Closing the gap in early diagnosis of autoimmune rheumatic diseases: discovery of novel biomarkers using high-density peptide microarrays

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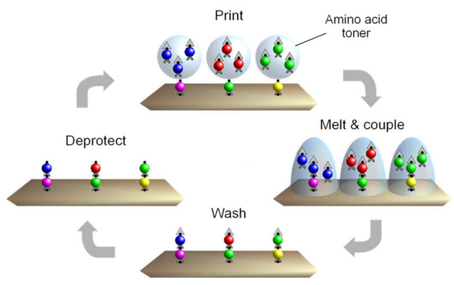
Abstract

Autoimmune rheumatic diseases such as rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are attributed to inflammation affecting the joints and connective tissues, with extra-articular manifestations in some disease types. Early diagnosis is the key to optimal therapeutic success in order to slow down disease progression or even prevent joint damage and hence irreversible disability. However, diagnosis in an early disease stage is often challenging. For example, up to 30% of early stage RA patients are negative for the current serological diagnostic measures using antibodies against cyclic citrullinated peptide antigens (ACPA) or rheumatoid factor (RF). A more comprehensive analysis of antigenic proteins and their underlying epitopes can ultimately provide the necessary information for the development of innovative serological tests with a higher sensitivity for early disease diagnosis.

To identify novel biomarkers in autoimmune rheumatic diseases, we apply high-density peptide microarrays that display large numbers of putative target proteins translated into overlapping linear and conformational peptides including posttranslational modifications such as citrullination and carbamylation. Using these highly diverse libraries covering tens of thousands of peptide antigens, we screened sera from different disease cohorts and healthy controls. Autoantibody responses against various peptides were identified, exhibiting a higher prevalence in previously seronegative early-stage patients. These autoantibodies can potentially serve as biomarkers for early diagnosis of autoimmune rheumatic diseases.

Technology



Benefits

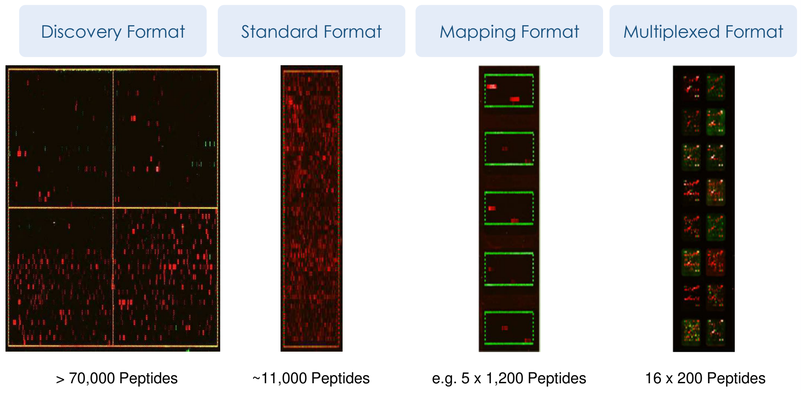
- very low material consumption
- high spot density / high content (1,200 peptides/cm²)
- digital printing flexibility, fast production times
- high peptide quality with routine double couplings

High-density PEPperCHIP® peptide microarrays are generated by digital laser printing on standard glass slides using a proprietary laser printer comprising 24 cartridges filled with individual amino acid toners. For array production, the amino acid toners are simultaneously printed with high precision on their respective positions on the glass slides.

Peptide synthesis is initiated by melting the toner particles at 90°C. Under these conditions, the amino acids are released and are available for coupling to the previous amino acid. The coupling cycle is completed by washing steps to remove excess building blocks and protecting groups. Finally, the array is ready for the next synthesis cycle with laser printing and coupling.

The benefits of this technique are an unique flexibility in terms of peptide content, a high spot density with up to 11,000 features per chip and low material consumption enabling the generation of customized peptide array at reasonable costs.

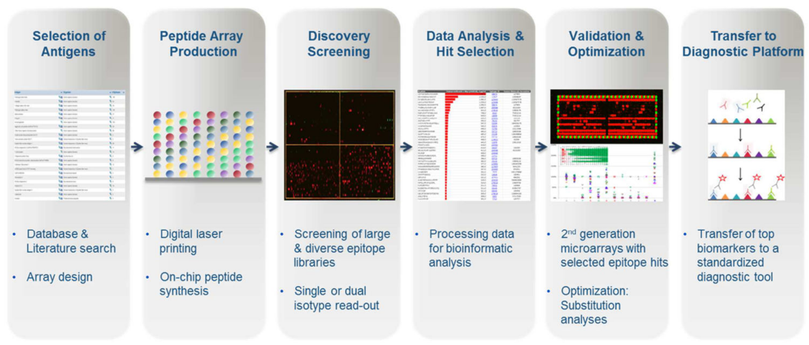
PEPperCHIP® Peptide Microarray Platform



Antibody-peptide interactions are analyzed by immuno-type assays in a high-throughput fashion on peptide microarrays. Depending on the application, various microarray formats are available:

