Reverse Autoreactivity Assay to Identify New Autoantigens in Autoimmune Liver Diseases

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Reverse Autoreactivity Assay to Identify New Autoantigens in Autoimmune Liver Diseases

Classical Autoreactivity Assays:

- Hypothesis driven approach
- Antibodies from patients screened on tissues or cells.
- Identification of most recognized antigens

Reverse Autoreactivity Assays:

- Proteome wide approach
- Antibodies from patients screened on arrays of thousands of proteins selected with the only criteria of being human, and in our case of being poorly known and dealing with the extracellular milieu (secreted or transmembrane).
- Characterization of most recognized antigens

Protein Array Strategy

Human genes ≅ 27000 genes

"External" human genes ≅ 8000 genes

Poorly known "external" human genes ≅ 3000

in silico

Cloning

Expression in *E. coli*

(2500 genes)

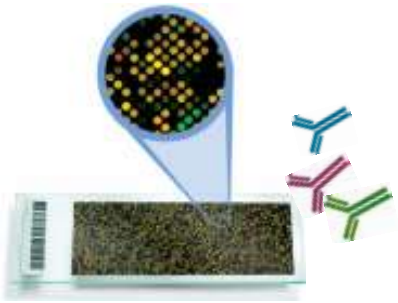
Purification

Recombinant proteins

(1700 proteins)

Custom Protein Library

Print Custom Protein Microarray



Sera from "any" patients groups

Identification of new autoantigens associated to the investigated diseases

- Protein characterisation
- Relevance for Pathogenicity
- Gene silencing (Si RNA)
- Engeneered animals (Tg, KO)

Target validation

Development of new

diagnosis/ prognosis kits

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Development of Protein Microarrays

