Patients with anaemia can shift from kidney to liver production of erythropoietin as shown by glycoform analysis

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The primary production site of erythropoietin (EPO) is shifted from the liver to the kidney shortly after birth. Under conditions of lost or reduced kidney production, it is valuable to measure the production capacity of the liver. However, there is a lack of urine or serum based methods that can distinguish endogenous EPO produced in different cell types. Here is presented a method based on chromatographic interaction with the lectin wheat germ agglutinin (WGA) that can distinguish presumably liver-produced EPO, found in anaemic patients receiving epoetin and darbepoetin, from kidney-produced EPO found in healthy individuals.

All the tested samples from haemodialysis patients with end-stage renal disease showed a presence of liver EPO. In some samples, the liver-produced EPO made up 90-100% of total EPO at a concentration of 8-10 ng/L in urine, which indicates that the liver has a quite high production capacity, although not adequate for the degree of anaemia.

This glycoform analysis has made it possible to affirm that some anaemic patients can increase their liver-production of EPO. The use of such a method can give better insight into the regulation of non-renal endogenous EPO production, a potential source of EPO intended to replace administration of exogenous EPO.

Key words: EPO, epoetin, darbepoetin, haemodialysis, lectin chromatography, WGA

Running title: Liver EPO Measured by Glycoform Analysis

1. Introduction

EPO is a glycoprotein hormone responsible for the homeostatic regulation of red cell production, which is up-regulated by hypoxia through a recently described oxygen sensing mechanism [1,2,3]. During fetal and early neonatal life, EPO is mainly produced in the liver [4], and production is shifted to the kidney only shortly after birth. Adults seem to have retained a hepatic production capacity of up to 10% of total EPO during normal conditions. Patients with end-stage renal disease require life-long therapy with recombinant EPO (rhEPO), but currently, therapeutic approaches for enhancing endogenous non-renal production of EPO is of high interest, as discussed in recent reviews [5,6]. Pharmacological manipulation of the hypoxia-inducible transcription factor (HIF) has been shown to stimulate endogenous EPO production [7]. Methods for up-regulating production of EPO from the liver seem especially attractive as an alternative to therapy with rhEPO when kidney production has ceased [8].

Studies on EPO expression in specific tissues have required ablation of organs, followed by measurement of the remaining EPO production activity in blood, or directly in tissue extracts. The early studies of the liver-to-kidney shift were based on measuring total EPO levels (with bio-assays and, subsequently, immunoassays) in response to anaemia in normal- and bilaterally nephrectomised fetal- and new-born sheep [9]. In studies on rats [10], the expression level of EPO mRNA was measured in the respective organs. Measurement of EPO mRNA has shown that the rat liver can contribute with over 33% of the produced EPO during short-term severe hypoxia, and up to 37% after severe haemorrhage [11]. For humans, increased liver EPO production has been indicated for anephric patients during some pathologic conditions affecting the liver. EPO concentrations in blood and haemoglobin levels were both increased [12]. A study of haemodialysis patients found that anaemic patients showed improved haemoglobin levels during hepatic regeneration after hepatitis virus infection [13].

There seems to be a common opinion that EPO expressed in different tissues, like that of kidney and liver, show no structural differences [5]. However, mammalian cells from different organs posttranslationally add a specific glycosylation signature to the produced glycoprotein [14]. Glycoproteomics methods, distinguishing differently glycosylated protein subpopulations, can thus reveal the production site for a protein, without requiring extraction from organs, just by measurements in serum samples.

