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**Specific effects exerted by B-lymphoproliferative diseases  
on peripheral T-lymphocytes protein expression**

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3 **Specific effects exerted by B-lymphoproliferative diseases on peripheral T-**  
4 **lymphocytes protein expression**  
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43 **Running title:** Peripheral T-lymphocyte as biosensor  
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## Summary

A proteomic approach was applied to study the protein expression profile of peripheral T-cells derived from patients at the onset of different B-lymphoproliferative diseases, since a rising interest in specific actions played by T-cells in such pathologies has emerged. Decreased levels of profilin-1 and cofilin-1 and increased levels of coronin1A and prohibitin were found in patients, compared with healthy controls. The protein-protein interaction network of these proteins was studied using a web-based bioinformatics tool, highlighting the actin cytoskeleton regulation as the main biological process involved in peripheral T-cells of such patients. Unsupervised cluster analysis of protein expression data shows that the recorded alteration of T-cell proteome was specifically induced by B-cells pathologies.

**Key words:** Proteomics, biosensor, T-cell, B-lymphoproliferative diseases

## Introduction

B-lymphoproliferative diseases represent a wide range of neoplastic syndromes: Hodgkin's lymphoma, aggressive and indolent lymphoma, chronic lymphocytic leukaemia, plasma cell dyscrasias. The need of stronger and reliable biomarkers for accurate diagnosis of such diverse pathologies increases together with the need of new tools for therapeutic monitoring and evaluation of residual disease. Transcriptomics and proteomics have arisen as promising tools to discover potential biomarkers and can considerably contribute to molecular characterization of different functional states of immune cells. Since various studies describe alteration of T-cells in patients affected by B-cells malignancies, suggesting a prognostic value of their counting and immunophenotyping (Heier *et al*, 2008; Hasselblom *et al*, 2007; Nadal *et al*, 2007; Glas *et al*, 2007), we studied the proteome profile of peripheral T-cells in patients affected by B-lymphoproliferative diseases at the onset. *The ex vivo study of circulating T cells presents the major advantage of analyzing a system with the minimal modification compared to the living condition. Though, proteomic profiling of blood cells through two-dimensional electrophoresis (2DE) needs to match the requirement of a suitable amount of protein with the ethical guidelines about blood draw for biomedical research. Choosing to study the total T lymphocytes population allowed us to obtain enough cells with a reasonable blood volume and to isolate untouched cell through negative selection. In these cells, statistical analysis revealed a set of proteins differentially expressed in diseased subjects compared to healthy subjects. The interaction network among these proteins was analyzed to detect molecular partners possibly involved in T cell functioning. Proteome profiles of T cells in different physiological or pathological contexts was also evaluated by unsupervised hierarchical clustering.*

## Materials and methods

### *Subjects*

Twelve patients (4 females and 8 males, mean age  $59\pm 15$ ) with various lymphoproliferative malignancies were selected for this study by the Haematology division of the Azienda Ospedaliera Sant'Andrea, Sapienza University of Rome, Italy. Among these, 4 were affected by diffuse-large-B-cell-lymphoma (DLBCL); 3 by follicular lymphoma (FL); 3 by classical Hodgkin's lymphoma (CHL); 2 by chronic lymphocytic leukaemia (CLL). The study was approved by the institutional Ethics Committee of the Hospital, and the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000. Blood harvest was drawn at disease onset before starting chemotherapy and steroid administration and after patients provided written informed consent. The control group was composed by 10 sex- and age- matched healthy volunteers.

### *T cells isolation*

About 25 ml of EDTA-anticoagulated peripheral blood were collected and immediately processed for purification of peripheral blood mononuclear cells (PBMC) by density gradient centrifugation over Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway), according to the manufacturer's protocol. T cells were then isolated by negative selection (mean purity  $\geq 95\%$ ) using a magnetic beads system (Pan T Cell Isolation Kit II, human, Miltenyi Biotec, Auburn, CA), according to manufacturer's instructions. Briefly, non-T cells are indirectly labelled with a cocktail of biotin-conjugated monoclonal antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and **glycophorin A**, as primary labelling reagent, and anti-biotin monoclonal antibodies conjugated to magnetic microbeads, as secondary labelling reagent. The magnetically labelled non-T cells are depleted by retaining them on a magnetisable separation column which is placed in a strong permanent magnet, while the unlabeled T-cells pass

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3 through the column. The PBMC and the isolated T-cells samples were counted on an Advia  
4  
5 Haematology Analyzer (Bayer Diagnostics, **Leverkusen, GE**).

### 6 7 8 9 *Protein separation*

10 Purified T cells were lysed in 1 ml Trizol/1 x 10<sup>6</sup> cells (Invitrogen, Carlsbad, CA, USA).  
11  
12 Protein fraction was precipitated with 80% (v/v) cold acetone followed by centrifugation  
13  
14 (20,000 g for 10 min at 4°C). Pellets were dissolved in the rehydration solution (8 M urea/4%  
15  
16 CHAPS) and protein content was determined by the Bradford assay. Equal amounts of  
17  
18 proteins derived from the healthy subjects were mixed to make a reference control pool.  
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### 26 *Two-dimensional (2D) gel electrophoresis*