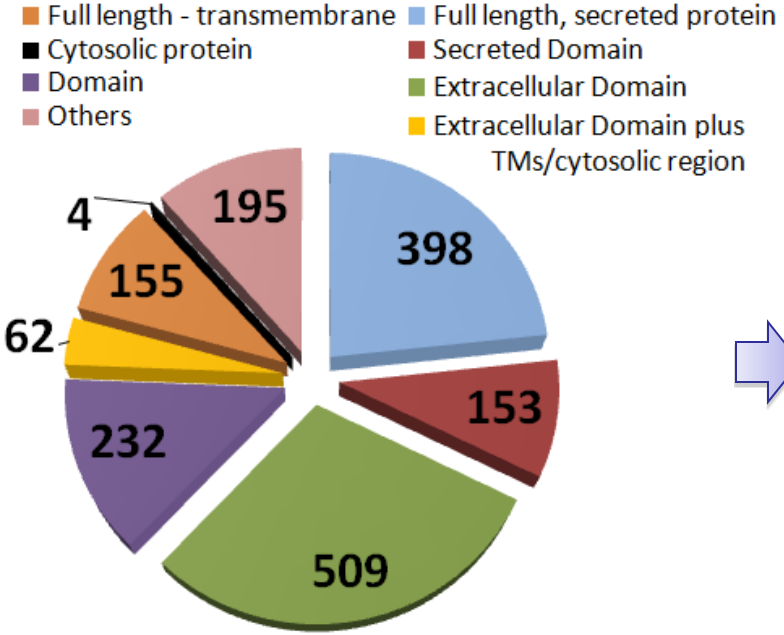
Platform

Protein Purifi Process

Gene Cloning and Expression in *E. Coli*

In silico Identification of the human genes of interest

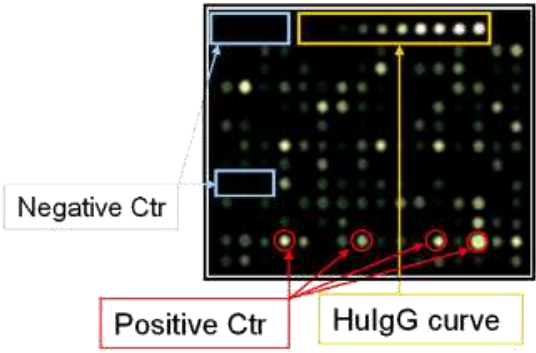
≅ 1700



Printing proteins on the slides

>1500 – recombinant human proteins

to identify
candidate autoantigens



Assay human sera on arrays

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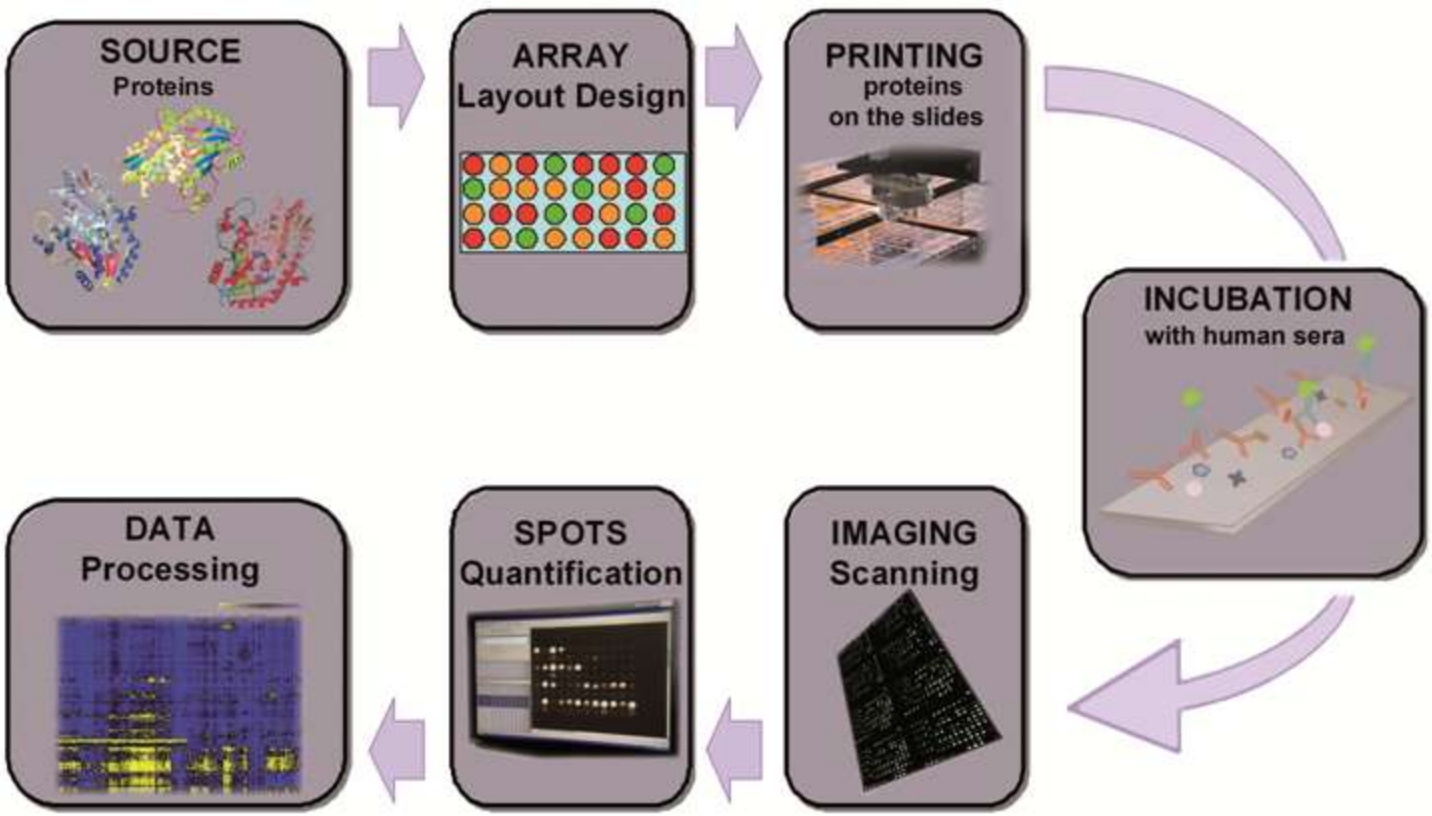
Advantages of Protein Microarray Technology

- High Throughput Screening of thousands of proteins simultaneously
- High Sensitivity (3-5 fold higher than other Ag-Ab detection techniques)
- Low amount (< 0.5 μl) of serum samples required



Bombaci M. *et al.*, 2010.

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Q: Is it possible to identify, with protein arrays, a new panel of auto-antigens that unequivocally marks autoimmune liver diseases ?

Assess sera from the following patients groups:

Discovery/Validation phase: 115 patients (sera assessed with protein arrays)
15 AI: Patients with autoimmune hepatitis (**AIH**; *n=8*) or primary biliary cirrhosis (**PBC** ; *n=7*)
48 HCV: Patients with HCV without auto-reactive antibodies
13 HCV+AI: Patients with HCV and Crioglobulinemia
38 HD: Healthy blood Donors

Assay phase: 80 patients (sera assessed with DELFIA assays, 96 well plate)
15 AI: Patients with autoimmune hepatitis (**AIH**; *n=13*) or primary biliary cirrhosis (**PBC** ; *n=7*)
13 HCV: Patients with chronic HCV without autoreactive antibodies
17 HCV+AI: Patients with chronic HCV and Crioglobulinemia
10 HBV: Patients with chronic HBV without auto-reactive antibodies
20 HD: Healthy blood Donors

AutoImmune Hepatitis (AIH)

An unresolving inflammation of the liver of unknown cause, characterized by:

- interface hepatitis and plasma cell infiltration
- hypergammaglobulinemia
- autoantibodies (ANA, SMA, LKM1, LC1, anti-actin, anti-ASGPR)

Pretty rare disease (prevalence 1-10/100.000), female sex predominant (~80%), pediatric and adult onset (peak incidence during the second and the fifth decade)

Genetic susceptibility: HLA *DRB1*0301*, HLA *DRB1*0401*

Responsive to immunosuppressive therapy

Primary Biliary Cirrhosis (PBC)

A chronic cholestatic granulomatous and destructive inflammatory disease of the intrahepatic bile ducts, characterized by:

- anti-mitochondrial antibodies - AMA - (~90%)
- intrahepatic cholestasis (increased Alk Ph, normal US scan)
- nonsuppurative cholangitis with destruction of interlobular and septal bile ducts

Varying prevalence (0.6-40/100.000, with a "polar-equatorial gradient"), female sex predominant (~90%), peak incidence during the fifth-sixth decade

Responsive to ursodeoxicholic acid

Validation Phase: Sera Stratification

Array Incubation with sera

Auto-Immunity	HCV +	HCV -	
+	HCV+AI (13)	AI (15)	28
-	HCV (48)	HD (39)	87
	61	54	**115**

HCV: Patients with HCV without autoreactive antibodies;
HCV+AI: Patients with HCV and Crioglobulinemia;
AI: Patients with autoimmune hepatitis (AIH; *n=8*) and primary biliary cirrhosis (PBC ; *n=7*)
HD: Healthy blood Donors

Strategy

Protein array

- > 1500 Human proteins
- >100 sera
- HD Vs Patients (HCV & AI)