For EPO, which appears in very low concentrations, the advent of sensitive bioassays [15] and immunoassays [16] were required to allow studies of pathophysiology and diagnosing anaemia and polycythaemia. Shortly thereafter, a novel methodology, using sensitive EPO immunoassay for measuring the distribution of EPO glycoforms separated by electrophoresis, made it possible to distinguish the presence of differently charged isoform populations of EPO [17]. It was found that

the liver-produced EPO in umbilical cord sera from new-born infants was distinguishable from serum EPO from adults, while rhEPO preparations showed a charge comparable to EPO in cord sera. Later on, the method proved to be useful for detecting illegal use of rhEPO in sports [18]. Separation of protein glycoforms can also be performed with lectins, the carbohydrate binders that can distinguish several types of specific structures. A study measuring the interaction between EPO carbohydrate structures and lectins found that the lectin wheat germ agglutinin was especially useful for distinguishing recombinant and endogenous EPO [19]. The interaction between EPO and WGA has been used in a rapid lateral flow test for identification of rhEPO doping in humans and in horses [20,21].

The present study evaluates if the analytical method, based on WGA affinity chromatography, is capable to measure the presence of endogenous non-renal EPO glycoforms in urine samples from anaemic patients treated with rhEPO.

2. Design and Methods

2.1. Reagents and chemicals

The water used in all experiments was prepared using Milli-Q Academic equipment (Millipore, Billerica, MA, USA). Reagents, unless specified, were of analytical grade and purchased from Sigma (St. Louis, MO, USA). BSA was obtained from Intergen Company (NY, USA).

2.2. Recombinant human erythropoietin

The pharmaceutical glycoprotein Eprex®, (recombinant epoetin α) was purchased from Janssen-Cilag AB (Sollentuna, Sweden) and Neorecormon® (recombinant epoetin β) was obtained from Roche Diagnostics GmBH (Mannheim, Germany). One IU of these preparations corresponds to 8.4 and 8.3 ng recombinant EPO for Eprex and Neorecormon, respectively. Aranesp®, the recombinant EPO analogue darbepoetin α, was purchased from Amgen (Thousand Oak, CA, USA).

2.3. Determination of EPO concentration in urine in order to select samples for analysis

The EPO concentration in urine specimens was measured by a lateral flow immunoassay for EPO (MAIIA Diagnostics, Uppsala, Sweden), as described previously [22]. From the 38 tested samples with values in the range < 0.5 ng/L to 798 ng/L, 15 patient samples above 7.5 ng EPO/L were selected for a WGA chromatography analysis.

2.4. Urine specimens selected for affinity purification and chromatographic analysis

Urine specimens from 9 healthy volunteer donors (men and women between 20 and 60 years old) and from 15 patients receiving erythropoiesis stimulating agents (ESAs) were analysed. Seven of the patients were maintaining haemodialysis (HD), while the other patients came from a

haematology clinic. The patients were subcutaneously (s.c.) injected at a rate from four times per week (4x w) to once every third week (3^{rd} w), with doses ranging from 2.000 to 30.000 IU (17-250 μg) for epoetin, and from 20 to 150 μg of darbepoetin α (Table 1). The samples were divided into aliquots and frozen at -20° C. The collection of samples was approved (D. No. 2005:307, 2006:062) by the local ethics committee.

2.5. Affinity purification of EPO from urine using anti-EPO monolith

Urine samples from 9 healthy individuals with EPO concentration in the range 4.5-51 ng/L, from 15 patients with samples in the range 7.6-798 ng/L, and from 4 urine samples spiked with Eprex (58 ng/L, 92% ESA), NeoRecormon (79 ng/L, 72% ESA and 97 ng/L, 96% ESA) and Aranesp (46 ng/L, 90% ESA) were affinity purified. The thawed urine specimens were gently turned end-over-end to distribute the precipitations evenly, and 20 mL urine samples were pH corrected to a pH of 7-8. The urine mixture was passed through a 13 mm ø wide and 0.15 mm long anti-EPO monolith column (prototype kit from MAIIA Diagnostics, Uppsala, Sweden), and EPO was captured and eluted to a final volume of 0.24 mL, in accordance with a previous description [23]. The average recovery of EPO was 64 % for the 28 urine specimens. The affinity purified EPO was stored at -20° C until it was used for WGA separation.