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28 Isoelectric focusing (IEF) was performed on an Ettan IPG-Phor system (**GE Healthcare**,  
29  
30 Uppsala, Sweden), at 16 °C and under a current limit of 50 µA/strip. Sixty µg of proteins in a  
31  
32 final volume of 350 µL of lysis buffer, containing 65 mM dithioerythritol (DTE), 0.5% (v/v)  
33  
34 ampholine pH 3-10 NL and a trace of bromophenol blue, were loaded onto 18 cm pH 3-10  
35  
36 NL Immobiline DryStrip (IPG strip, **GE Healthcare**, Uppsala, Sweden). The strip re-hydration  
37  
38 step was carried out at 16 °C at a constant voltage of 30V for 4 h and additional 50V for 5 h.  
39  
40 Damp electrode pads were positioned under the re-hydrated strip over the electrodes. The IEF  
41  
42 step was performed using the following parameters: 400 V, 2h; 800 V, 1h; 1200 V, 2h; 3000  
43  
44 V, 3h; 8000 V, 6-8 h, until the total voltage reached 70 kVh. Immediately after the IEF run,  
45  
46 IPG strips were equilibrated for 12 min in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50  
47  
48 mM Tris-HCl, pH 6.8, 2% (w/v) DTE, and for 5 min in a similar solution, with a trace of  
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50 bromophenol blue, in which 2% DTE was replaced with 2.5% (w/v) iodoacetamide. The  
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52 second dimension electrophoresis was run on 9-16% linear gradient polyacrylamide gels (18  
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54 cm x 20 cm x 1.5 mm) at 40 mA/gel constant current at 10 °C for approximately 5 h until the  
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56 dye front reached the bottom of the gel. Gels were stained with colloidal Coomassie blue and  
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3 acquired on a BioRad GS-800 Calibrated Imaging Densitometer (Bio-Rad, Veenendaal, The  
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5 Netherlands).  
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#### 10 *Differential analysis of gel images and statistics*

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12 The sample from each patient was run in triplicate, while proteins from the 10 control  
13 subjects were pooled and run in six technical replicates. Image analysis was performed using  
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15 the Bio-Rad PDQuest software, version 7.1.0. Spot quantity was normalized to the total  
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17 density in valid spots. Only valid spots, defined as spots with a CV  $\leq$  5% inside each technical  
18  
19 replicate group, were included in the analysis. For each spot, the mean quantity  $\pm$  SD obtained  
20  
21 in the pool of healthy subjects was compared with the mean quantity  $\pm$  SD obtained averaging  
22  
23 spot quantities in all patients. A 2-fold change and a t-test  $p$ -value  $<$  0.05 were applied to  
24  
25 select protein spots differentially expressed among healthy subjects and patients.  
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#### 34 *Protein identification by MALDI-ToF mass spectrometry*

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36 Protein spots of interest were manually excised from the gel, washed with high-purity  
37  
38 water and 50% acetonitrile/water and dehydrated with 100% acetonitrile. The gel slices were  
39  
40 swollen at room temperature in 20  $\mu$ l of 40 mM  $\text{NH}_4\text{HCO}_3$ /10% acetonitrile containing 25  
41  
42 ng/ $\mu$ l trypsin (Trypsin Gold, mass spectrometry grade, Promega). After 1 h, 50  $\mu$ l of 40 mM  
43  
44  $\text{NH}_4\text{HCO}_3$ /10% acetonitrile were added and digestion proceeded overnight at 37 °C. The  
45  
46 generated peptides were extracted with 50% acetonitrile/5% trifluoroacetic acid (TFA, 2  
47  
48 steps, 20 min at room temperature each), dried by vacuum centrifugation, suspended in 0.1%  
49  
50 TFA, passed through micro ZipTip C18 pipette tips (Millipore, Bedford, MA, U.S.A.) and  
51  
52 directly eluted with the spectrometer matrix solution (10 mg/ml  $\alpha$ -cyano-4-hydroxycinnamic  
53  
54 acid in 50% acetonitrile/1% TFA). Mass spectra of the tryptic peptides were obtained using a  
55  
56 Voyager-DE MALDI-ToF mass spectrometer (Applied Biosystems, Foster City, CA, USA).  
57  
58 Peptide mass fingerprinting database searching was performed using MASCOT searching  
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60

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3 engine (<http://www.matrixscience.com>) in the NCBI Inr/Swiss-Prot databases. Parameters were  
4  
5 set to allow one missed cleavage per peptide, a mass tolerance of 0.5 Da, and considering  
6  
7 carbamido-methylation of cysteines as a fixed modification and oxidation of methionines as a  
8  
9 variable modification. The criteria used to accept identifications included the extent of  
10  
11 sequence coverage, number of matched peptides and probabilistic score, as detailed in Table  
12  
13  
14  
15 1.

### 16 17 18 19 20 *Interaction network analysis*

21  
22 Protein-protein interaction analysis was performed using STRING (Search Tool for the  
23  
24 Retrieval of Interacting Genes/Proteins) software version 8.1, which allows selection of  
25  
26 different prediction methods (Jensen *et al*, 2009). To get a more rigorous prediction, the  
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28 database was queried using only experimental data as prediction method and limiting the  
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30 search to no more than 100 molecular interactors, with a required minimum confidence score  
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32 of 0.4 (medium level).  
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### 39 *Hierarchical clustering of proteomic data*

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41 Unsupervised analysis of proteomic data was performed by the GeneSpring GX 7.3  
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43 expression analysis software (Agilent Technologies, **Santa Clara, CA, USA**). For each  
44  
45 analyzed patient, the average quantization of spot values inside its replicate group, reported as  
46  
47 the ratio to the average of the control replicate group, was imported as expression data.  
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49 Analysis included T-cell proteome profiles of: 12 B-lymphoproliferative disorders, 10  
50  
51 polycystic ovary syndrome (PCO), 5 congenital adrenal hyperplasia (CAH), 2 monoclonal  
52  
53 gammopathy of undetermined significance (MGUS), healthy subjects (single sample  
54  
55 representative of ten pooled control subjects, as described above). Clustering was performed  
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60 according to an average linkage algorithm using the following parameters: similarity measure

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3 by Pearson correlation, similar branches merged with a separation ratio of 1, minimum  
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5 distance 0,001.  
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## 10 **Results**

