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Validation of Prostate Cancer Biomarkers and Inflammation: A Proteomics Study

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BACKGROUND

- Despite the improvements in clinical and surgical practice, prostate cancer (PCa) remains one of the most widespread cancers in males.
- Cancer survival rates depend on the early detection of the disease: currently, PCa diagnosis is performed using digital rectal exploration (DRE), trans-rectal ultrasound guided prostate biopsy (TRUS), and by the measurement of serum PSA levels.
- The serum marker currently used for the diagnosis of PCa is the prostate-specific antigen (PSA), which is not particularly reliable, having a predictive value estimated at 25-35% in the range of 2.6 – 10 ng/mL.
- Benign conditions such as prostatitis and benign prostatic hyperplasia (BPH) and after biopsy can lead to an increase in PSA levels causing false positive results.

BACKGROUND

- Cancer and inflammation are closely linked, cancer patients show both local and systemic changes in inflammatory parameters.

- In some cancer types, inflammatory conditions are present before a malignant change occurs; otherwise, in different type of cancers, an oncogenic alteration generates an inflammatory microenvironment that induces the development of tumors.

- Differently from the previous publications, we considered the benign states vs the pathological ones focusing on the co-existence of inflammation, since research underlined a tight link between chronic inflammation and endothelial activation in both PCa and BPH.

AIM

- A more specific and reliable early diagnostic markers for prostate cancer (PCa) is highly desirable with the aim of improving accuracy for the detection, monitoring and distinction between benign conditions and PCa.

- In our study, serum protein profiles were investigated by proteomics analysis in order to identify distinctive protein profiles and possible biomarkers able to discriminate patients between PCa and benign prostatic hyperplasia (BPH), being inflammation the focus of our effort.

Methods

- Patients with clinical suspect of PCa undergoing trans-rectal ultrasound guided prostate biopsy (TRUS) were enrolled into the study.

- Biopsy specimens were examined in order to grade and classify the tumor, identify BPH and detect inflammation.

- Surface Enhanced Laser Desorption/Ionization-Time of Flight-Mass Spectrometry (SELDI-ToF-MS) and two-dimensional gel electrophoresis (2-DE) coupled with Liquid Chromatography-MS/MS (LC-MS/MS) were used to analyze immuno-depleted serum samples from patients with PCa and BPH.

Methods

- Serum samples were depleted of high-abundant proteins by immuno-chromatography and the depleted samples were analysed by SELDI-ToF-MS.

- This is a sensitive proteomic technique that analyses proteins on a large scale in a relatively short time and therefore it is of help for the preliminary screening of complex samples and for biomarkers search.

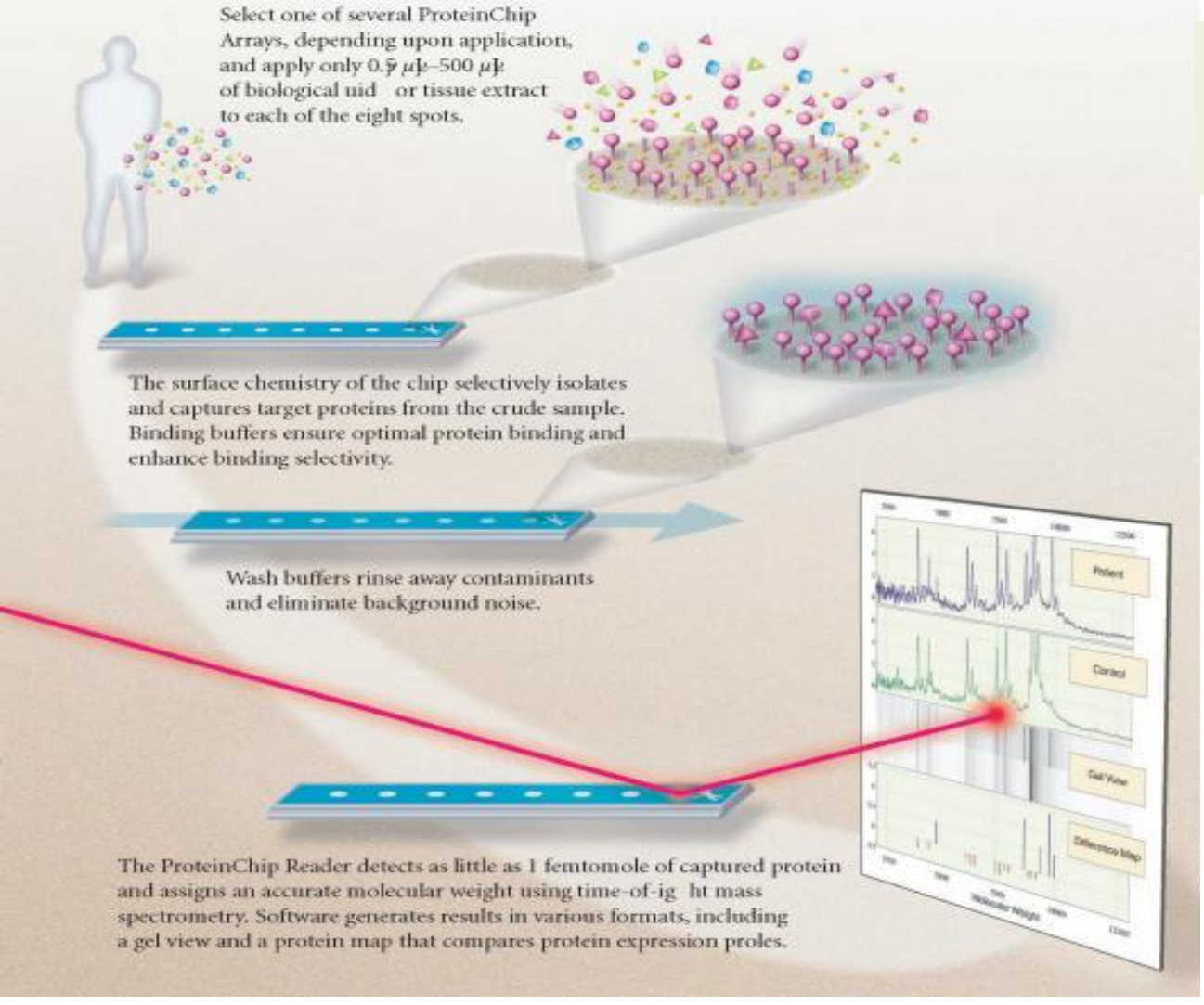
- Subsequently, samples were analysed by 2-DE coupled with LC-MS/MS, in order to precisely identify relevant proteins.

