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## **Shifted isoelectric profiles of endogenous erythropoietin possibly due to different physiological origin**

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### *Abstract*

We have initiated a project aimed at investigating possible physiological explanations for variations in endogenous EPO isoform distribution, and to look at ways of distinguishing these from the profiles of recombinant epoetins. Preliminary results show that the isoelectric profiles of EPO from cord blood have a more basic *pI* range than that of adult serum EPO; consistent with the hypothesis that EPO produced in the liver is less acidic than EPO produced in the kidney. Using the EPO MAIIA WGA-tool, hyper basic Chinese epoetins showed WGA-affinities similar to epoetin alfa and beta, while both the EPO from patients and the tested effort urines displayed aberrant WGA-affinities. The EPO from the patient samples had, just like the effort urines, basic bands 3-5 as the most intense after isoelectric focusing. This shift in EPO isoform distribution seen in certain physiological conditions may be due to hepatic involvement.

### *Introduction*

Lately, the anti-doping community has been challenged by the entry of new epoetins with deviating IEF-profiles; i.e. the Asian epoetins with hyper basic isoelectric profiles, epoetin delta (Dynepo), and the PEGylated epoetin beta named CERA (Continues Erythropietin Receptor Activator). Further, a shift in the isoelectric EPO profile towards more basic *pI* has been reported for some urine samples after strenuous exercise<sup>1</sup>. In addition, we and other labs have analyzed urines with hyper basic EPO-profiles, but unfortunately, we cannot from their isoelectric distribution alone judge whether they are due to misuse of hyper basic recombinant epoetins or whether they represent an extreme version of effort urine or some other physiological condition.

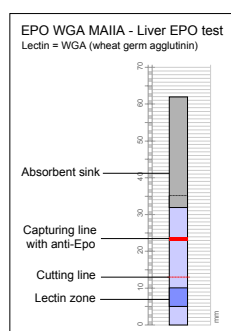
EPO from umbilical cord blood and serum from certain kidney patients has previously been reported to be less negatively charged than normal serum EPO<sup>2</sup>. Cord blood

contains mainly EPO produced in the liver since he switch from hepatic to renal production of EPO starts at the end of third trimester of pregnancy but is far from completed at birth<sup>3</sup>.

### Materials and Methods

Affinity purification of EPO from serum: Monoclonal EPO-antibodies (clone 9C21D11 from R&D and clone 3F6 from MAIIA Diagnostics) were covalently bound to a matrix (either Affi-Gel Hz Hydrazide Gel from BioRad or rec-Protein G-Sepharose 4B from Invitrogen) prior to affinity purification. 3-4 ml serum (umbilical cord serum or normal serum) was incubated with the covalently bound antibodies for 1h and eluted from the matrix using either a low pH buffer (0.2 M glycine-HCl, pH 2.5) or a high pH buffer (50 mM NaOH, 10 mM glycine, 0.625 M NaCl, pH 12.1). Immediately neutralized, the purified EPO-fractions were concentrated by ultra filtration using Microcon YM-30 filters (MWCO 30 kDa), prior to either isoelectric focusing (IEF) or sodium dodecyl sulphate electrophoresis (SDS-PAGE).

Gel Electrophoresis and Double-blotting: Isoelectric focusing (IEF) and the double-blotting procedure were performed as described<sup>4</sup>. SDS-PAGE was performed using 10 % mini ready gels in MOPS running buffer with samples in 1x NuPAGE sample buffer (all Invitrogen). Proteins were transferred to the first PVDF membrane using the XCell II Blot Module Kit (Invitrogen).