### 11 *Differential analysis of protein expression*

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13 The experimental design is depicted in Fig. 1. T-cells were isolated from peripheral blood  
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15 of 10 healthy subjects (control) and 12 patients affected by B-cells lymphoproliferative  
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17 disorders (comprising Hodgkin's lymphoma, diffuse large B-cell lymphoma, chronic  
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19 lymphocytic leukaemia and follicular lymphoma). Protein samples from control subjects were  
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21 pooled and analyzed by 2D-electrophoresis, which allows separation and semi-quantitative  
22  
23 detection of different protein species. Previous studies found no evidence of systematic bias  
24  
25 triggered by sample pooling for 2D-electrophoresis and showed that it is useful in reducing  
26  
27 the variance and increasing the number of technical replicates, which is often limited by the  
28  
29 low amount of available biological samples (Weinkauff *et al*, 2006; Karp *et al*, 2009). Also,  
30  
31 data acquired in our laboratory (data not shown) showed no significant sex- or age-related  
32  
33 differences among 2D maps of peripheral T-cells from healthy people. Thus, a "healthy" map  
34  
35 of peripheral T-lymphocytes was obtained (Fig. 2).  
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43  
44 Comparison of this reference map with the maps obtained from protein samples of each  
45  
46 patient affected by B-lymphoproliferative disorders allowed to detect in all patients three  
47  
48 spots with decreased and two spots with increased expression levels. These spots were  
49  
50 identified by mass spectral analysis as: profilin-1 (two spots with different isoelectric point)  
51  
52 and cofilin-1, in the first case, and coronin1A and prohibitin, in the second (Table 1 and Fig.  
53  
54 2). Both profilin-1 and cofilin-1 appear in the 2DE maps of T-cells as two spots (Fig. 2),  
55  
56 where the more acidic ones can be attributed to protein phosphorylation (Navakauskiene *et al*,  
57  
58 2004; Lee *et al*, 2006). However, whereas both isoforms of profilin-1 display decreased levels  
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3 in T-cells of the patients, only the phosphorylated form of cofilin-1 is decreased, while the  
4  
5 level of the unphosphorylated isoform is similar to the control.  
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#### 10 *Protein interaction network*

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12 To get insight into the biological significance of differential expression of the proteins  
13 identified by proteomic analysis, we performed a search of their molecular partners using the  
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15 STRING 8.1 software (<http://string-db.org/>). STRING is a database which integrates protein-  
16  
17 protein interaction data from different sources and generates a list of known and/or predicted  
18  
19 partners (interaction network) of the input proteins, including both physical and functional  
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21 associations. Query of the database using only experimental data as prediction method  
22  
23 produced the interaction network shown in Fig 3. A list of the proteins involved in this  
24  
25 network is reported in the **Supplementary Table 1**.  
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#### 34 *Hierarchical clustering*

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36 Qualitative assessment of the separation power of proteome profiling can be achieved by  
37  
38 unsupervised analysis to organize expression data into similar groups. Hierarchical clustering  
39  
40 was applied to compare the protein profiles of peripheral T-cells derived from patients  
41  
42 affected by B-lymphoproliferative diseases, healthy controls and subjects affected by non-  
43  
44 related pathologies: mainly polycystic ovary syndrome (PCO) and congenital adrenal  
45  
46 hyperplasia (CAH), the profiles of which were acquired in our proteomic database during a  
47  
48 previous study (Borro *et al*, 2007). Clustering of samples by similarity generates the  
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50 dendrogram shown in Fig. 4. The algorithm automatically created five principal branches,  
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52 each clusters composed by all samples associated with the same condition.  
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## Discussion

Proteome analysis allows evaluation of the modifications of the protein expression profile of a cell type that can be determined by peculiar conditions. The proteins identified as differentially expressed in healthy or pathological states might represent the main players as well as downstream and/or minor actors in the biological pathways involved in a given condition: hence, the functional link between expression levels of a protein and the observed biological effects often appears not immediately evident. Protein expression data need various levels of interpretation to achieve a comprehensive explanation and to produce information relevant to clinical research and practice.

Results of proteome profiling of circulating T-lymphocytes in patients affected by B-cells lymphoproliferative disorders are discussed below according to different approaches to data interpretation: i) review of known function of the differentially expressed proteins in relation with T-cell functions; ii) analysis of protein interaction networks; iii) unsupervised hierarchical clustering of expression data.

### *Differentially expressed proteins*

Cofilin-1 belongs to a family of ubiquitous actin-binding proteins (Maciver *et al*, 2002) which is negatively regulated by phosphorylation of a serine residue (Moriyama *et al*, 1996). A decreased expression of the phosphorylated form of cofilin-1 was found, while the level of the unphosphorylated form is similar in patients and controls. In resting human peripheral blood T-lymphocytes, cofilin is mainly phosphorylated and thus inactive, while following co-stimulation via accessory receptors (e.g., CD2 or CD28) undergoes activation through dephosphorylation (Lee *et al*, 2000; Samstag *et al*, 1991).

Profilin-1 is an abundant protein that binds monomeric actin in a 1:1 complex and catalyzes exchange of the actin-bound nucleotide (ATP/ADP). Formerly known as an inhibitor of actin polymerization, profilin has been shown to also promote actin