Clinical data of enrolled patients

	PCa (n = 31)	BPH (n = 30)
Median age (years)	67	68
PSA (range ng/mL)	0.20 – 25.00	0.80 – 34.36
Gleason Score		
G < 7	14	/
G ≥ 7	17	/
Tumor clinical stage		
T1	5	/
T2	20	/
T3	6	/
Inflammation		
Absence	10	11
Presence	21	19

SELDI

Surface-Enhanced Laser Desorption Ionization



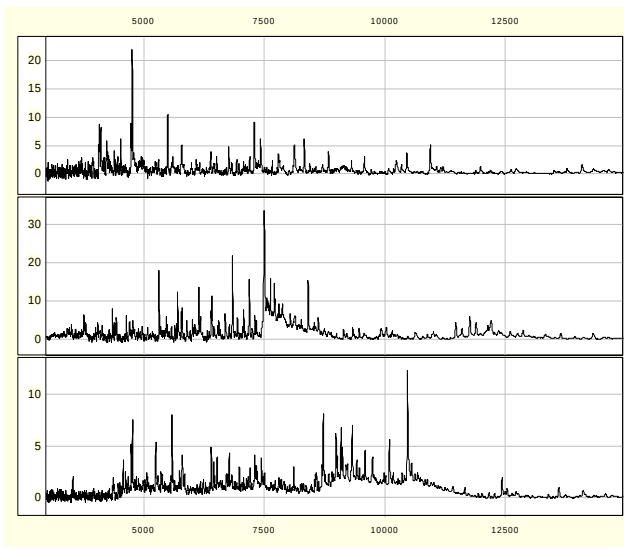
Components of SELDI Array Tecnology

. ProteinChip Arrays

. ProteinChip Reader

. Software

SELDI-TOF-MS and ProteinChip technology



Proteomic analysis results of PCa patients versus BPH patients

- The analysis was first carried out using SELDI-ToF-MS and the H50 ProteinChip surface, irrespective of the presence of inflammation in the total PCa (n=31) and BPH (n=30) patients.

- Under this condition, no differential expression of protein peaks was evident between PCa samples (n=31) and BPH (n=30).

Table 1. Differentially expressed peaks detected by SELDI-ToF-MS in the PCa (10 patients) *vs* BPH (11 patients) excluding patients with inflammation

Peak	m/z	PCa Intensity peak	BPH Intensity peak	*t*-test p-value
Increased				
1	2325	4.29	1.32	0.002
2	2348	3.97	1.18	0.006
3	2373	3.27	1.10	0.005
4	2581	1.34	0.33	0.002
5	3104	2.19	0.88	0.007
Decreased				
1	6624	17.63	24.54	0.037
2	6837	2.37	3.19	0.010
3	9352	1.84	2.38	0.033
4	9922	0.44	0.66	0.048
5	13775	1.21	1.67	0.049
6	14031	2.76	4.98	0.001
7	14106	1.67	2.66	0.005
8	14473	0.55	0.85	0.0003
9	14763	0.57	0.76	0.002
10	22668	0.06	0.10	0.003
11	28052	2.05	3.90	0.003
12	28242	1.42	2.33	0.011
13	29018	0.48	0.93	0.003
14	45350	0.78	1.23	0.002
15	56390	0.84	1.32	0.026

20 differentially expressed protein peaks were identified; in particular, **5 peaks increased** (m/z 2325, 2348, 2373, 2581, 3104) and **15 peaks decreased** (m/z 6624, 6837, 9352, 9922, 13775, 14031, 14106, 14473, 14763, 22668, 28052, 28242, 29018, 45350, 56390) **in PCa compared to BPH.**

Table 2. Differentially expressed peaks detected by SELDI-ToF-MS in the PCa patients with inflammation *vs* PCa without inflammation

Peak	m/z	PCa with inflammation Intensity peak	PCa without inflammation Intensity peak	*t*-test p-value
Increased				
1	*9352*	2.26	1.84	0.050
2	*9922*	0.64	0.45	0.040
3	21739	0.08	0.05	0.019
4	*29018*	0.75	0.49	0.043
Decreased				
1	*2325*	2.51	4.29	0.025
2	*2348*	2.28	3.97	0.044
3	*3104*	1.24	2.19	0.025
4	3215	1.49	1.96	0.024
5	17471	2.67	3.25	0.047

9 protein peaks differentially expressed were detected: **4 peaks increased** and **5 peaks decreased** in the presence of inflammation.

6 protein peaks (**italic**) coincided with **6 of the 20 peaks** differentially expressed in the comparison between **PCa and BPH in the absence of inflammation.**

Table 3. Differentially expressed peaks detected by SELDI-ToF-MS in the BPH patients with inflammation *vs* BPH without inflammation

Peak	m/z	BPH with inflammation Intensity peak	BPH without inflammation Intensity peak	*t*-test p-value
Increased				
1	[2325]	3.28	1.32	0.016
2	[2348]	3.34	1.84	0.013
3	[2373]	2.93	1.10	0.009
4	[2581]	1.06	0.33	0.007
5	[3104]	1.74	0.88	0.037
Decreased				
1	6433	9.65	12.61	0.009
2	[6624]	18.39	24.54	0.017
3	[6837]	2.51	3.19	0.018
4	[9352]	1.92	2.38	0.037
5	[14031]	3.49	4.98	0.012
6	[14106]	2.00	2.66	0.036
7	[14473]	0.68	0.85	0.033
8	[22668]	0.07	0.10	0.011
9	[28052]	2.82	3.90	0.033
10	[45350]	0.94	1.23	0.037

15 protein peaks differentially expressed were detected: **5 peaks increased** and **10 peaks decreased** in the presence of inflammation.