DISCOVERY PHASE

Focused Protein array

- 24 Human proteins
- > 100 sera
- HD Vs HCV Vs AI

VALIDATION PHASE

DELFIA Assay

- 17 Human proteins
- > 50 sera
- HD Vs HCV Vs AI

ASSAY PHASE

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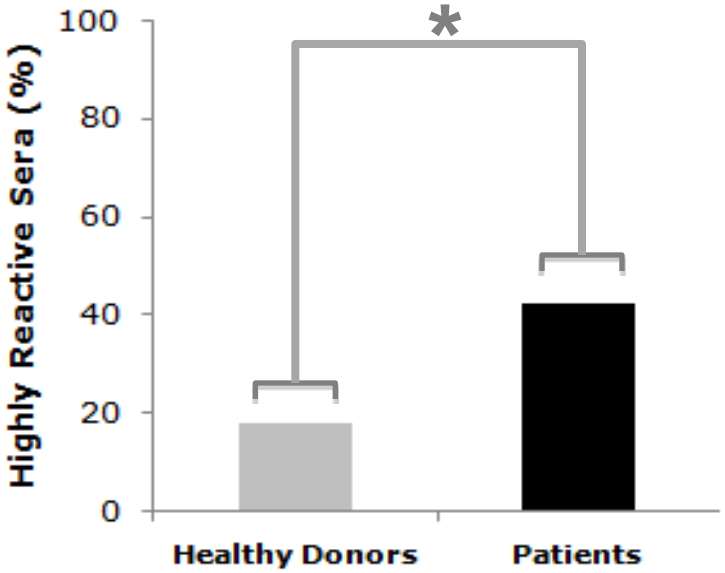
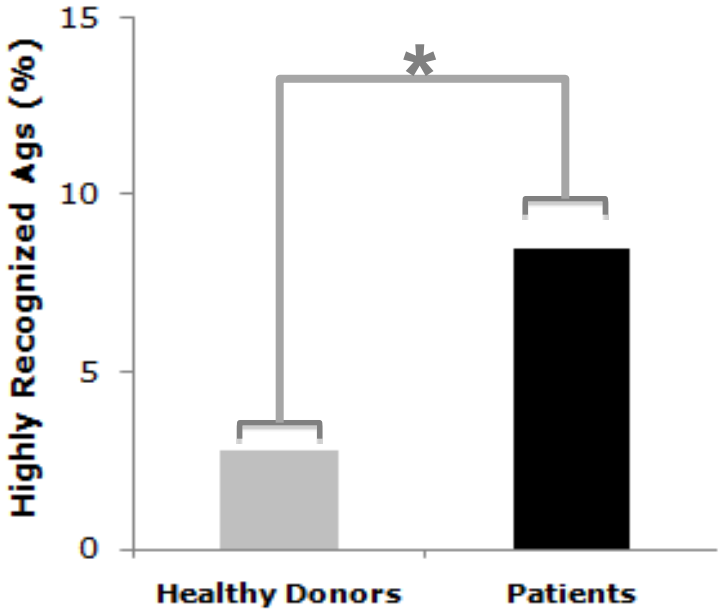
Sera from Patients (HCV, HCV+AI and AI all together) Show Autoreactivity higher than Healthy Donors