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3 depolymerization and to bind many ligands other than actin, so playing an important role in  
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5 processes like cell motility, development, signalling and membrane trafficking (Yarmola *et al*,  
6  
7 2006). We detected decreased levels in both total and phosphorylated fractions of profilin-1.  
8  
9 Phosphorylation has been shown to promote actin binding activity of profilin (Sathish *et al*,  
10  
11 2004), but a detailed description of the influence of phosphorylation on binding to other  
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13 molecular partners of profilin is missing.  
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17 Coronin-1A, that showed increased expression in T lymphocytes from patients affected by  
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19 B-cells disorders, is also involved in cytoskeleton dynamics through its interaction with actin,  
20  
21 and is preferentially expressed in haematopoietic cells (Utrecht *et al*, 2006). In mouse,  
22  
23 coronin-1 has been shown to be essential for T-cell survival and its absence resulted in a deep  
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25 defect in  $Ca^{2+}$  mobilization, interleukin-2 production, T-cell proliferation and survival  
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27 (Mueller *et al*, 2008). Recently, coronin-1A-deficient mice was found to have specific  
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29 alterations in terminal development and survival of  $\alpha\beta$ T cells as well as defects in cell  
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31 activation and cytokine production following T-cell receptor (TCR) triggering (Mugnier *et al*,  
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33 2008).  
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41 Identification of four out of five differentially expressed spots as proteins involved in actin  
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43 dynamics is significant, and possibly reflects the commitment of T cells to execution of  
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45 effectors functions in patients affected by B cells disorders. In fact, T lymphocytes are highly  
46  
47 dynamic cells and reorganization of the actin cytoskeleton is a key process devoted to  
48  
49 signalling, differentiation, migration through tissues and completion of immune functions.  
50  
51 Cytoskeleton remodelling is actually crucial for interaction of T cells with antigen presenting  
52  
53 cells (APCs), TCR signalling, endocytosis and release of cytokines and cytotoxic granules  
54  
55 (Sechi *et al*, 2002; Samstag *et al*, 2003). **In particular, actin cytoskeleton reorganization is**  
56  
57 **crucial for the formation of the immunological synapse, a complex cellular structure**  
58  
59 **mediating signalling between interacting T cell and APC (Reichardt *et al*, 2010). Recent data**  
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3 showed that interaction with the tumour microenvironment induces defective T cell  
4 immunological synapse formation in different B cell malignancies (FL, DLBCL, CLL) and  
5 that lenalidomide, a currently used immunomodulatory drug, can reverse this effect (Ramsay  
6 *et al*, 2008, 2009). This finding hints molecular pathways involved in actin dynamics as  
7 useful targets of immunotherapeutic strategies aimed to enhance T cell anti-tumour response.  
8 To be noted that regulators of cytoskeleton functions represent key determinants of disease  
9 progression in the lymphoma B cell itself, as recently demonstrated in a mouse model using  
10 RNA interference (Meacham *et al*. 2009).  
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23 The differential expression of prohibitin (up-regulated in T cells of patients affected by B-  
24 cells disorders) can not be simply explained by a single biological process (as cytoskeleton  
25 regulation), since prohibitin (PHB) localizes in multiple cellular compartments and takes part  
26 in many molecular functions: from acting as scaffolding protein at the plasma membrane level  
27 to its role as chaperone protein in mitochondria, to modulation of transcriptional activity by  
28 interacting, directly or indirectly, with various transcription factors (p53, Rb, E2F) (Mishra *et*  
29 *al*, 2006). However, PHB was found to be preferentially expressed in non-proliferating  
30 thymocytes and mature splenic T cells, suggesting a possible role in the maturation of T-  
31 lymphocytes (Dixit *et al*, 2003); it was also detected as an up-regulated factor upon activation  
32 of primary human T-cells (Ross *et al*, 2008). Thus, up-regulation of prohibitin might be  
33 regarded as a further marker of T-cells commitment in presence of B-lymphoproliferative  
34 diseases.  
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### 53 *Protein interaction network*

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55 Functional links among the four differentially expressed proteins are evidenced by  
56 analysis of their interaction network. Looking at Fig. 3, actin clearly emerges as the central  
57 node of the protein-protein interaction network, and member of the RhoA/ROCK/LIM-kinase  
58 pathway (as *ROCK1*, *LIMK1*, *LIMK2*, *VASP*, *PIK3R1*), implicated in cytotoxic lymphocyte  
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3 activation through cytoskeletal reorganization (Lou *et al*, 2001; Khurana, *et al*, 2003) are  
4 highly represented. Furthermore, the interrelations among prohibitin and the other network  
5 nodes are highlighted. A link between prohibitin and T-cell activation through actin  
6 reorganization is given by Raf-1, a MAP3K which functions downstream of the Ras family of  
7 membrane-associated GTPases. Activation of the Ras/Raf/MEK/ERK signalling pathway  
8 follows the activation of the tyrosine kinase Lck during the engagement of the T-cell antigen  
9 receptor (TCR).  
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19 A further link between prohibitin and actin dynamics is constituted by factors as  
20 *SMARCA4*, *HDAC1*, *SIN3A*, involved in gene regulation through chromatin remodelling.  
21 *SMARCA4* codes for Brg (Brahama related gene), a subunit of the SWI/SNF chromatin  
22 remodelling complex. It is one of the mammalian multi-protein machines, able to modify  
23 chromatin structure with an ATP-driven process, and known to act as a regulator of the  
24 immune system, from lymphocyte development to immune responses (Chi, 2004). The  
25 relation between actin organization and the SWI/SNF complex is well described in the review  
26 of Farrants (2008).  
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38 Chromatin structure is also affected by histone acetylation, a reversible post-translational  
39 modification acting as an epigenetic factor for gene regulation in mammalian cells (Haberland  
40 *et al*, 2009). The level of acetylation is determined by the balance between the opposing  
41 activities of histone acetyltransferases (HAT) and histone deacetylases (HDAC). Histone  
42 deacetylation in the promoters of growth regulatory genes was shown to be implicated in  
43 cancer pathogenesis (Toyota *et al*, 2005) and inhibitors of deacetylating activity are emerging  
44 as a new class of drugs for treatment of cancer, including haematological malignancies  
45 (Stimson *et al*, 2009). *HDAC1*, one of the prohibitin proximal interactors, is a histone  
46 deacetylase whose recruitment to chromatin is mediated by SIN3, a factor that in mouse  
47 appears to be essential for T cell development (Cowley *et al*, 2005). Besides regulation of  
48 gene expression by modification of histones, acetylation can directly affect protein activity.  
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3 Interestingly, the fork-head transcription factor Foxp3+, the most specific marker of Treg  
4 cells, is a protein regulated by acetylation, and recent data (Yang *et al*, 2008; Wang *et al*,  
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Interestingly, the fork-head transcription factor Foxp3+, the most specific marker of Treg cells, is a protein regulated by acetylation, and recent data (Yang *et al*, 2008; Wang *et al*, 2009) have shown that HDAC inhibitors enhance the numbers and suppressive functions of Foxp3+ regulatory T-cells.

#### *T-cell as a biosensor of specific conditions*

Aim of this study was the evaluation of the influence played by the onset of a B-cell lymphoproliferative disease on peripheral T-cell homeostasis. We detected an alteration in the level of protein factors which can be directly linked to regulation of different effectors functions of T-cells through cytoskeleton reorganization. Furthermore, protein-protein interaction network analysis suggests that modified expression of these factors could affect (or could be an effect of) other processes, such as regulation of gene and protein activity by acetylation, of proved importance in immune response. Still, our findings need to be assessed for specificity to get some value as candidate markers for evaluation of disease state.