14 protein peaks (in square brackets) coincided with 14 of the 20 peaks differentially expressed in the comparison between PCa and BPH in the absence of inflammation.

Table 4. Comparison of peaks intensities differentially expressed detected by SELDI-ToF-MS in PCa *vs* BPH

Peak	m/z	Intensity peak			
		PCA (n = 31)		BPH (n = 30)	
		Inflammation		Inflammation	
		Absent (n=10)	**Present** (n=21)	**Absent** (n=11)	**Present** (n=19)
1	2325	4.30	2.51	1.32	3.28
2	2348	3.97	2.28	1.84	3.34
3	2373	3.28	1.95*	1.10	2.93
4	2581	1.34	0.85*	0.33	1.06
5	3104	2.20	1.24	0.88	1.74
6	6624	17.63	18.93*	24.54	18.39
7	6837	2.37	2.54*	3.19	2.51
8	9352	1.84	2.26	2.38	1.92
9	9922	0.44	0.64	0.66	0.52*
10	**13775**	1.21	1.58*	1.67	1.57*
11	14031	2.76	3.74*	4.98	3.49
12	14106	1.67	2.14*	2.66	2.00
13	14473	0.55	0.73*	0.85	0.68
14	**14763**	0.57	0.66*	0.76	0.71*
15	22668	0.06	0.08*	0.10	0.07
16	28052	2.05	3.06*	3.90	2.82
17	**28242**	1.42	1.88*	2.33	1.81*
18	29018	0.48	0.75	0.93	0.68*
19	45350	0.78	1.00*	1.23	0.94
20	**56390**	0.84	1.24*	1.32	1.09*

SELDI-ToF-MS analysis demonstrated that **only 4 peaks**, highlighted differentiate PCa from BPH, since their expression is not altered by the presence of inflammation. **The remaining 16 peaks** (also found differentially expressed in presence of inflammation) seem to be strongly related to inflammation, hence they can not be used as markers of PCa .

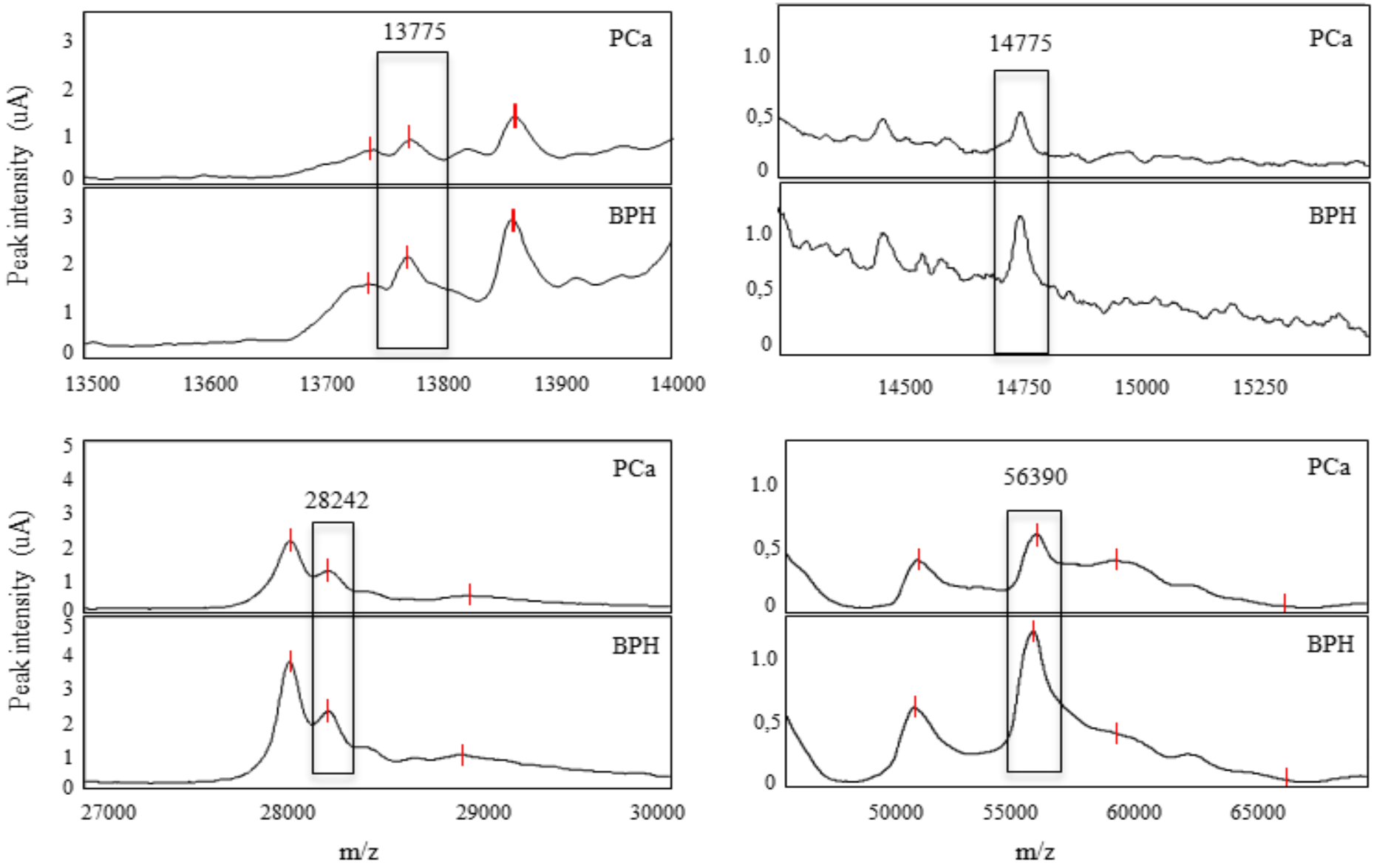


Figure 1. Representative spectra obtained by SELDI-ToF-MS analysis concerning the 4 statistically significant peaks detected with H50 ProteinChip Array.