*Pval < 0,01

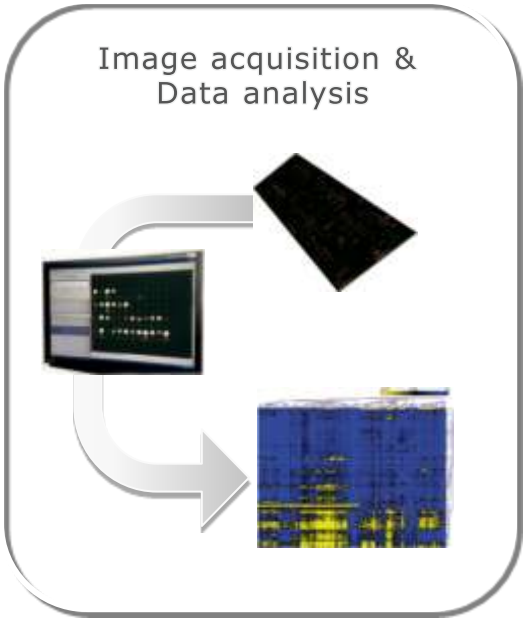


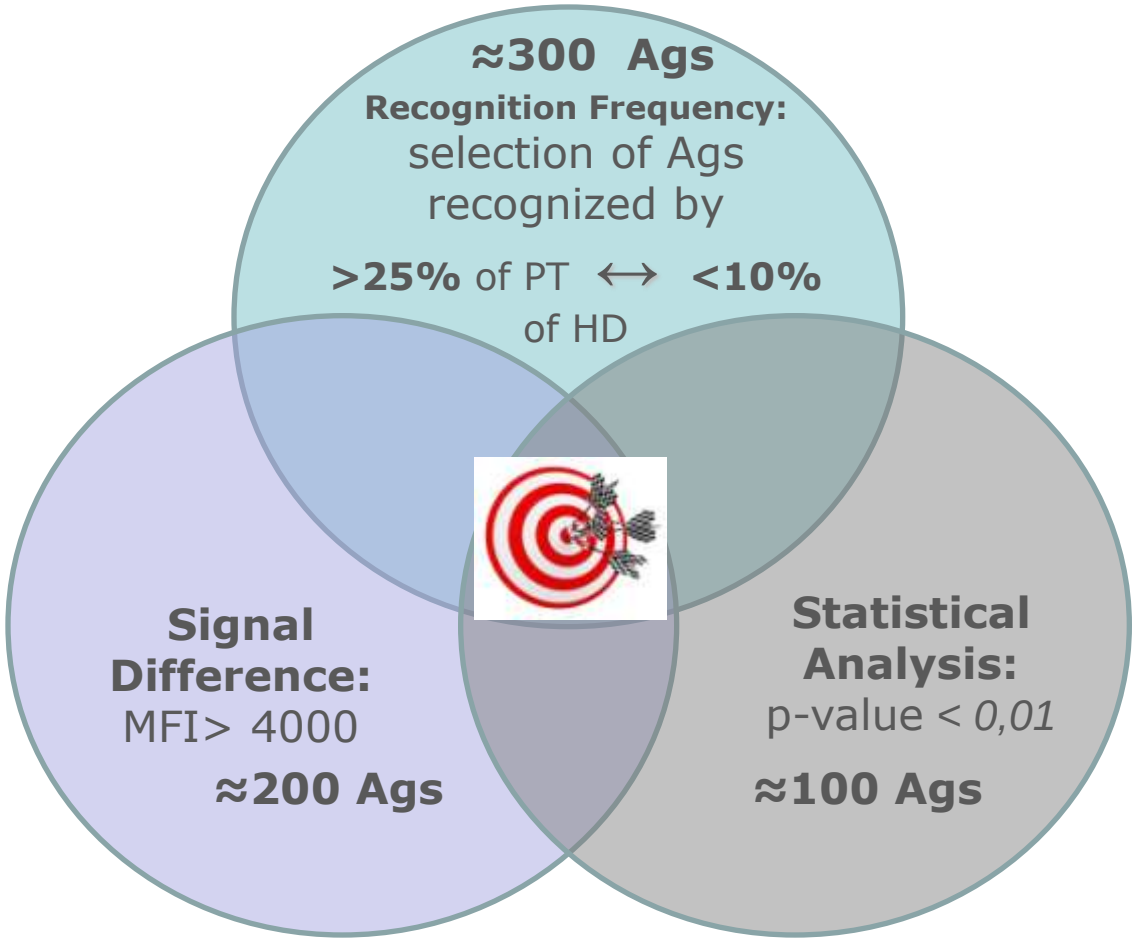
% of sera reacting with > 3% Antigens

% of Ags recognized by at least 15% of the sera

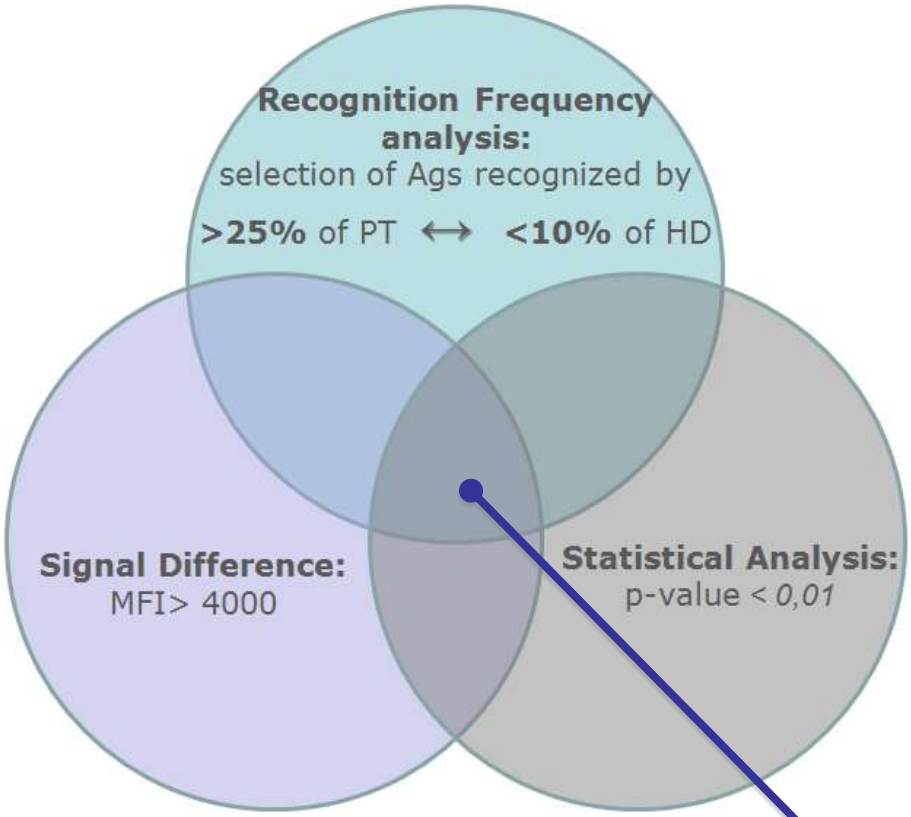
P.S.: A certain level of B lymphocytes autoreactivity is physiologic

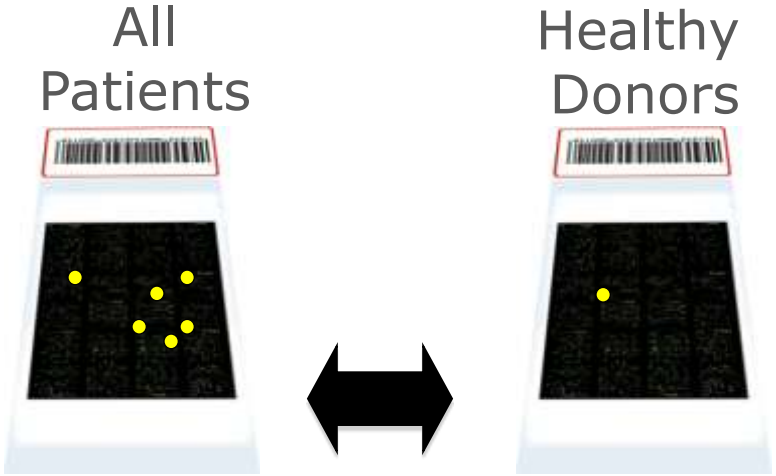
MULTIPLE CRITERIA TO SCORE POTENTIAL AUTOANTIGENS





A total of 24 distinct human proteins were selected as autoantigens for further analysis





Auto-antigens selection

24/1600

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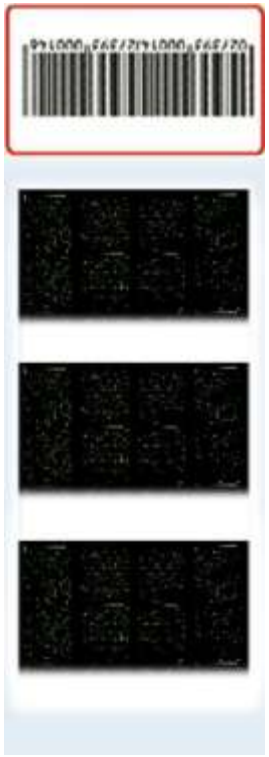
Focused Protein Microarray Validation

