Impact of hydroxycarbamide and interferon-α on red cell adhesion and membrane protein expression in polycythemia vera

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Methods

Sample preparation and nLC-MSMS ITRAQ multiplex analysis

Ghosts were resuspended in 0.5 M TriEthyl Ammonium Bicarbonate (TEAB) buffer containing 0.1% Rapigest and heated for 5 min at 95°C. Protein concentration was determined by Bradford assay (Biorad). 100 µg of each sample were reduced 30 min at 56°C with 30 mM dithiothreitol and alkylated with 70 mM chloroacetamide for 30 min at room temperature before digestion during 3h at 37°C using 1 µg trypsin (Promega). Rapigest was removed by acidification with 2 µl of trifluoroacetic acid and centrifugation. Peptides were desalted on UptiTip C18 (Interchim Uptima BI5280) and dried. They were solubilized in 500 mM TEAB and labeled according to the protocol of the iTRAQ Reagents 8plex Application Kit Protein (ABSciex). PV1, PVHC1, PV2, PVHC2, PV3, PVHC3, CT1 and CT2 samples were labeled with isobaric 8plex tag 113, 114, 115, 116, 117, 119, 121 and 118, respectively. Peptide labeling efficiency was controlled by mass spectrometry before pooling and drying.

Excess iTRAQ reagent was removed by retaining peptides on a Strong Cation Exchange column (SCX) as instructed by the manufacturer, and eluted peptides were desalted using a Sep-Pak C18 column (Waters). They were then separated by isoelectrofocalisation on 13 cm pH 3-10 strips using an Agilent 3100 Off-Gel fractionator following manufacturer's instructions. After focusing, each fraction was collected and peptides trapped in the strip were extracted with 200 µl of 50% methanol in 1% formic acid. Methanol-extracted peptides were pooled with their respective fraction and dried in a vacuum concentrator. After solubilization in 10 µl of 10% Acetonitrile (ACN), 0.1% Trifluoroacetic Acid (TFA) in milliQ-H_2O, 1µl of each fraction was analyzed in LC-MS-MS using an Ultimate 3000 Rapid Separation liquid chromatographic system coupled to a hybrid LTQ-Orbitrap Velos mass spectrometer (both from Thermo Fisher Scientific). Briefly, peptides were loaded and washed on a C_{18} reverse

phase precolumn (3 μm particle size, 100 Å pore size, 75 μm i.d., 2 cm length from Thermo).

The loading buffer contained 98% milliQ-H_2O, 2% ACN and 0.1% TFA. Peptides were then

separated on a C_{18} reverse phase resin (2 μm particle size, 100 Å pore size, 75 μm i.d.,15 cm

length from Thermo) with a 97 min binary gradient from 99% A (0.1% formic acid in milliQ-

H_2O) to 7% B (80% ACN, 0.085% formic acid in milliQ-H_2O) in 33 min then to 40% B in 64

min. The mass spectrometer acquired data throughout the elution process and operated in a

data dependent scheme with full MS scans acquired with the Orbitrap, followed by

fragmentation of the 10 most abundant ions detected in the MS scan in two distinct

fragmentation modes for each selected precursor ion: (1) LTQ MS/MS CID fragmentations

for sequence information and sensitivity and (2) Orbitrap MS/MS HCD fragmentations for

reporter ion. Mass spectrometer settings were: full MS (AGC: $1x10^6$, resolution: $6x10^4$, m/z

range 400-2000, maximum ion injection time: 500 ms); MS/MS (minimum signal threshold:

500, isolation width: 2Th, dynamic exclusion time setting: 30s, Ion Trap MSn AGC Target:

$5x10^3$ and maximum injection time: 200 ms, FTMS MSn AGC Target: $5x10^4$ and maximum

injection time: 200 ms). The fragmentation was permitted for precursor with a charge state

>1.

Proteome discoverer 1.3 was used to generate .mgf files for precursors with a signal to noise

ratio >3 and for merging the corresponding HCD and CID peak lists. The data was analyzed

by Protein Pilot version 4.5 (ABSciex) using the human database from Uniprot swiss-prot

(October 2014, 20,268 sequences). Data were processed with the following criteria:

iTRAQ8plex (peptide-labeled on lysines and N-termini), trypsin cleavage specificity,

carbamidomethylated cysteins, "Orbi-FT MS (1-3ppm) LTQ MSMS" and "Rapid" search

effort. A 5% protein local FDR as threshold was applied for protein identification. FDR

calculations were performed using a reverse database. Finally, 12459 Peptide spectrum

matches, corresponding to 2664 unique peptidic sequences, allowed the identification of 375

proteins. Protein quantifications were obtained using unshared peptides (annotated "auto").