Further studies to validate SELDI-ToF-MS analysis results

- Results obtained by SELDI-ToF-MS analysis, suggested that inflammation could be a confounding factor in the identification of protein profiles able to discriminate PCa and BPH.

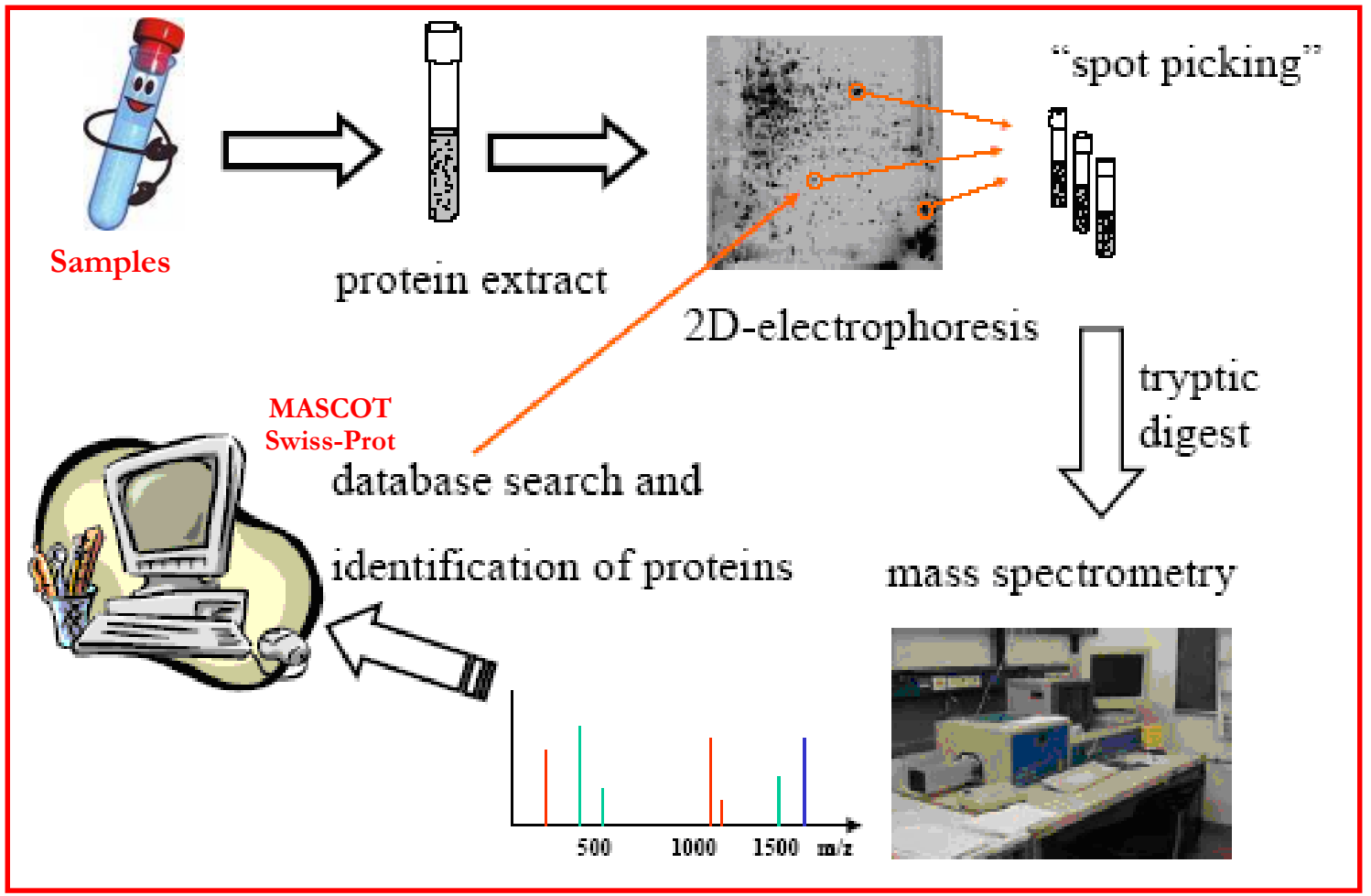
- Proteomic analysis was performed to verify this data, by 2-DE coupled with LC-MS/MS.

2-DE & MS

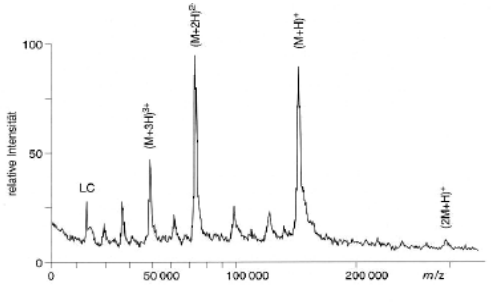
"Solubilization buffer"

Strip pH 3-10
Gel a gradiente
"Silver stain"

PDQuest analysis software



PROTEOMICS ANALYSIS



LC-Mass spectrum

ESI-Q-Tof-MS/MS (Agilent Technologies)

ESI = Electro Spray Ionization
Q = Quadrupole
ToF = Time of Flight
MS/MS = Tandem mass

