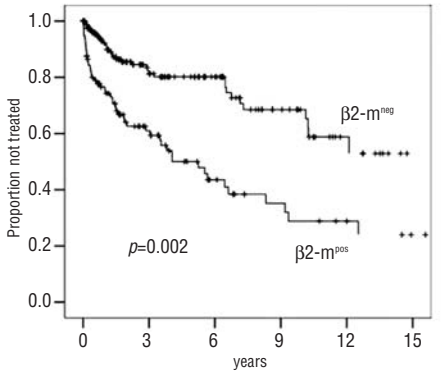
A



B

Risk categories	Univariate analysis HR (95% C.I., *p* value)
No factor	1
One factor	1,5 (0.7-3.4, *p*=ns)
Two factor	5.0 (2.5-10.2, *p*<0.0001)
Three factors	15.4 (7.3-32.5, *p*<0.0001)

C

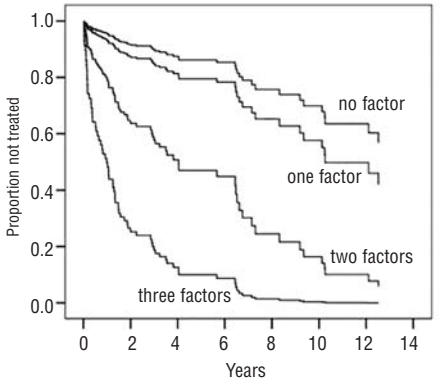


Figure 1. (A) **Kaplan–Meier estimates of treatment-free survival (TFS). Comparison between β2-m^neg patients versus β2-m^pos patients.** (B) **Cox univariate analysis for the predictive value of marker combinations for the risk categories.** (C) **Cox derived estimated TTT curves according to the combination of the three prognostic factors.**

by lymphocytes and it is expected that its levels steadily increase together with the progressive expansion of the leukemic clone suggesting a close correlation between stage (which measures tumor burden) and β2-m levels. Although a correlation with disease stage likely exists, there was a substantial proportion of patients with high β2-m levels already at Binet A stage (low tumor burden). Possibly, CLL cells from these patients are more activated *in vivo* and shed more abundant β2-m. Taken all the above into consideration, the data indicate that the role of β2-m as a prognostic tool should be re-evaluated possibly in prospective studies involving large patient cohorts.

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Funding: supported from Associazione Italiana Ricerca sul Cancro (AIRC) (to FM and MF) and Fondazione 'Amelia Scorza' Onlus, Cosenza, Italy.

Acknowledgments: we would like to acknowledge Dr. Vincenzo Callea, Prof Luca Baldini, Dr Ugo Consoli and Dr Serena Matis for their contribution and useful suggestions. We thank Laura Veroni and Brigida Gulino for precious secretarial assistance.

Key words: β2-microglobulin, CD38, IgVH mutational status, CLL, prognosis.

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Citation: Gentile M, Cutrona G, Neri A, Molica S, Ferrarini M, and Morabito F. Predictive value of B2-microglobulin (B2-m) levels in chronic lymphocytic leukemia since Binet A stages. Haematologica 2009; 94:887-888. doi:10.3324/haematol.2009.005561

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Detection of continuous erythropoietin receptor activator in blood and urine in anti-doping control

Anti-doping control of erythropoietin (Epo) relies on the differentiation by isoelectric profile of natural endogenous hormone from the recombinant hormone used for doping. The first and second generations of recombinant Epo were detectable in urine.[1,2] The third generation, Continuous Erythropoietin Receptor Activator (CERA), was obtained by linking a methoxy polyethylene glycol to epoetin β, a first generation rHuEPO, resulting in significantly greater stability in blood. CERA, approved in Europe in July 2007, was

expected to be quickly misused in sport. Several questions were thus raised about its detectability. We demonstrate here that detection in blood poses no problem and that partial excretion in urine occurs after some physical exercise. Our analytical results from the Tour de France 2008 anti-doping control, which tested both urine and blood samples, serve as an illustration.

Staining revealed that the isoelectric pattern of pure CERA was composed of seven bands with apparent isoelectric points in the pI 4.8-5.2 range (Figure 1). This was surprising since CERA that results from pegylation of epoetin β via the amino group of lysine residues was expected to be more acidic than epoetin β. One explanation could be a shielding effect of the PEG shell on some surface charges of epoetin. In any case, the isoelectric pattern clearly differentiated CERA from natural Epo.

However, analysis of Epo isoelectric patterns in urine or plasma as previously described[2,3] required two monoclonal anti-human Epo antibodies. Briefly, 18 mL of urine or 1 mL of plasma were first submitted to a preparation step including immunoaffinity chromatography using clone 9C21D11 and ultrafiltration at a 30-kDa molecular weight cut-off. The retentates from ultrafiltration were then submitted to isoelectric focusing and Epo was detected by double-blotting, using clone AE7A5.[4,5] The final result was a chemiluminescent image of the isoelectric patterns of Epo.

Due to the polymer chain in the molecule, the CERA structure might not be recognized by the antibodies used in this method. In fact, use of AE7A5 only (double-blotting only) and both AE7A5 and 9C21D11 (immunochromatography and double-blotting) provided patterns identical to that obtained without antibody (stained gel) (Figure 1).Thus, although the amino terminus of the polypeptidic chain is one of three major pegylation sites, the epitope of AE7A5, which is within the first 26 amino acids, was still accessible to this antibody and the epitope of the 9C21D11 antibody (not identified) was also not affected by pegylation. The sensitivity of the method was determined to be less than 40 pg applied onto the IEF gel (corresponding to initial 50 pg/mL in 1 mL serum and 3 pg/mL in 18 mL urine).