2.6. WGA-Sepharose column

The WGA-Sepharose was prepared by reacting WGA (Medicago, Uppsala, Sweden) with NHS-Sepharose (GE Healthcare, Uppsala, Sweden) by adding 4.3 mg WGA to one mL of sedimented gel and proceeding in accordance with the instructions from the gel supplier.

2.7. Separation of EPO isoforms using WGA-Sepharose

About 1 mL of diluted sample containing 120-400 pg of anti-EPO affinity purified urine EPO was applied to a 0.9 mL HR 5/10 column of WGA-Sepharose, connected to an ÄKTAexplorer 10 automatic chromatography system (GE Healthcare, Uppsala, Sweden). The sample also contained 0.2 mg BSA/ml, 20 mM bis-tris pH 6.4, 0.1 % tween 20, 0.02 % NaN_3, 8 μM pepstatin A and 1/500 protease inhibitor cocktail (Sigma P8340). The chromatographic separation was performed with a starting buffer containing 20 mM TRIS pH 7.5, 0.15 M NaCl, 0.1% tween 20, 0.05% NaN_3 and 1/500 protease inhibitor. The flow rate was 0.5 mL/min and sample application and washing with a starting buffer was performed during 7 minutes. The desorption gradient of competing sugar derivative was formed by mixing the starting buffer with the same buffer containing N-acetyl glucose amine (GlcNAc). A linear gradient from 0 to 15 mM GlcNAc was formed during 10 minutes, and 15 mM and 50 mM GlcNAc were applied during 2 and 3 minutes, respectively. During the separation, 55 fractions with a volume of 0.35 mL were collected in microtiter wells.

2.8. Lateral flow immunoassay for quantification of EPO

Each one of the 55 fractions per applied sample from the WGA chromatography were measured with EPO immunoassay. An aliquot of 50 µL of the 0.35 mL fractions was used for analysis. The EPO lateral flow immunoassay kit (EPO Quantification Kit, art. No 100, MAIIA Diagnostics), was kindly provided and used as described by the producer.

2.9. Calculations from the WGA-Sepharose chromatography

The desorption profile was obtained by calculating the percentage of EPO (%EPO) in each of the 55 collected fractions between 3.15 to 22.75 mL elution volumes, using the sum of the fractions for determination of total amount of EPO. The median elution volume (mL) that was required to elute 50 % of EPO was calculated for each sample.

Patient samples containing ESA were indicated by calculating the strong-to-medium WGA reacting EPO ratio (SWRE-ratio) by summing the % EPO for the elution volumes from 3 fractions (1.05 mL) between 19.25 to 19.95 mL (ESA position) and from 3 fractions between 12.95 to 13.65 mL (normal endogenous EPO position) and calculate the ratio ($\%EPO_{(19.25\text{-}19.95)}$ / $\%EPO_{(12.95\text{-}13.65)}$).

Samples with deviating endogenous EPO were identified by calculating the weak-to-medium WGA reacting EPO ratio (WWRE-ratio) by summing the % EPO for the elution volumes from 3 fractions (1.05 mL) between 9.1 to 9.8 mL and from 3 fractions between 12.95 to 13.65 mL (normal endogenous EPO position) and calculate the ratio ($\%EPO_{(9.1\text{-}9.8)}$ / $\%EPO_{(12.95\text{-}13.65)}$).

Estimation of the percentage of non-normal EPO populations was performed by comparing the sample desorption profile with the generated profile with the best fit, obtained by mixing, in known proportions, the distribution values for the two endogenous EPO populations and for epoetin or darbepoetin.

2.10. Statistics

Values are means ± one standard deviation (SD) if not otherwise stated. Differences between groups were examined by Student´s *t*-test or Mann-Whitney test and statistical significance was accepted at $P< 0.05$.