Mass Lynx software

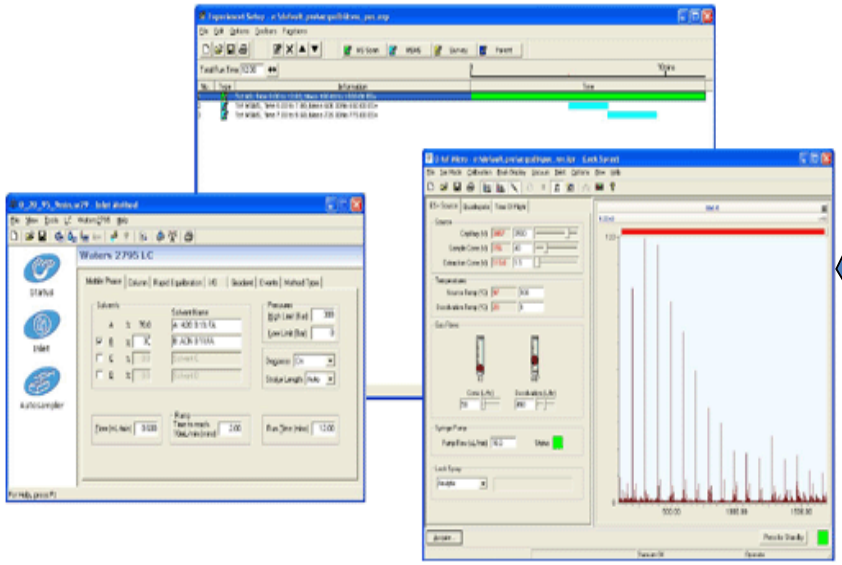


Figure 2.
Bi-dimensional proteome maps of serum samples from **PCa without (A)** and **with inflammation (B),** and **BPH in absence (C)** and **presence of inflammation (D).**

Inflammation-free PCa vs PCa with inflammation were first compared (**first comparison**); then, **BPH** was considered in **the absence or presence** of inflammation (**second comparison**), and finally the two conditions were compared with the exclusion of inflammation (**third comparison**).

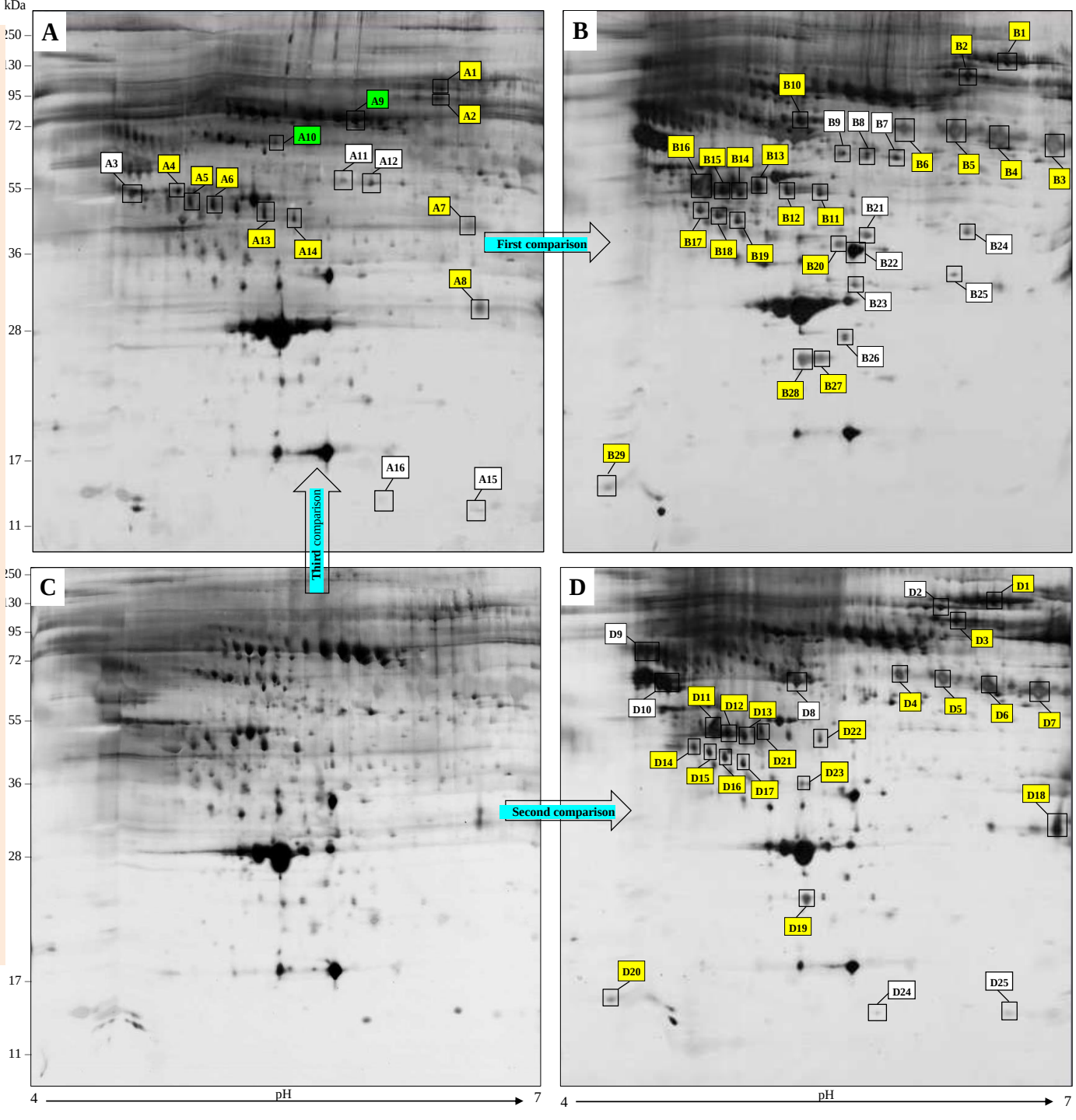


Figure 2.
Bi-dimensional proteome maps of serum samples from **PCa without (A) and with inflammation (B),** and **BPH in absence (C) and presence of inflammation (D).**

Yellow tags indicate the overlapped proteins detected in presence of inflammation in both **PCa (B)** and **BPH (D)** conditions.

