated antigen 11 in different cohorts of CCP neg. samples.
Cut-off: 97% percentile of healthy controls

Conclusion

In this proof-of-concept Serotag® -Screen using citrullinated human antigens 25 novel ACPA-candidates were discovered showing increased reactivity compared to non-citrullinated antigens. 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. 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