- **Discovery format**: > 70,000 individual peptides; suitable for screening of large, diverse epitope libraries covering up to 100 proteins; applied for biomarker and target binder discovery
- **Standard format**: ~ 11,000 individual peptides; routinely used for epitope mapping. Custom peptide microarrays or standard chips e.g. PEPperCHIP® Autoimmune Epitope Microarray
- **Mapping format:** several identical array copies on a single chip; ideal for parallel screening of multiple samples; used for epitope mapping of single protein antigens, detailed epitope characterization or biomarker validation
- **Multiplexed format:** up to 16 array copies on a single chip; ideal for assay development or hit validation studies with sample cohorts

General Workflow Biomarker Discovery



Biomarker Discovery Approach:

- The process starts with the design of the antigen library. Antigen proteins are translated into sets of overlapping peptides and synthesized on linear or conformational discovery microarrays.
- The discovery screening is performed as immunoassay with single or dual isotype read-out.
- Most relevant biomarker candidates are selected for further validation and optimization on 2nd generation microarrays.
- Finally, the most comprehensive biomarkers are validated by standard diagnostic formats such as ELISA.

RA discovery screening revealed a strong and differential antibody response in SN early-stage patients

PEPperCHIP® SeroRA Discovery Library Content

- Antigens of human and pathogen origin
- Library size: > 100,000 peptides with citrulline and homocitrulline variants

Study Outline Discovery Screening

- Control Cohort (healthy (HC) and psoriatic arthritis (PsA))
- Disease Cohort (early-stage seronegative (SN; RF⁻/CCP⁻) and seropositive (SP) RA patients, late-stage RA patients)
- Cohort Screening with pooled sera
- Dual isotype read-out (IgG and IgA)

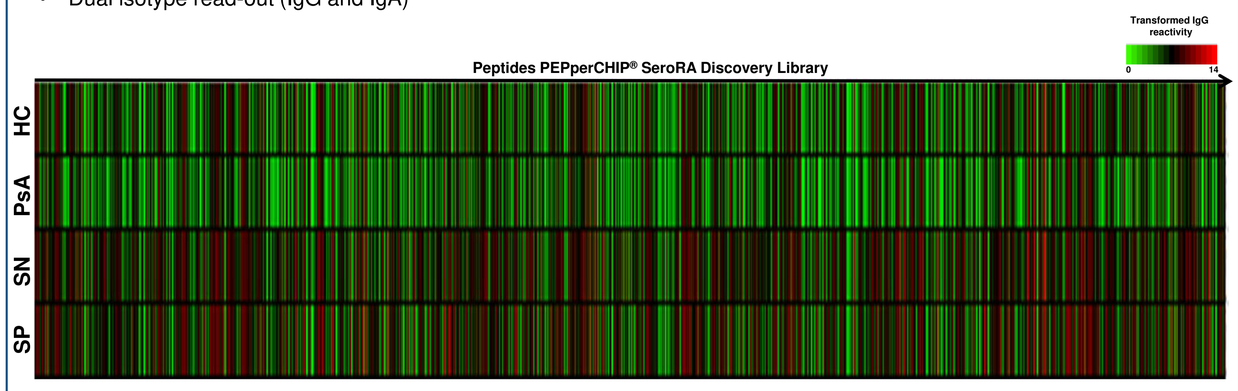


Figure 1: Differential IgG antibody response in previously seronegative early-stage RA patients. The heat-map shows the mean fluorescence intensity for each individual peptide detected for the respective screening cohort.. For a better visualization, peptides with a mean cut-off value of >500 FU in the SN patient group are shown. Before calculating the mean, raw fluorescence intensity values were vsn transformed.

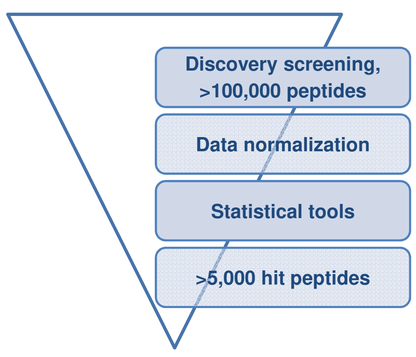


Figure 2: Schematic overview of the applied data analysis process. After raw data normalization, significant peptides were determined applying a 2-sided Welch-2-sample test (5% FDR, LFC ≥ 5).

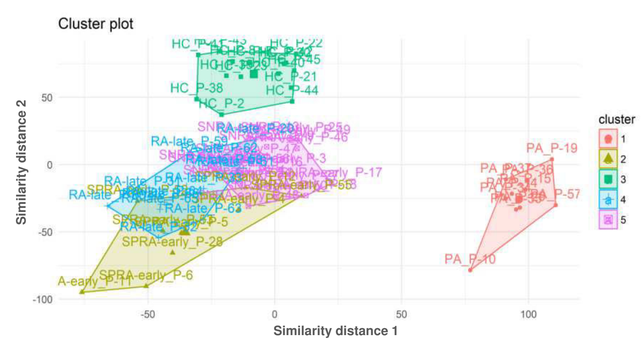


Figure 3: k-means clustering of top hit peptides stratified subjects into control and disease-related clusters. Clusters of healthy (HC), psoriatic arthritis (PsA) and all rheumatoid arthritis (RA) conditions can be clearly distinguished as separate groups.