Some of these proteins were also revealed in PCa in absence of inflammation (**A, third comparison**). **Green labels** represent proteins not previously identified in the first and second comparisons, namely in presence of inflammation.

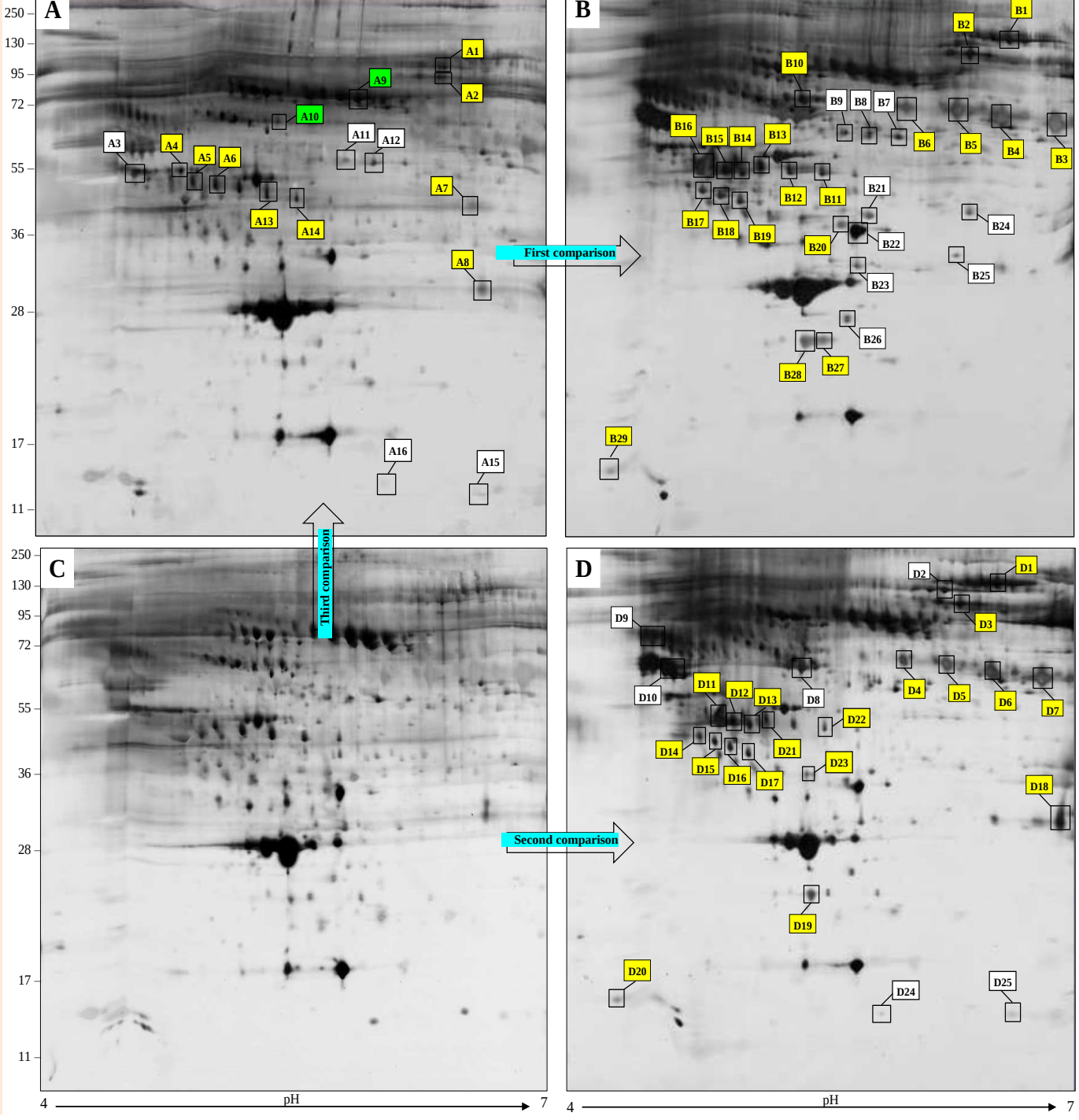


Table 5. Differentially expressed proteins in PCa without inflammation *vs* PCa with inflammation (UniProtKB database)

Spot n°.	Acc. n°.	Protein full name	Mass (Da)	Score	N° matchs/ signif. matchs	N° seq./ signif. seq.	Expression change
B1	P00751	***Complement factor B***	86847	445	212/60	33/19	↑
B2	P00734	***Prothrombin***	71475	455	75/39	15/7	↑
B3	P02749	***Beta-2-glycoprotein 1***	39584	458	81/42	17/8	↑
B4	P02749	***Beta-2-glycoprotein 1***	39584	288	69/32	14/12	↑
B5	P02749	***Beta-2-glycoprotein 1***	39584	122	55/17	11/8	↑
B6	P02749	***Beta-2-glycoprotein 1***	39584	35	28/4	6/3	↑
B7	P36955	**Pigment epithelium-derived factor**	46454	142	46/13	11/8	↑
B8	P36955	**Pigment epithelium-derived factor**	46454	65	42/12	13/5	↑
B9	P36955	**Pigment epithelium-derived factor**	46454	51	35/8	13/7	↑
B10	Q14624	***Inter-alpha-trypsin inhibitor heavy chain H4***	103521	375	152/47	30/13	↑
B11	P00738	***Haptoglobin***	45861	110	83/21	17/7	↑
B12	P00738	***Haptoglobin***	45861	236	99/28	18/8	↑
B13	P00738	***Haptoglobin***	45861	138	97/16	18/6	↑
B14	P25311	***Zinc-alpha-2-glycoprotein***	34465	104	24/9	10/4	↑
B15	P01024	***Complement C3 (fragment)***	188569	104	49/8	21/4	↑
B16	P01024	***Complement C3 (fragment)***	188569	2354	365/180	38/27	↑

In the presence of inflammation, **the first comparison** showed **29 spots** differentially expressed corresponding to **17** unique proteins (**Table 5 and Figure 2B**),

Protein names in italic: proteins found also in the comparison between **BPH without inflammation and BPH with inflammation** (**Table 6**).