The 24 proteins identified as potential autoantigens (in All patients Vs HD) were used to print a focused protein microarray



Incubation with sera

← HD

← HCV +/- AI

←AI

Auto-Immunity	HCV +	HCV -	
+	HCV+AI (13)	AI (15)	28
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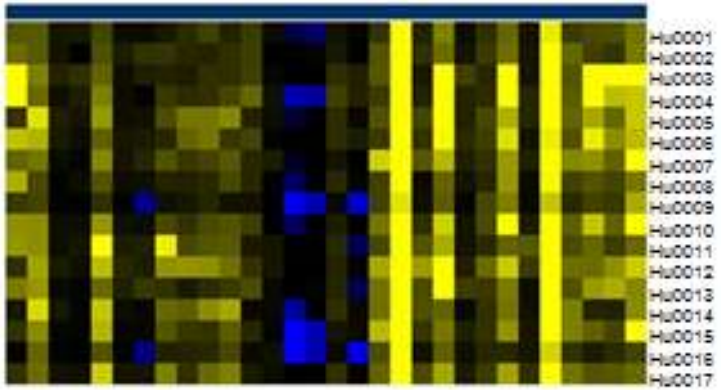
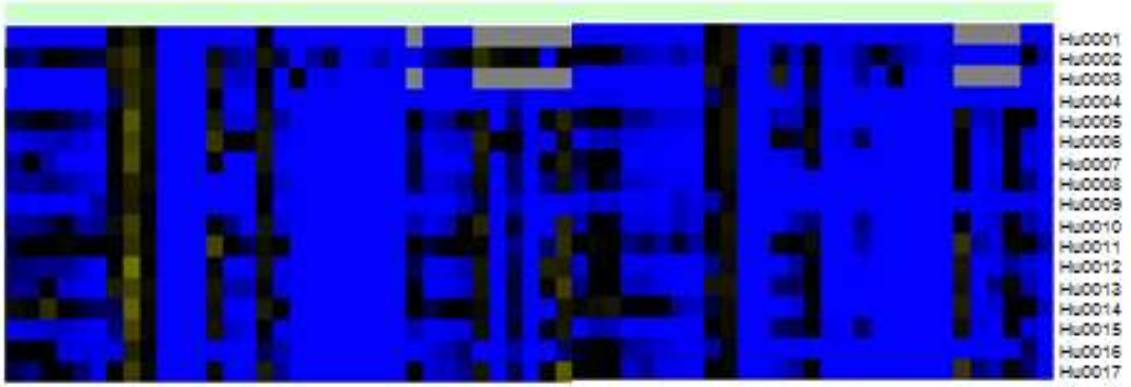
	61	54	**115**

Heat map representations of sero-reactivity against the 17 auto-antigens in the three sera groups