Coupling conventional proteome analysis to unsupervised clustering techniques represents a good platform to investigate specificity of T-cells protein profile for a defined condition. Unsupervised clustering analysis allows classification of a huge bulk of raw data in groups organized by similarity. This approach takes advantage from the intrinsic function of the method that includes all the data in the analysis, whereas in a classic proteome analysis only data above stringent parameters are selected: this can lead to loss of some information.

The protein expression data obtained by the 2DE maps of T-cells derived from patients affected by B-cell diseases were matched to those obtained from patients affected by non-related pathologies and to the reference data from healthy subjects. Hierarchical clustering of our sample cohort showed that all samples sharing the same physio-pathological condition (i.e., healthy controls, B-lymphoproliferative disorder patients, MGUS, PCO, or CAH patients) are clustered in a separate branch of the dendrogram, meaning that T-cells can record

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3 different environmental conditions and that the overall protein profile contains the  
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5 information able to distinguish among them.  
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8 To be noted that in peripheral T-cells, cofilin-1 has been shown to be differentially  
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10 expressed also in PCO and CAH syndromes (Borro *et al*, 2007) and that the specificity of T-  
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12 cell proteome variation is given by the combination of factors which modify their expression  
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14 concomitantly. The clinical impact of proteomics relies on its power to indicate many  
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16 candidate factors for prognostic and/or diagnostic purposes, but it is crucial to evaluate their  
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18 potential predictive strength jointly, not separately. We suggest analysis of protein-protein  
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20 interaction network as a powerful tool to extract information underlying the results of  
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22 classical differential proteomics, thus overcoming the detection limits of the 2DE technology.  
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24 Furthermore, it provides a functional interpretation of the data based on the overall of the  
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26 actual knowledge which may guide the selection of the more promising markers for further  
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28 evaluation.  
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34 Peripheral T-lymphocytes represent easily available living biosensors, and proteins whose  
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36 expression level changes significantly in specific pathological states, and their proximal  
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38 interactors, might represent new molecular markers for follow-up testing of the disease state.  
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40 All patients enrolled in this study were at the on-set of the disease, and the identified proteins  
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42 differentially expressed can be regarded as markers of T-cell activation, according to many  
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44 recent works reporting alterations of T-cells functionality, number and functional class  
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46 balance in B-lymphoproliferative disorders (Mittal *et al*, 2008; Ramsay *et al*, 2008, 2009; Cox  
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48 *et al*, 2008; Bari *et al*, 2009). Besides, interaction network analysis suggests candidate  
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50 biomarkers which escaped direct characterization by proteomics techniques.  
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55 The identified protein signatures may surely derive from unbalance of the normal  
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57 (healthy) repertoire of specific T cell subsets in patients affected by B cell malignancies, but  
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59 the possibility to detect and quantify them also in the total T cell population is an appealing  
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suggestion for biomarkers research.

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3     **In conclusion**, we believe it interesting to evaluate the potential prognostic and/or  
4 diagnostic value of monitoring the expression level of some of the **described** factors during  
5 the therapy.  
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## 10 11 12 13 14 15     **Acknowledgements**

16  
17     This work was supported in part by grants from ‘Ministero dell’Università e della Ricerca’  
18 and the Sapienza University of Rome.  
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For Peer Review

## Legends to the figures

**Figure 1.** Experimental work-flow.

**Figure 2.** Representative 2DE map of a protein sample from peripheral T-lymphocytes (pooled healthy subjects). Proteins are separated according to their isoelectric point (pH range 3-10) and molecular mass (Mw, range 10-200 kDa). Proteins of interest are circled.

**Figure 3.** Protein-protein interaction network view. Stronger associations are represented by thick lines. Each protein is labelled with the gene name, as reported in [Supplementary Table 1](#).

**Figure 4.** Dendrogram derived by hierarchical clustering of protein expression data. Each column represents a sample; each horizontal row represents the average quantity of a protein spot inside the replicate group of the corresponding sample. The rows' colour indicates the expression level according to the left bar: red indicates over-expression, yellow indicates basal expression and blue indicates under-expression. Proteins differentially expressed in B-lymphoproliferative disorders are marked on the side. Pink box: PCO sample; yellow box: MGUS sample; blue box: CAH sample; red box: B-lymphoproliferative disease patients; azure box: healthy pool sample.

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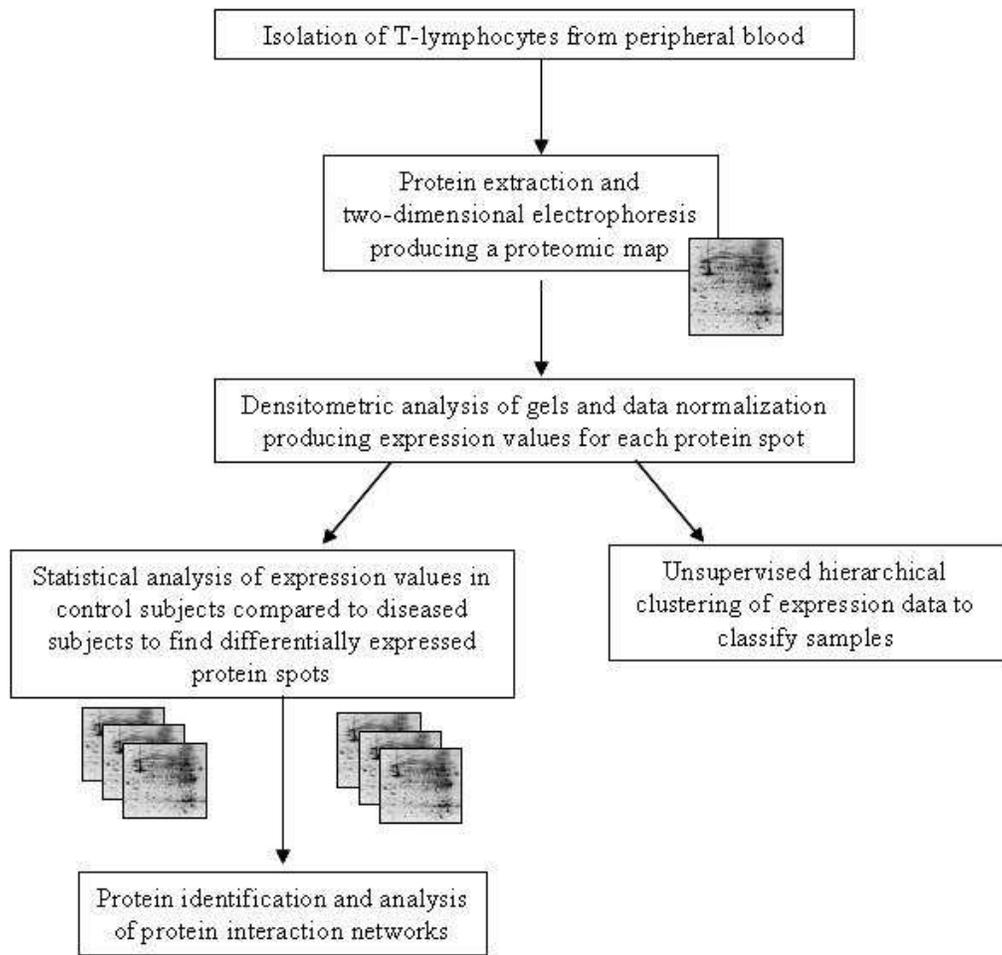