Table 5. Differentially expressed proteins in PCa without inflammation *vs* PCa with inflammation (UniProtKB database) Continued

B17	P10909	***Clusterin***	53031	241	57/16	10/4	↑
B18	P10909	***Clusterin***	53031	138	31/12	7/3	↑
B19	P10909	***Clusterin***	53031	202	55/22	11/7	↑
B20	Q14624	***Inter-alpha-trypsin inhibitor heavy chain H4***	103521	41	13/3	2/2	↑
B21	P02649	**Apolipoprotein E**	36246	398	125/43	27/13	↑
B22	P02766	**Transthyretin**	15991	1086	165/71	16/11	↑
B23	P02743	**Serum amyloid P-component**	25485	304	43/20	8/6	↑
B24	O75636	**Ficolin-3**	33395	205	71/24	12/7	↑
B25	P36980	**Complement factor H-related protein 2**	31543	70	25/8	6/4	↑
B26	O95455	**Apolipoprotein M**	21582	49	22/3	7/3	↑
B27	P02753	***Retinol binding protein 4***	23337	230	42/15	7/5	↑
B28	P02753	***Retinol binding protein 4***	23337	1071	154/74	9/8	↑
B29	P02656	***Apolipoprotein C-III***	10846	84	10/4	2/4	↓

In the presence of inflammation, the first comparison showed **29 spots differentially expressed corresponding to 17 unique proteins (Table 5 and Figure 2B),**

Protein names in italic: proteins found also in the comparison between **BPH without inflammation and BPH with inflammation (Table 6).**

Table 6. Differentially expressed proteins in BPH without inflammation *vs* BPH with inflammation

Spot n°.	Acc. n°.	Protein full name	Mass (Da)	Score	N° matchs/ signif. matchs	N° seq./ signif. seq.	Expression change
D1	P00751	*Complement factor B*	86847	501	196/57	34/15	↑
D2	P06396	**Gelsolin**	86043	301	141/37	25/14	↑
D3	P00734	*Prothrombin*	71475	168	42/14	12/5	↑
D4	P02749	*Beta-2-glycoprotein 1*	39584	39	15/2	5/2	↑
D5	P02749	*Beta-2-glycoprotein 1*	39584	41	32/15	7/4	↑
D6	P02749	*Beta-2-glycoprotein 1*	39584	331	72/30	13/9	↑
D7	P02749	*Beta-2-glycoprotein 1*	39584	204	70/33	13/7	↑
D8	P02774	**Vitamin-D binding protein**	54526	1679	283/149	32/28	↑
D9	P01011	**Alpha-1-antichymotrypsin**	47792	324	54/24	11/8	↑
D10	P02765	**Alpha-2-HS-glycoprotein**	40098	273	96/38	11/9	↑
D11	P01024	*Complement C3 (fragment)*	188569	700	210/73	33/16	↑
D12	P01024	*Complement C3 (fragment)*	188569	1500	300/126	42/23	↑
D13	P25311	*Zinc-alpha-2-glycoprotein*	34465	66	15/6	6/3	↑
D14	P10909	*Clusterin*	53031	83	21/4	8/2	↑
D15	P10909	*Clusterin*	53031	245	42/14	8/4	↑
D16	P10909	*Clusterin*	53031	159	39/14	8/6	↑

The second comparison (BPH in the absence or presence of inflammation) showed **25 spots differentially expressed corresponding to 15 unique proteins (Table 6 and Figure 2D).**

Protein names in italic: proteins found also in the comparison between **PCa without inflammation and PCa with inflammation (Table 5).**

Table 6. Differentially expressed proteins in BPH without inflammation *vs* BPH with inflammation. Continued.

D17	P10909	*Clusterin*	53031	174	25/11	6/3	↑
D18	P01024	*Complement C3 fragment*	188569	834	119/57	16/10	↑
D19	P02753	*Retinol binding protein 4*	23337	585	134/64	9/9	↑
D20	P02656	*Apolipoprotein C-III*	10846	231	7/7	2/2	↑
D21	P00738	*Haptoglobin*	45861	524	102/43	15/10	↓
D22	P00738	*Haptoglobin*	45861	348	90/34	17/9	↓
D23	Q14624	*Inter-alpha-trypsin inhibitor heavy chain H4*	103521	222	30/19	7/6	↓
D24	P0DJI8	**Serum amyloid A-1 protein**	13581	269	26/13	10/5	↓
D25	P0DJI8	**Serum amyloid A-1 protein**	13581	360	29/16	10/5	↓

The second comparison (BPH in the absence or presence of inflammation) showed **25 spots differentially expressed corresponding to 15 unique proteins (Table 6 and Figure 2D).**

Protein names in italic: proteins found also in the comparison between **PCa without inflammation and PCa with inflammation (Table 5).**

Common proteins in both PCa and BPH in the presence of inflammation

Ten unique proteins, corresponding to 20 and 19 spots in the **first and second comparison** respectively, were found to be **common to both PCa and BPH in the presence of inflammation** (**yellow labels in Figure 2B and in Figure 2D, respectivel**y).

Seven of these proteins were found increased in both conditions:

1. **Complement factor B ↑**
2. **Prothrombin ↑**
3. **Beta-2-glycoprotein 1 ↑**
4. **Complement C3 fragment↑**
5. **Zinc-alpha-2-glycoprotein↑**
6. **Clusterin ↑**
7. **Retinol binding protein↑**

1. **Apolipoprotein CIII decreased in PCa ↓ and increased in BPH ↑**
2. **Inter-alpha-trypsin inhibitor heavy chain in PCa ↑ and decreased in BPH↓**
3. **3. Haptoglobin increased in PCa ↑ and decreased in BPH↓**

Third comparison

When the two conditions were compared **in the absence of inflammation** (**third comparison**), **9 unique** proteins differentially expressed, corresponding to 16 spots, were found in PCa *vs* BPH (**Figure 2A and Table 7**).