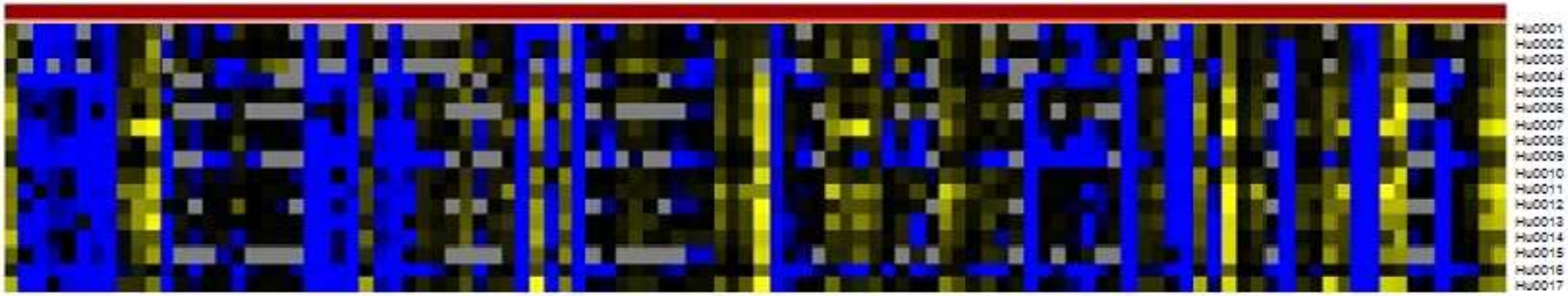
AI



HD



HCV +/- cryo

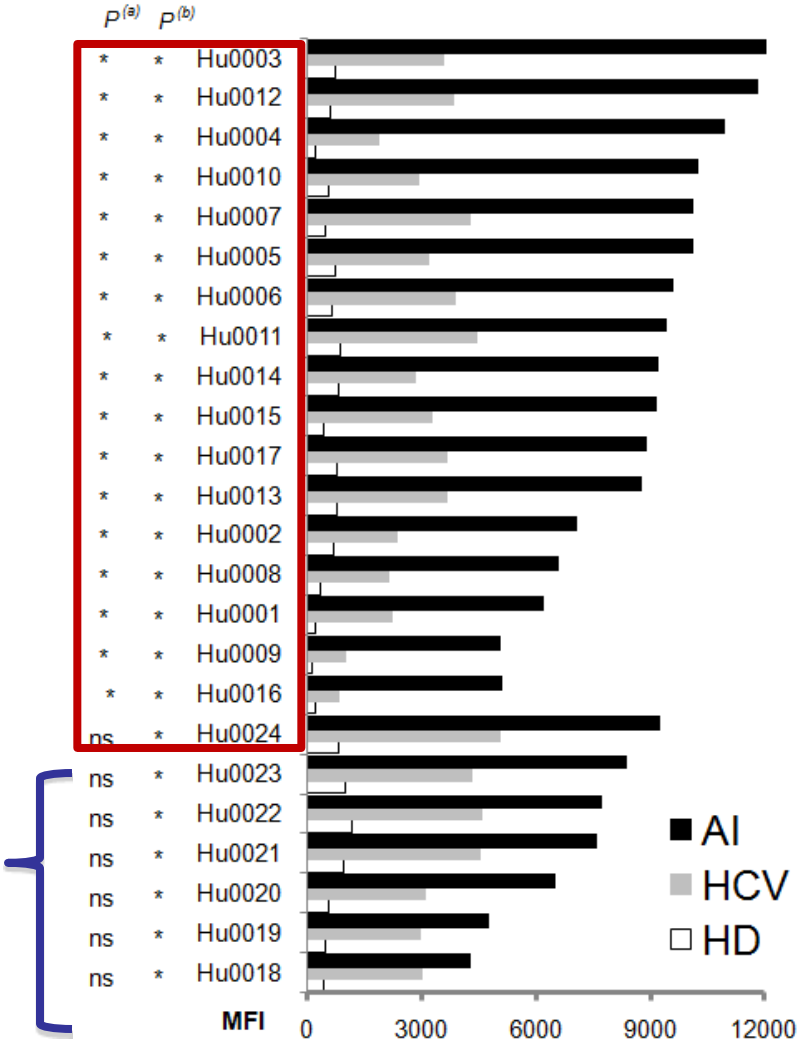


17/24 proteins display significant autoreactivity when comparing Autoimmune (AI) with HCV (+/- Cryo) patients or with HD

* p val<0.01
T-test

(a) AI *vs* HCV

(b) AI *vs* HD



VALIDATION OF THE 17 AUTO-ANTIGENS WITH NEW SERA PANELS OF PATIENTS FROM DIFFERENT CENTERS ASSESSED WITH A DIFFERENT ASSAY

DELFIA
(fluorescent ELISA)

Incubation with sera

Auto-Immunity	HCV +	HCV -	
+	HCV+AI (17)	AI (20)	37
-	HCV (13)	HD (20)	33
	30	40	**70**

Additional controls sera: *HBV (N = 10)*

Additional control Proteins:

ASGR-1
CYP2D6

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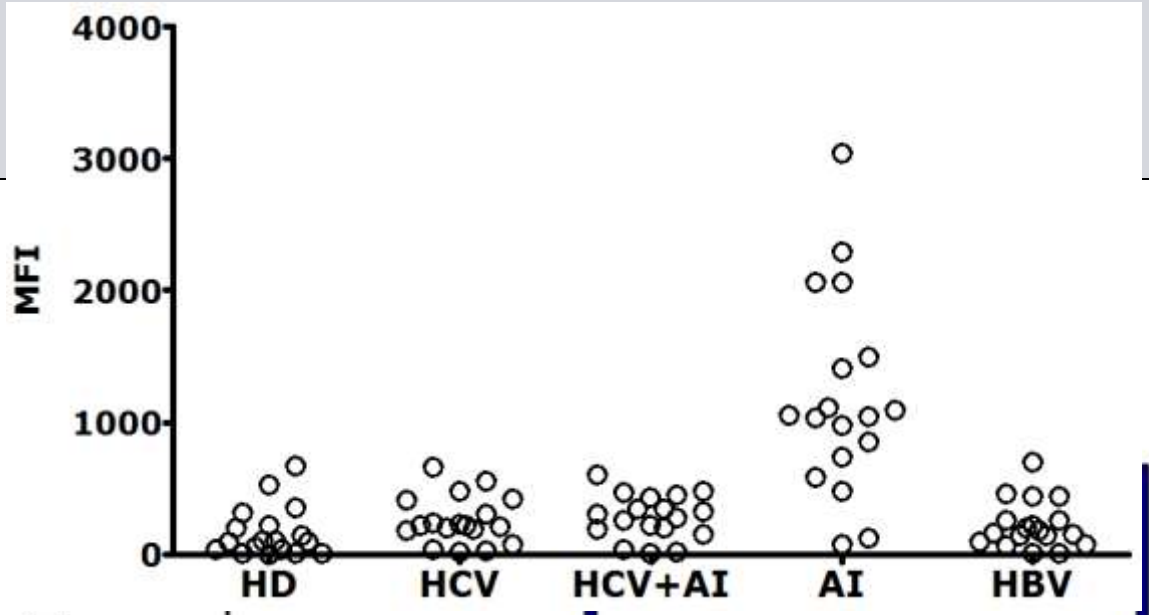
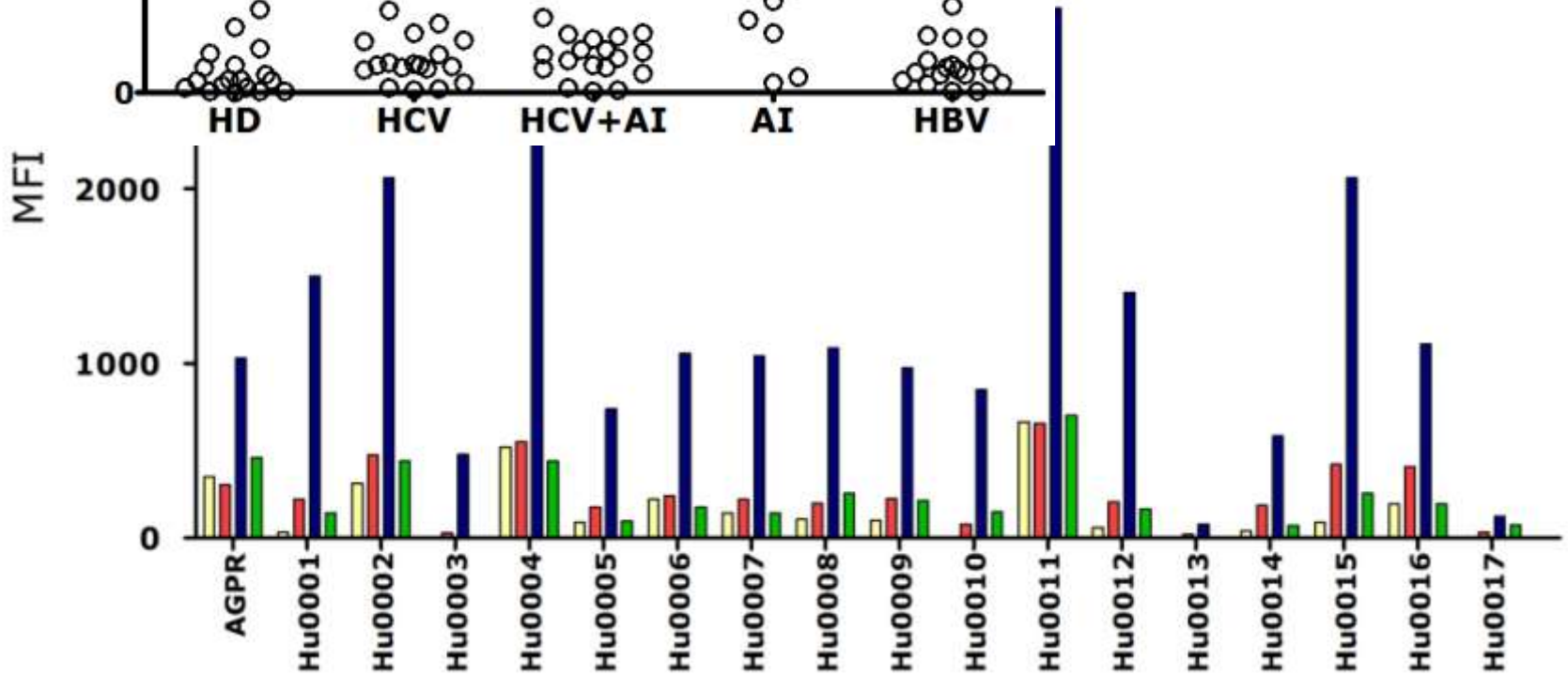
Assay Phase: DELFIA DATA