Figure 1. Experimental work-flow.  
24x23mm (600 x 600 DPI)



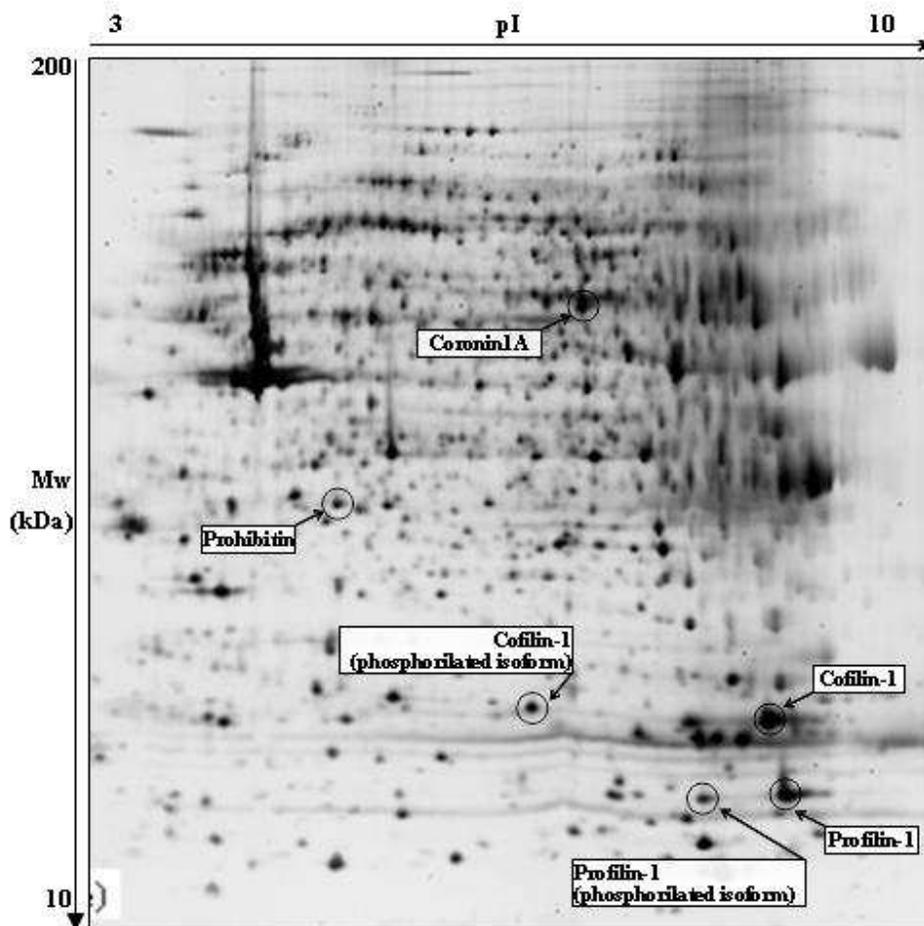


Figure 2. Representative 2DE map of a protein sample from peripheral T-lymphocytes (pooled healthy subjects). Proteins are separated according to their isoelectric point (pH range 3-10) and molecular mass (Mw, range 10-200 kDa). Proteins of interest are circled.  
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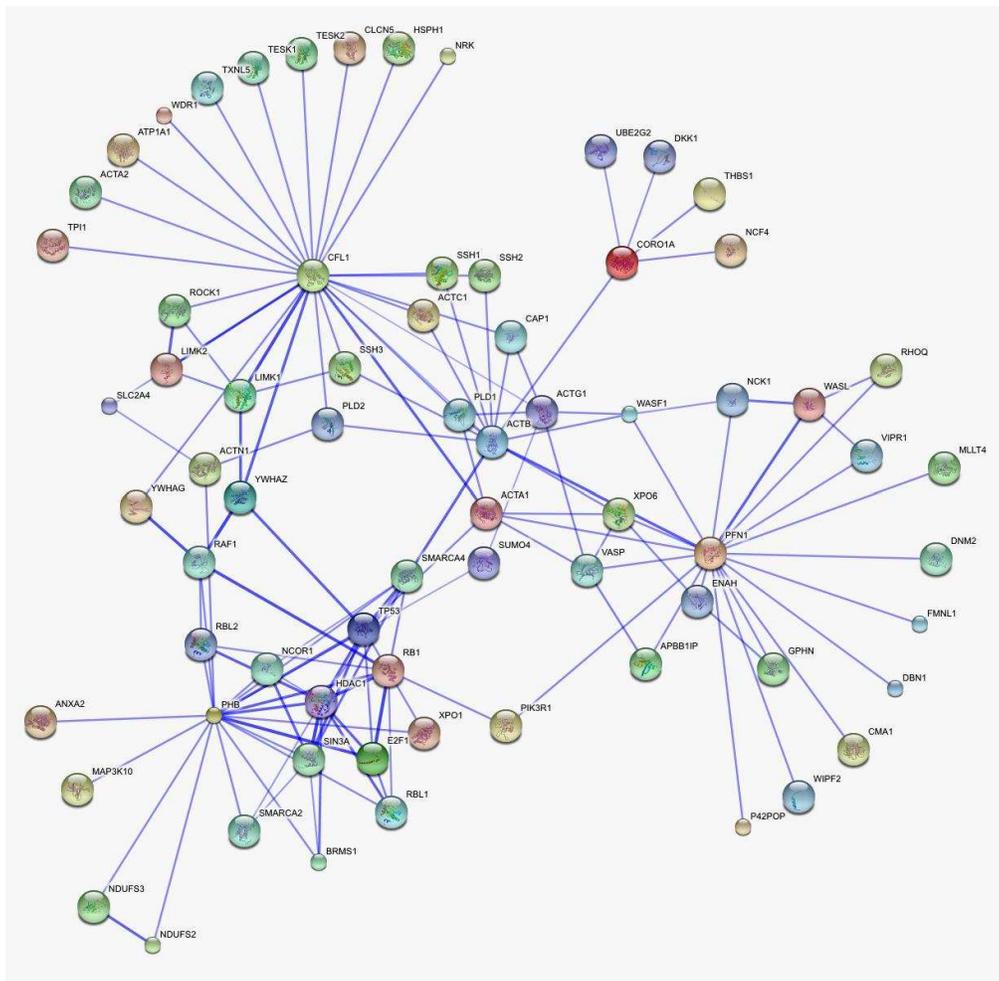