Supplementary Figure 1

A

B
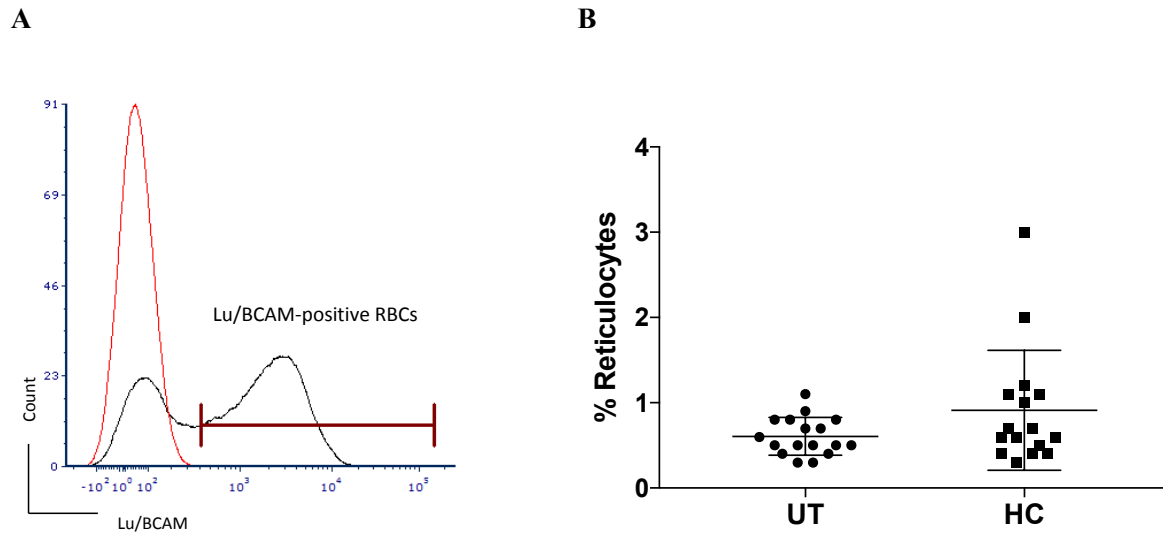
Supplementary Figure 1: Flow cytometry analysis of Lu/BCAM expression, and percentage of reticulocytes in PV patients. (A) Representative flow cytometry diagram of Lu/BCAM expression on the surface of PV red blood cells (RBCs) showing the typical double population of Lu/BCAM-negative and Lu/BCAM-positive RBCs. Red curve: RBCs incubated with control isotype antibody. (B) Flow cytometry quantification of the percentage of reticulocytes in 17 untreated (UT) and 16 hydroxycarbamide-treated (HC) PV patients.

Supplementary Table 1. ITRAQ ratios of proteins with increased (i) or decreased (d) expression at the membrane of PV RBCs and effect of HC treatment. ITRAQ ratios for proteins up- or down-regulated in RBC membranes between PV and control CT2 (PV/CT) and the effect of HC during treatment compared to CT2 (PVHC/CT). Proteins from ER are in bold (i.1, i.2, i.3, i.5, i.7, i.9 and i.11). CT1/CT2 represents the ratio of each protein between the 2 control samples used in this study. CT: control; PVHC: blood sample from HC-treated patient.

| Protein | Gene | # | PV / CT | | | PVHC / CT | | | CT / CT |
			PV1 / CT2	PV2 / CT2	PV3 / CT2	PV1HC / CT2	PV2HC / CT2	PV3HC / CT2	CT1 / CT2
Calnexin	**CANX**	**i.1**	**20.3**	**16.3**	**6.8**	**9.3**	**13.1**	**7.4**	**1.0**
Peroxiredoxin-4	**PRDX4**	**i.2**	**7.3**	**5.2**	**5.3**	**2.3**	**5.9**	**2.8**	**1.1**
Calreticulin	**CALR**	**i.3**	**5.9**	**6.2**	**5.5**	**3.0**	**10.2**	**1.8**	**1.0**
Lu/BCAM	BCAM	i.4	2.9	3.2	3.6	34.4	21.3	33.7	0.9
GRP78	**HSPA5**	**i.5**	**3.3**	**3.8**	**2.9**	**2.0**	**5.6**	**0.8**	**0.8**
Abhydrolase domain-containing protein 16A	ABHD16A	i.6	3.1	10.3	3.1	4.2	24.0	14.1	1.0
14-3-3 protein beta/alpha	YWHAB	i.7	3.1	1.8	2.5	4.1	0.8	0.1	1.1
Protein ERGIC-53	**LMAN1**	**i.8**	3.0	2.7	2.1	2.7	3.1	2.1	1.1
Neutral alpha-glucosidase AB	**GANAB**	**i.9**	**1.7**	**2.7**	**2.0**	**0.7**	**2.7**	**1.0**	**0.8**
Thioredoxin-related transmembrane protein 1	TMX1	i.10	2.0	2.2	2.0	2.2	2.0	1.4	1.1
Endoplasmic reticulum resident protein 44	**ERP44**	**i.11**	**1.4**	**1.3**	**1.5**	**1.3**	**1.5**	**0.8**	**1.0**
Equilibrative nucleoside transporter 1	SLC29A1	d.1	0.7	0.5	0.1	0.4	0.4	0.4	1.0
Plasma membrane calcium-transporting ATPase 4	ATP2B4	d.2	0.5	0.7	0.5	0.9	1.0	1.2	1.1
Membrane transport protein XK	XK	d.3	0.4	0.5	0.2	0.9	0.8	0.8	0.9
Intercellular adhesion molecule 4	ICAM4	d.4	0.4	0.4	0.2	0.5	0.2	0.4	1.0
Aquaporin-3	AQP3	d.5	0.3	0.7	0.2	0.1	0.8	0.5	1.1
Kell blood group. metallo-endopeptidase	KEL	d.6	0.3	0.7	0.3	1.2	1.0	2.1	1.1

Ratio

≤ 0.4	≤ 0.7	1	≥ 1.3	≥ 1.7	≥ 2.0	≥ 3.0

Supplementary File 2. Table of the proteomic analysis data. The table includes detailed results of the proteomic analysis of RBC membranes from PV patients and control donors, in terms of peptides, coverage and identified proteins.