ASGPR: Asiagoglycoprotein





DELFIA: Statistical Analyses

Newman-Keuls Multiple Comparison Test	*P < 0.05*
HD vs AI	**Yes**
HD vs HCV+AI	No
HD vs HCV	No
HD vs HBV	No
HBV vs AI	**Yes**
HBV vs HCV+AI	No
HBV vs HCV	No
HCV vs AI	**Yes**
HCV vs HCV+AI	No
HCV+AI vs AI	**Yes**

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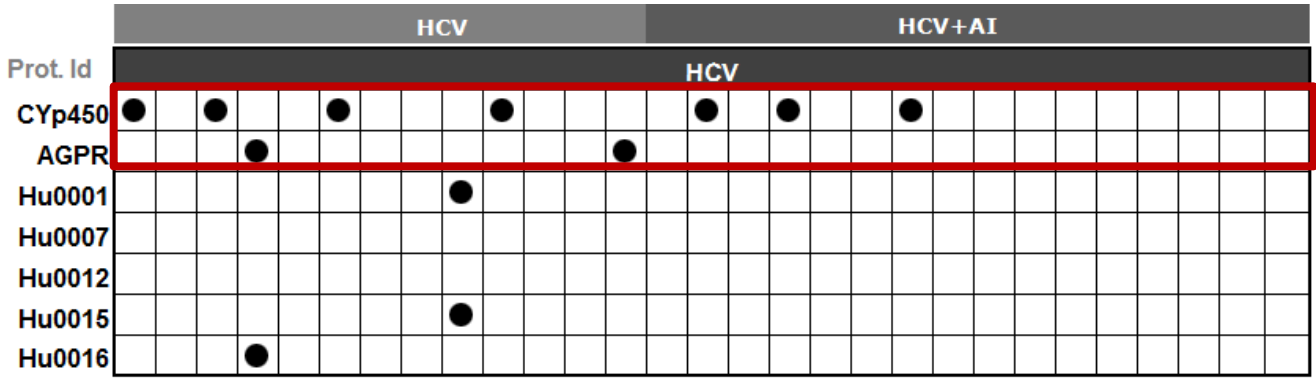
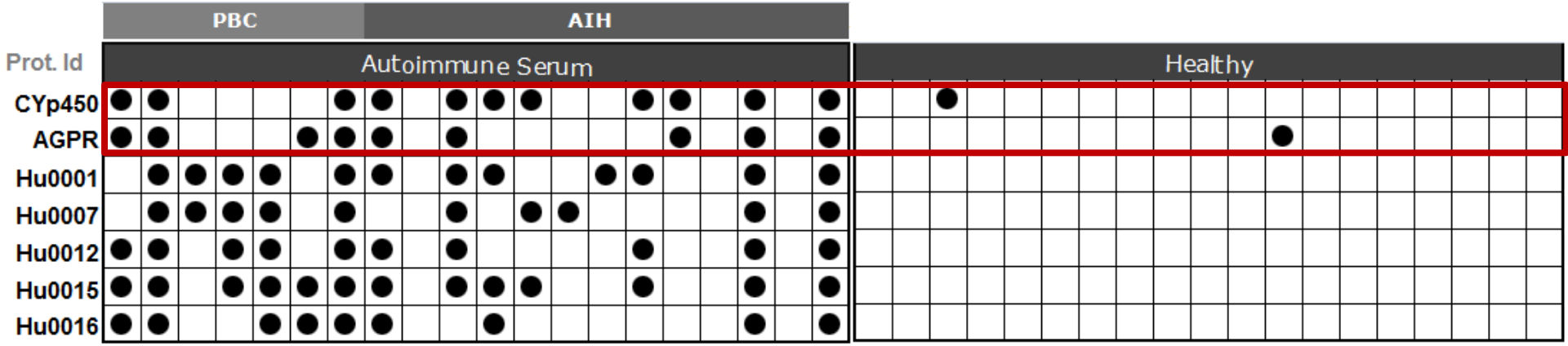
Five new candidates antigens showed sensitivity value for the autoimmune samples that ranged from **45% to 65%**

Prot. Id	sensitivity[a]	Specificity[b] (HD)	Specificity (HCV)	Specificity (HBV)	Accuracy $_{HD}$	*P values
Hu0001	60%	100%	97%	100%	79%	< 0.0001
Hu0007	50%	100%	100%	100%	74%	0.0004
Hu0012	50%	100%	100%	100%	74%	0.0004
Hu0015	65%	100%	97%	100%	82%	< 0.0001
Hu0016	45%	100%	97%	100%	72%	0.0012
CYP2D6	58%	94%	75%	89%	82%	< 0.0001
AGPR-1	45%	95%	93%	80%	69%	0.0084

(a)Sensitivity is defined as % of positive autoimmune patients.
(b)Specificity is defined as % of negative healthy donors.
*P values of autoimmune patients *versus* healthy calculated with $\chi 2$ test.