Figure 3. Protein-protein interaction network view. Stronger associations are represented by thick lines. Each protein is labelled with the gene name, as reported in Supplementary Table 1.  
51x50mm (600 x 600 DPI)

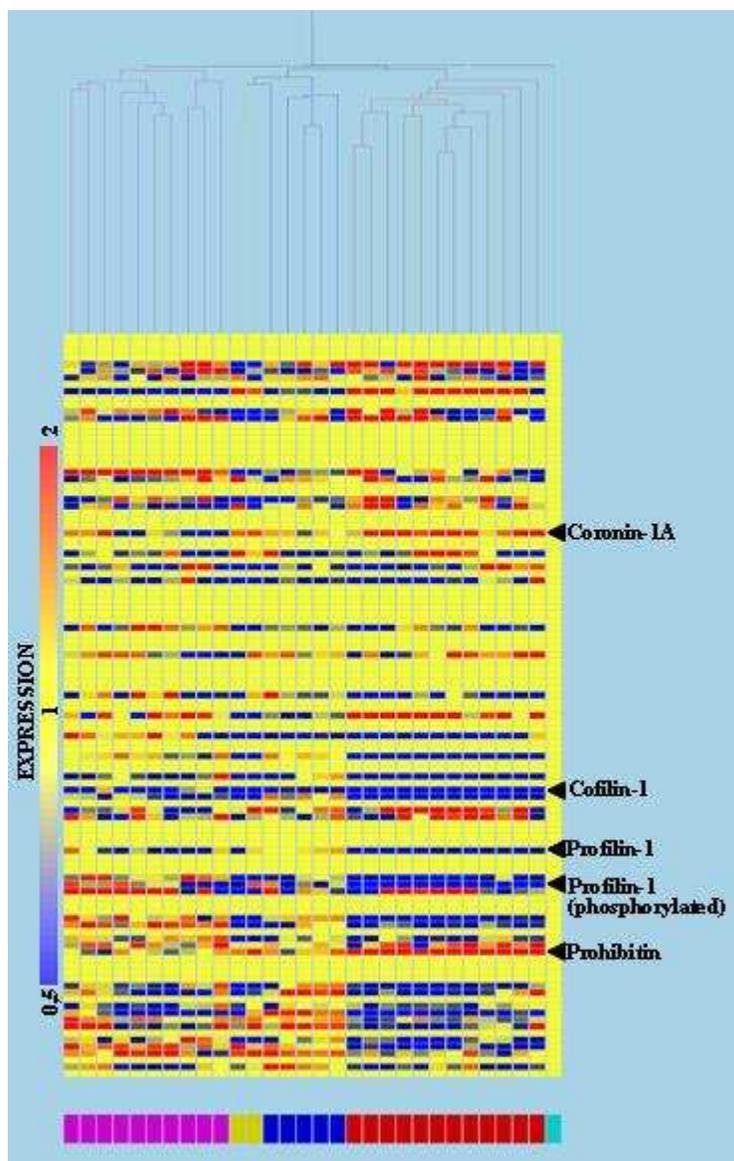


Figure 4. Dendrogram derived by hierarchical clustering of protein expression data. Each column represents a sample; each horizontal row represents the average quantity of a protein spot inside the replicate group of the corresponding sample. The rows' colour indicates the expression level according to the left bar: red indicates over-expression, yellow indicates basal expression and blue indicates under-expression. Proteins differentially expressed in B-lymphoproliferative disorders are marked on the side. Pink box: PCO sample; yellow box: MGUS sample; blue box: CAH sample; red box: B-lymphoproliferative disease patients; azure box: healthy pool sample.

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**Table 1.** Proteins identified as differentially expressed. Experimental parameters allowing unambiguous identification by mass spectrometry are reported.

Protein name	UniProtKB <sup>a</sup> (Accession no)	Theoretical pI/Mw	Score <sup>b</sup>	No. of matching peptides	Sequence coverage (%)	Mean spot quantity ± SD <sup>c</sup> (x10 <sup>-3</sup> )		FC <sup>d</sup>	p <sup>e</sup>
						Healthy subjects (n=10, pooled)	Patients (n=12)		
Profilin-1	P07737	8.5/14.9	90	8	62	13.09±0.61	5.82±2.28	-2.2	4.7 x10 <sup>-5</sup>
Profilin-1 (phosphorylated)	P07737	8.5/14.9	90	8	62	3.11±0.13	0.41±0.24	-7.6	1.67x10 <sup>-7</sup>
Cofilin-1 (phosphorylated)	P23528	8.2/18.7	69	7	30	5.33±0.25	0.91±0.45	-5.8	6.5 x10 <sup>-6</sup>
Coronin-1A	P31146	6.2/50.9	97	10	26	17.13±0.84	120.38±76.85	+7.0	4.5 x10 <sup>-3</sup>
Prohibitin	P35232	5.6/29.8	87	7	37	3.27±0.15	36.97±30.30	+11.3	1.9 x10 <sup>-3</sup>

<sup>a</sup>Protein Knowledgebase (UniprotKB) is a main protein database freely available at <http://au.expasy.org>

<sup>b</sup>Mascot score represents the probability that the observed match is a random event. Protein scores greater than 61 are significant (p < 0.05).