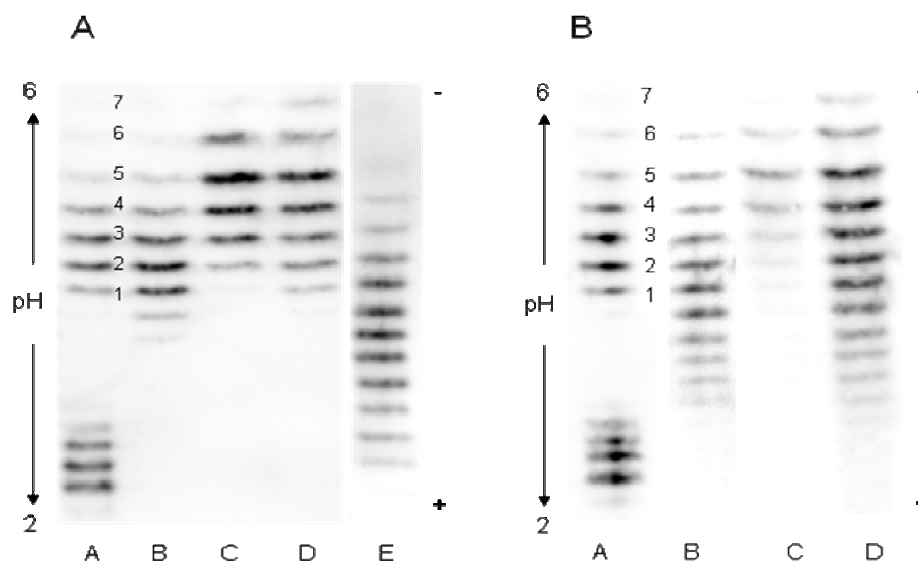


EPO MAIIA WGA: Affinity purified EPO isoforms were separated by difference in migration speed in a 5 mm zone of WGA in a thin micro-column. Only the most rapidly migrating EPO isoforms will reach the anti-EPO zone. The amount of bound EPO in this line is detected by anti-EPO immobilized to carbon black nano-strings and the blackness quantified with an image scanner. The percentage of fast migrating EPO

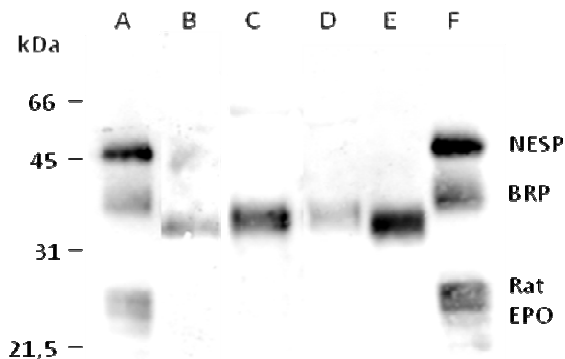
isoforms is calculated by also measuring the total amount of all EPO isoforms.

### Results and Discussion

The isoelectric profiles of some Chinese epoetins and epoetin omega deviate from the profiles of epoetin alfa, beta and delta, by being hyper basic with isomer bands 3-6 as the most intense (Fig. 1 A). The so far tested cord sera have a more basic isoform distribution than the EPO profiles in sera from healthy adults (Fig. 1B). A similar basic profile shift was seen in urine from some patients with kidney dysfunction (Fig. 3).