Is there room for a diagnostic application ?





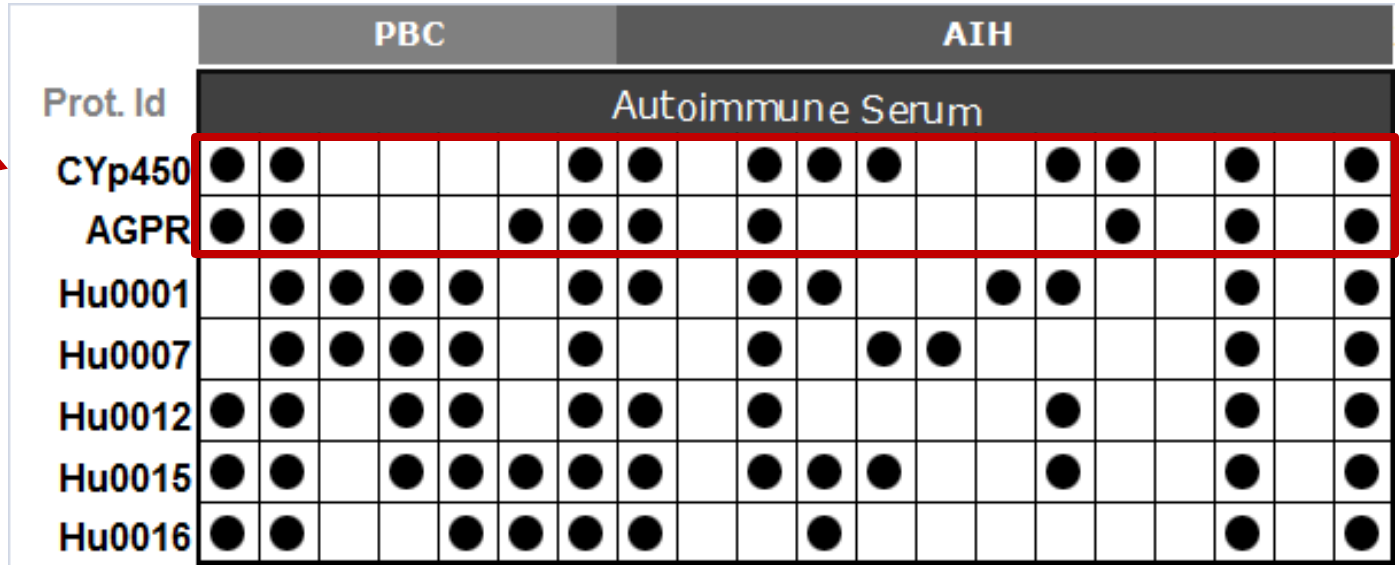
Threshold is $MFI_{HD}+3SD_{HD}$

SEROLOGY WITH COMBINATION OF THE FIVE AUTO-ANTIGENS IDENTIFIES **80% OF PATIENTS WITH AIH OR PBC**

With Comparable sensitivity of the five antigens for AIH and PBC

Known antigens

5 new antigens in combination as diagnostic markers



	sensitivity[a]	specificity[b]	accuracy	P values
*5-COMB	80%	100%	90%	<0.0001

Combo serology:

Little advantage in adding AGPR and CYP2D6 to the five new autoantigens

New five Auto-Ags

Known Auto-Ags

Known Auto-Ags

	sensitivity[a]	specificity[b]	accuracy	^P values
5-COMB	80%	100%	90%	<0.0001
AGPR+CYP2D6	63%	89%	74%	0.0019
5-COMB + AGPR+CYP2D6	85%	89%	87%	<0.0001

[a]Sensitivity is defined as % of positive autoimmune patients.
[b]Specificity is defined as % of negative healthy donors.
^P values of autoimmune patients *versus* healthy calculated with $\chi 2$ test.

CONCLUSIONS

❖ A panel of 17 (poorly known) potential novel autoantigens identified in patients with liver autoimmune diseases (AIH & PBC) by protein array

❖ 5 of the 17 novel autoantigens validated in patients with liver autoimmune diseases with individual sensitivities that ranged from 45% to 65% by DELFIA method. The combined assessment of the five autoantigens displays 80% sensitivity and 100% specificity

❖ Comparable sensitivity of the five antigens for AIH and PBC, so apparently these are markers of liver autoimmunity

❖ Superior Sensitivity and Specificity (Vs HD, HCV and HBV) compared to benchmarks (CYP2D6, NP & ASGPR)

❖ Protein Microarray technology has the potential to rapidly identify new biomarkers useful to improve the diagnosis and/or prognosis of autoimmune diseases, and at the same time to identify new pathogenetic proteins

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IN PROGRESS

Characterisation of the five new auto-antigens: distribution, structure and putative functions (possible pathogenetic role). Now, they are poorly known gene products, simply annotated as ORFs.

These new autoantigens could be readily applied for "real-life" use in diagnosis and/or prognosis of AIH and PBC

Confirm with Delfia assay the value of the 17 newly identified markers of liver autoimmunity in other liver diseases (e.g., PSC)

Protein microarray technology used to identify novel proteins that are target of auto-antibodies in other diseases of interest (not necessarily autoimmune)

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