These results proved that, technically, the usual method for Epo analysis could detect CERA. However, due to its hydrodynamic volume that is critical for glomerular filtration, CERA detection in urine, the main biological medium for anti-doping control, seemed unlikely.

Anti-doping control of recombinant Epo during the Tour de France 2008 was performed as usual from urine. CERA was clearly detected in two urine samples (both from the same athlete) reported as positive. Other samples from this same athlete and 3 others gave rise to faint CERA images and were considered highly suspicious. Blood samples taken before the race and mid-race were retrospectively analyzed. All eight plasma samples (two per athlete) from the 4 athletes having positive or suspicious urine samples gave rise to very clear CERA images. This confirmed that only a small part of CERA was excreted from blood into urine. However, strenuous exercise enhances protein excretion in urine by increasing glomerular permeability and decreasing tubular reabsorption.[6-8] CERA excretion in urine is affected by exercise as well. This is illustrated by the case of the

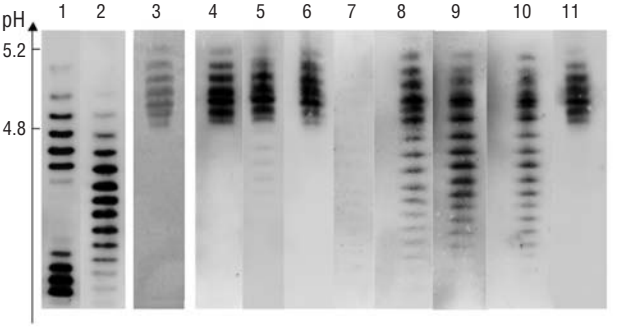

Figure 1. Isoelectric patterns of Epo. All the patterns are chemiluminescent images except in lane 3. Lane 1, mixture of Epoetin beta, a first generation rHuEpo (up), and Darbepoetin alfa, a second generation rHuEpo (down); lane 2, natural urinary Epo; lane 3, pure CERA (Coomassie Blue stained gel); lane 4, pure CERA analyzed by double-blotting only; lane 5, CERA added to a urine sample and analyzed by immunochromatography and double-blotting. Note that in addition to the characteristic pattern of CERA, some less intense bands corresponding to the endogenous Epo of this sample were detected in a more acidic area. The next lanes correspond to samples from an athlete of the Tour de France 2008 (see Figure 2 for details of chronology): lane 6, first plasma sample (presence of CERA); lane 7, first urine sample (no Epo detected, neither natural nor CERA); lane 8, second urine sample (presence of CERA); lane 9, third urine sample (traces of CERA); lane 10, fourth urine sample (presence of CERA); lane 11, second plasma sample (presence of CERA). Note the presence of additional isoforms to those of CERA in lanes 8 to 10, corresponding to the natural endogenous Epo in urine. This latter is not visible in plasma samples (lanes 6, 11) due to the very intense signal of CERA, which is present in a much greater proportion in plasma than in urine.

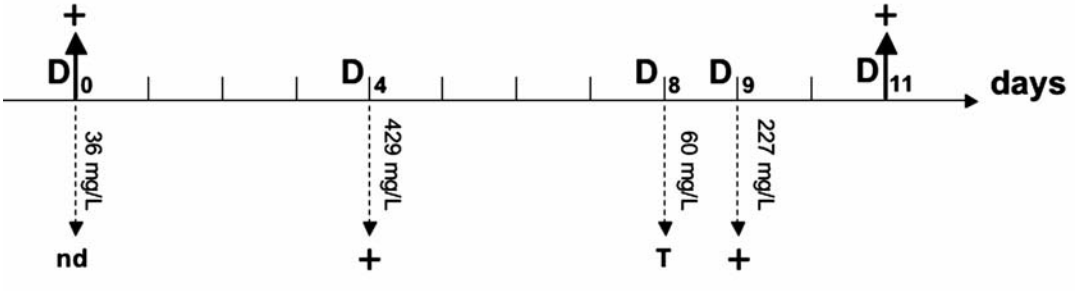

Figure 2. Chronology of sampling in the athlete reported positive for CERA from urine samples during the Tour de France 2008. The days of sampling are indicated by up and down arrows for blood and urine, respectively. The ends of the arrows point to the results of the analyses: +, positive; T, traces; nd, no detectable Epo (neither natural nor CERA). D_0 corresponds to the day before the start of the race. Along the down arrows are indicated the protein levels of the urine samples. The isoelectric patterns of Epo obtained from these samples are shown in Figure 1.

athlete reported positive from two urine samples. Two positive blood samples were taken 11 days apart while four urine samples were tested for Epo during this period (Figure 2). Sample 1 was totally devoid of Epo (natural or CERA) and sample 3 revealed only traces of drug. Both samples presented physiological protein levels of 36 and 60 mg/L, respectively (Coomassie Plus Protein Assay Reagent, Pierce). The presence of CERA was clearly established in samples 2 and 4, which presented high total protein levels (429 and 227 mg/L, respectively).

In conclusion, the IEF test for anti-doping control of Epo is perfectly able to detect CERA in blood and urine, from a technical point of view. However, the poor drug excretion in urine must be taken into account for anti-doping control purposes and tests may have to be performed in blood.

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Key words: erythropoietin receptor activator (CERA), urine, anti-doping control.

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Citation: Lasne F, Martin L, Martin JA, de Ceaurriz J. Detection of continuous erythropoietin receptor activator (CERA) in blood and urine in anti-doping control. Haematologica 2009; 94:888-890. doi: 10.3324/haematol.2009.006809

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