Novel epitopes in previously seronegative early-stage RA patients

SeroRA Validation Library Content

- > 5,000 peptide hits selected from Discovery Screening

Study Outline Validation Screening

- Control Cohort (healthy and psoriatic arthritis)
- Disease Cohort (early-SN, early-SP and late RA patients)
- Cohort Screening with individual sera (> 300 sera)
- Dual isotype read-out (IgG and IgA)

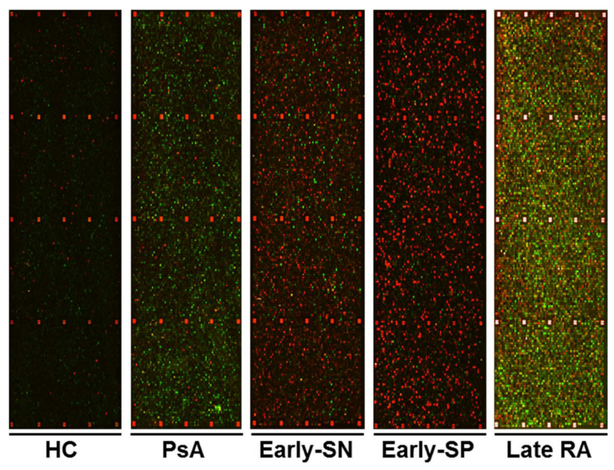


Figure 4: Validation screening demonstrates a differential staining pattern. Representative staining images of all patient groups are shown. Validation arrays were incubated with respective sera overnight at 4°C. Secondary detection was done using anti-human IgG DyLight680 and anti-human IgA DyLight800. Read-out was performed using a LI-COR Odyssey Imaging System. Polio peptide spots served as control.

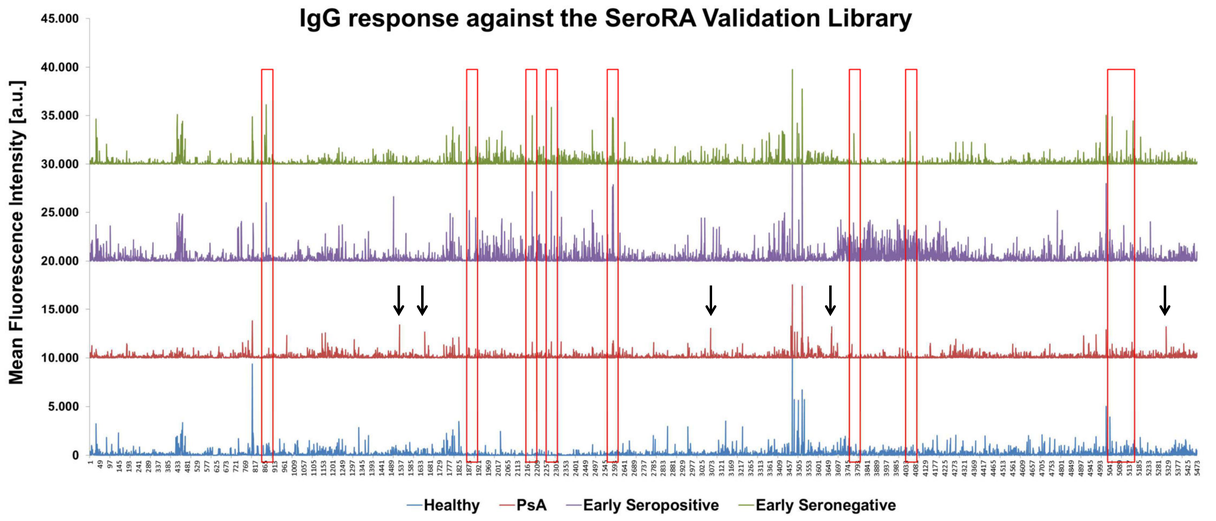


Figure 5: Validation of newly identified epitopes in previously seronegative RA and psoriatic arthritis patients. Mean fluorescence intensity units (y-axis) were plotted against the peptides of the SeroRA validation library (x-axis). For a better comparison, the IgG responses of HC, PsA, early-stage seropositive and early-stage seronegative were combined in a single plot, and the baseline of PsA, early-stage seropositive and early-stage seronegative were leveled to 10.000 (PsA, red line), 20.000 (early-stage Seropositive, purple line) and 30.000 (early-stage seronegative, green line) fluorescence units, respectively. Blue line: Healthy control (HC). Unique epitopes are highlighted with red boxes (for early-stage RA patients) and black arrows (for PsA patients), respectively.

Summary

- Largest library for the identification of prognostic RA peptides
- Strong and differential autoantibody response in early-stage autoimmune rheumatic diseases
- Novel epitopes in previously seronegative RA patients identified
- Discovery of unique psoriatic arthritis epitopes

Outlook

- Technology platform for analysis of stage and serotype dependent autoimmune responses on discovery arrays covering up to 100 autoantigen proteins
- Multiplexed high throughput screening of large samples sets
- Discovery of diagnostic, prognostic and predictive biomarkers for the development of IVD and CDx