4 proteins increased

- **Prothrombin,**
- **Complement C4-B,**
- **fragments of Complement C3**
- **Zinc-alpha-2-glycoprotein**

5 were decreased

- **Hemopexin,**
- **Antithrombin-III,**
- **Pigment epithelium-derived factor,**
- **Haptoglobin**
- **Serum amyloid A-1 protein).**

Table 7. Proteins differentially expressed in the absence of inflammation in PCa *vs* BPH

Spot n°.	Acc. n°.	Protein full name	Mass (Da)	Score	N° matchs/ signif. matchs	N° seq./ signif. seq.	Expressi chang
A1	P00734	*Prothrombin*	71475	122	29/11	8/5	↑
A2	P00734	*Prothrombin*	71475	31	17/2	6/2	↑
A3	P0C0L5	Complement C4-B (fragment)	194170	2320	117/90	20/17	↑
A4	P01024	*Complement C3 (fragment)*	188569	1531	134/78	54/36	↑
A5	P01024	*Complement C3 (fragment)*	188569	209	27/12	15/6	↑
A6	P00738	*Zinc-alpha-2-glycoprotein*	45861	1037	95/61	20/17	↑
A7	P01024	*Complement C3 (fragment)*	188569	46	9/3	6/3	↑
A8	P01024	*Complement C3 (fragment)*	188569	69	24/8	7/5	↑
A9	P02790	**Hemopexin**	52385	1160	310/114	30/21	↓
A10	P01008	**Antithrombin-III**	53025	542	136/50	26/13	↓
A11	P36955	Pigment epithelium-derived factor	46454	166	33/15	10/8	↓
A12	P36955	Pigment epithelium-derived factor	46454	422	61/35	12/10	↓
A13	P00738	*Haptoglobin*	45861	437	52/31	8/8	↓
A14	P00738	*Haptoglobin*	45861	871	90/52	20/15	↓
A15	P0DJI8	Serum amyloid A-1 protein	13581	111	17/8	8/2	↓
A16	P0DJI8	Serum amyloid A-1 protein	13581	244	34/22	9/6	↓

- **Protein names in italic** were found in PCa and BPH in the presence of inflammation (inflammation linked proteins). This can be clearly explained since a certain degree of inflammation is always present in PCa.
- **Protein names in bold:** proteins not previously identified in presence of inflammation. Hemopexin is a heme-binding serum protein indicated to be of diagnostic value in hepatocellular carcinoma patients. Antithrombin-III is a member of the serpin family and functions as an inhibitor of thrombin and enzymes involved in clotting moreover, it has been demonstrated to possess a potent antiangiogenic activity and antitumor action.

Table 7. Proteins differentially expressed in the absence of inflammation in PCa *vs* BPH

Spot n°.	Acc. n°.	Protein full name	Mass (Da)	Score	N° matchs/ signif. matchs	N° seq./ signif. seq.	Expressi change
A1	P00734	*Prothrombin*	71475	122	29/11	8/5	↑
A2	P00734	*Prothrombin*	71475	31	17/2	6/2	↑
A3	P0C0L5	Complement C4-B (fragment)	194170	2320	117/90	20/17	↑
A4	P01024	*Complement C3 (fragment)*	188569	1531	134/78	54/36	↑
A5	P01024	*Complement C3 (fragment)*	188569	209	27/12	15/6	↑
A6	P00738	*Zinc-alpha-2-glycoprotein*	45861	1037	95/61	20/17	↑
A7	P01024	*Complement C3 (fragment)*	188569	46	9/3	6/3	↑
A8	P01024	*Complement C3 (fragment)*	188569	69	24/8	7/5	↑
A9	P02790	**Hemopexin**	52385	1160	310/114	30/21	↓
A10	P01008	**Antithrombin-III**	53025	542	136/50	26/13	↓
A11	P36955	Pigment epithelium-derived factor	46454	166	33/15	10/8	↓
A12	P36955	Pigment epithelium-derived factor	46454	422	61/35	12/10	↓
A13	P00738	*Haptoglobin*	45861	437	52/31	8/8	↓
A14	P00738	*Haptoglobin*	45861	871	90/52	20/15	↓
A15	P0DJI8	Serum amyloid A-1 protein	13581	111	17/8	8/2	↓
A16	P0DJI8	Serum amyloid A-1 protein	13581	244	34/22	9/6	↓

Our finding of a significantly lower expression of **Antithrombin-III** in PCa than the BPH indicates that the local anti-angiogenic activity of Antithrombin-III may be partially lost in advanced stages of PCa.

CONCLUSIONS

- The comparison of the protein profile between PCa and BPH by 2-DE LC-MS/MS showed several differentially expressed proteins, the majority of which could be related to the inflammatory process and not to the pathological condition.
- These results confirm those obtained by SELDI-ToF-MS analysis although it is not possible to perform a direct correspondence between the two techniques because the analytical conditions are different (pre-analytical sample treatment, detection of proteins in different mass range, use of selective chromatographic surface with the SELDI-ToF-MS technology).

CONCLUSIONS

- This study emphasizes the importance of inflammation to identify specific markers capable to differentiate PCa from BPH.

- Using two different proteomic techniques, we have clearly demonstrated that, **in the presence of inflammation**, the majority of the differentially expressed protein peaks detected by SELDI-ToF-MS and protein spots revealed by 2-DE LC/MS analysis cannot be considered discriminating markers of PCa.

CONCLUSIONS

- Therefore, the inflammatory process masks the detection of some proteins, which are the real differential targets between the malignant and benign condition.

- Our results indicate that inflammation might be a confounding parameter during the proteomic research of candidate biomarkers of PCa and some possible biomarker-candidate proteins are strongly influenced by the presence of inflammation, hence only a well-selected protein pattern should be considered for potential marker of PCa.

Acknowledgements

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