<sup>c</sup>Spot quantity is the total density of a defined spots in a gel image. Since the mean spot quantity for healthy subjects derives from technical replicates of a pooled sample, the associated SD is smaller compared to the SD computed for the patients' group (see Materials and methods).

<sup>d</sup>FC: Fold-change variation of protein expression level, calculated dividing the average spot quantity in all patients by the average spot quantity in the healthy subjects pool.

<sup>e</sup>p < 0,05 represents a statistically significant change in protein expression level, according to the Student's t-test.

**Supplementary Table 1.** List of the proteins interacting with profilin-1, cofilin-1, coronin-1A and prohibitin, as generated by the STRING software version 8.1 (<http://string-db.org/>).

Gene Symbol	Protein name	Interaction Score <sup>a</sup>
CORO1A	Coronin-1A	-
PFN1	Profilin-1	-
CFL1	Cofilin-1	-
PHB	Prohibitin	-
E2F1	Transcription factor E2F1	0.976
LIMK1	LIM domain kinase 1	0.976
YWHAZ	14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1)	0.867
ACTB	Actin, cytoplasmic 1	0.855
TP53	Cellular tumor antigen p53	0.855
HDAC1	Histone deacetylase 1	0.853
ACTA1	Actin, alpha skeletal muscle (Alpha-actin-1)	0.851
WASL	Neural Wiskott-Aldrich syndrome protein (N-WASP)	0.851
LIMK2	LIM domain kinase 2	0.851
RB1	Retinoblastoma-associated protein (PP110)	0.849
WDR1	WD repeat protein 1	0.656
XPO1	Exportin-1	0.633
TPI1	Triosephosphate isomerase	0.633
CLCN5	Chloride channel protein 5	0.633
ANXA2	Annexin A2	0.633
NCF4	Neutrophil cytosol factor 4	0.633
P42POP	Myb protein P42POP	0.633
YWHAG	14-3-3 protein gamma (Protein kinase C inhibitor protein 1)	0.633
ATP1A1	Sodium/potassium-transporting ATPase alpha-1 chain	0.633
ACTC1	Actin, alpha cardiac muscle 1	0.633
PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha	0.633
THBS1	Thrombospondin-1	0.633
MAP3K10	Mitogen-activated protein kinase kinase kinase 10	0.633
CMA1	Chymase precursor	0.633
NRK	Nik-related protein kinase	0.633
RHOQ	Rho-related GTP-binding protein RhoQ precursor	0.633
ACTN1	Alpha-actinin-1	0.633
XPO6	Exportin-6	0.631
NDUFS2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2	0.631
HSPH1	Heat-shock protein 105 kDa	0.631
SSH1	Protein phosphatase Slingshot homolog 1	0.631
SSH3	Protein phosphatase Slingshot homolog 3	0.631
SSH2	Protein phosphatase Slingshot homolog 2	0.631
NDUFS3	Protein-tyrosine phosphatase mitochondrial 1	0.631
MLLT4	Afadin (Protein AF-6)	0.631
GPHN	Gephyrin	0.63
ROCK1	Rho-associated protein kinase 1	0.627
APBB1IP	Amyloid $\beta$ A4 precursor protein-binding family B member 1- interacting protein	0.625
TESK2	Dual specificity testis-specific protein kinase 2	0.625
ACTA2	Actin, aortic smooth muscle	0.625

SIN3A	Paired amphipathic helix protein Sin3a	0.625
BRMS1	Breast cancer metastasis-suppressor 1	0.625
SMARCA4	Probable global transcription activator SNF2L4	0.625
TESK1	Dual specificity testis-specific protein kinase 1	0.625
DNM2	Dynammin-2	0.625
NCOR1	Nuclear receptor corepressor 1	0.625
SMARCA2	Probable global transcription activator SNF2L2	0.625
RAF1	RAF proto-oncogene serine/threonine-protein kinase	0.625
VASP	Vasodilator-stimulated phosphoprotein	0.625
TXNL5	Thioredoxin-like protein 5	0.623
RBL1	Retinoblastoma-like protein 1	0.623
CAP1	Adenylyl cyclase-associated protein 1	0.623
WASF1	Wiskott-Aldrich syndrome protein family member 1	0.623
PLD1	Phospholipase D1	0.623
FMNL1	Formin-like protein 1 (Leukocyte formin)	0.623
VIPR1	Vasoactive intestinal polypeptide receptor 1	0.623
WIPF2	WAS/WASL interacting protein family member 2	0.623
DBN1	Drebrin	0.623
NCK1	Cytoplasmic protein NCK1 (NCK adaptor protein 1)	0.623
ENAH	Protein enabled homolog	0.623
PLD2	Phospholipase D2	0.623
RBL2	Retinoblastoma-like protein 2	0.623
DKK1	Dickkopf-related protein 1 precursor (Dkk-1)	0.571
UBE2G2	Ubiquitin-conjugating enzyme E2 G2	0.561
SLC2A4	Solute carrier family 2, facilitated glucose transporter member 4	0.54
SUMO4	Small ubiquitin-related modifier 4 (SUMO-4)	0.492
ACTG1	Actin, cytoplasmic 2	0.447

<sup>a</sup>Interaction score is automatically generated by the software. For details about the applied prediction algorithms see: Jensen, L.J., Kuhn, M., Stark, M., Chaffron, S., Creevey, C., Muller, J., Doerks, T., Julien, P., Roth, A., Simonovic, M., Bork, P. & von Mering, C. (2009) STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Research*, 37, D412-416.