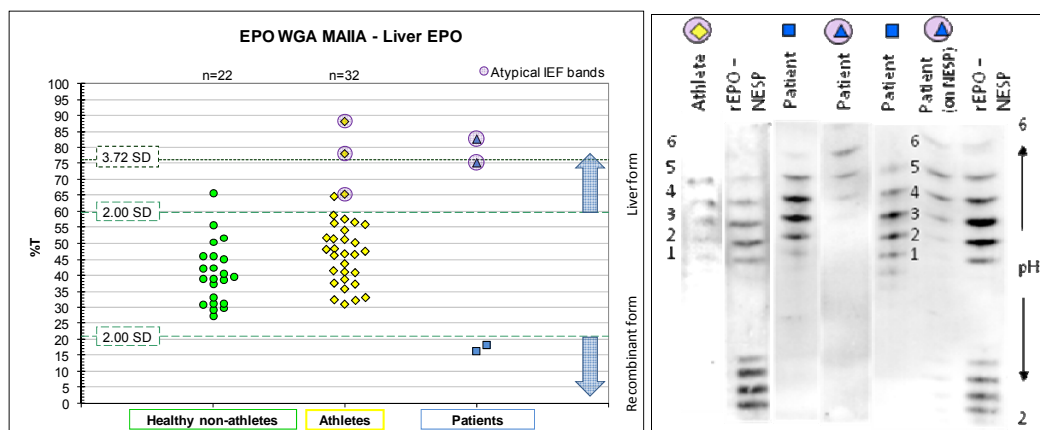


**Figure 1 A. Isoelectric profiles of EPO analogues.** Lane (A) Biological reference preparation (BRP; epoetins alfa and beta, 1:1) and Darbepoetin alfa (NESP), (B) Dynepo (epoetin delta), (C) Epomax (epoetin omega), (D) EPIAO (China) and (E) Second International Biological Standard of human urinary erythropoietin, for comparison. Bands in the recombinant area are numbered 1-7, with band 1 representing the least basic BRP isoform. Three out of four tested Chinese epoetins had hyper basic profiles (not shown).  
**Figure 1 B. Comparison of the isoelectric profiles of EPO from normal adult and umbilical cord serum.** Lane (A) BRP and NESP, (B) natural endogenous EPO profile from adult serum, and (C-D) natural endogenous EPO profiles from umbilical cord serum. Note that the isoforms of cord sera EPO are shifted towards more basic *pI* than adult serum EPO.



**Figure 2. SDS-PAGE separation of EPO standards and serum and urine specimens.** Images of (A) and (F) rat EPO, BRP and Darbepoetin alfa (NESP), (B) urine EPO from patient with kidney disease treated with NESP (corresponds to  $\Delta$  in Figure 3), (C) cord serum EPO, (D) normal serum EPO and (E) Second International Biological Standard of human urinary erythropoietin (NIBSC). Note that all endogenously produced EPO has an apparent lower *M<sub>w</sub>* compared to epoetin alfa + beta (BRP).

One of these patients was treated with Aranesp (faintly visible in 2<sup>nd</sup> lane from the right, Fig.3), so the prescribed epoetin cannot explain the basic pattern. Interestingly, some athlete samples taken during a large international competition displayed hyper basic EPO profiles where the most intense isoforms corresponded to bands 3-6 (Fig. 3). EPO MAIIA WGA was capable of separating the EPO from these particular athletes and patients from different recombinant epoetins (hyper basic Chinese epoetins, CERA and epoetin alfa, beta and delta) due to their differences in WGA affinity. We therefore believe a possible explanation may be that these particular patient and athlete samples contain a greater proportion of EPO produced



**Figure 3. Urine specimens from patients and athletes tested with the IEF doping test and the EPO WGA MAIIA-liver EPO test.**

**Patients:** Two patients receiving Neorecormon due to non-renal anaemia (■), showed low %T and band 2-3 most intense in IEF, as expected. Two patients receiving NESP or Neorecormon to adjust anaemia due to renal dysfunction (▲), showed high %T (82.8, 75.3) and the most intense isoform were basic bands 4-6 and 3-5, respectively, in the IEF testing. **Athletes:** Three specimens out of the 32 MAIIA-liver tested specimens from athletes during a competition showed MAIIA values above 2SD from the non-athletes specimens. These specimens showed also atypical bands (4-5 or 3-4 most intense) in the IEF method. The fourth specimen with MAIIA value above 2SD was not detectable in the IEF method. The binding to WGA is strongest for the carbohydrates attached to Aranesp followed by Neorecormon > endogenous kidney EPO > endogenous liver EPO. To obtain high resolution between endogenous renal and liver isoforms, a low concentration of competing sugar derivative was chosen for this EPO WGA MAIIA-liver variant compared to the EPO WGA MAIIA doping test.

in the liver. It is possible to distinguish between rEPO and endogenous EPO from both cord serum and kidney disease when separated on SDS-PAGE gel. The cord and patient EPO has similar mobility to normal serum EPO; a lower apparent MW than both rEPO (Fig. 2) and the Chinese epoetins (not shown). Whether strenuous physical exercise can increase hepatic EPO production and/or secretion is to date not known. These results open up the possibility that the shift in EPO isoform distribution seen in certain physiological conditions may be due to hepatic involvement.

#### *Acknowledgements:*

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