3. Results

3.1. EPO desorption profile for wheat germ agglutinin (WGA) affinity chromatography

Endogenous urine EPO from healthy individuals, rhEPO (epoetin α and β), and the EPO analogue darbepoetin α all bound to the lectin ligand WGA, immobilized on a Sepharose column. During desorption, it was found that they showed different desorption profiles (see Fig. 1A) when utilizing gradient elution with a low concentration of the competing sugar derivative GlcNAc. Fig. 1B shows the results from some of the patients. The majority of the patient samples showed EPO desorption profiles with a strong WGA binding, in accordance with the ESA used for treatment of their anaemia. However, some of the patients showed a form of EPO with weaker reaction to WGA compared to endogenous EPO from healthy individuals. The profile for EPO from a urine sample collected from patient 2 showed presence of both the weak WGA reacting EPO forms and injected darbepoetin. Table 1 shows the percentage of weak and strong reacting EPO populations, defined by the desorption profile, in the samples.

3.2. Identifying EPO populations by their median EPO elution volume

For endogenous EPO (n=9), epoetin preparations (n=5) and darbepoetin preparations (n=2), the median EPO elution volumes were 13.48 ± 0.23, 14.79 ± 0.35, 17.05 ± 0.20 (mL, mean ± SD), respectively (Fig. 2). The epoetin and darbepoetin preparations were significantly (P<0.001) differentiated from endogenous EPO, as well as from each other.

The EPO chromatography results for the 15 patients receiving rhEPO or darbepoetin showed that 60% (9/15) of the ESA treated patients had median elution volumes above the 97.8% confidence limit (CL), indicating presence of ESA. Samples from 27% (4/15) of the patients had very low elution volumes (11.3 ± 0.12 mL, n=4), clearly deviating from both endogenous and recombinant EPO. However, for the results from the urine sample from patient 2, containing both weak reacting forms and darbepoetin, as shown in Fig. 1B, the median elution value indicated only the presence of ESA with a median elution value of 14.86 mL.

3.3. Identifying specific EPO populations by their weak or strong WGA reacting EPO ratios

It was clear, after analysing the urine specimen from patient 2, that presence of three different EPO populations in a sample required ratio-calculations at selected positions in the WGA-Sepharose elution profile to describe the EPO population distribution. The presence of weak and strong WGA reacting EPO forms was estimated as a ratio relative to the normal endogenous EPO form. The best resolution was obtained when using the positions at 9.45 mL (weak reaction), 13.3 mL (normal endogenous form), and 19.6 mL (strong reaction) for calculation. The positions are

indicated with arrows in Fig. 1B. The ratio results for both weak WGA reaction EPO (WWRE-ratio) and strong WGA reaction EPO (SWRE-ratio) are found in Fig. 3.

Presence of ESA was revealed by the estimation of strong reacting EPO forms, which gave values of 3.06 ± 0.45 in SWRE-ratio for darbepoetin preparations (n=2), compared to 0.75 ± 0.17 in samples prepared with epoetin (n=5) added to buffer or urine ($P<0.001$). The samples from 9 healthy individuals showed a SWRE-ratio of 0.26 ± 0.053 and the values for both darbepoetin and epoetin were significantly different ($P<0.001$). Among the samples from the 15 ESA treated patients, 67% (n=10) had SWRE-ratio values outside the one-tailed 97.8% CL, in the range 0.57-1.90, indicating presence of epoetin or darbepoetin. The results for each individual patient are found in Table 1.

Weak WGA reacting forms appearing as the dominant EPO form (>50%) were found in the urine for patients 15, 14, 5, 12 and 2, having high WWRE-ratio values of 1.33 ± 0.27 (mean ± SD). These values are significantly different ($P<0.005$) from the endogenous values (0.13 ± 0.030) from 9 healthy individuals. The weak WGA reacting endogenous EPO form was present (above 97.8% CL) in 53% (8/15) of the patient samples. Four of the patients had both deviating EPO and ESA in the urine. One of them was patient 2 with a SWRE-ratio of 2.40 and a WWRE-ratio of 1.06. The ratio results for patient 2 is thus in accordance with the information obtained from the desorption profile in Fig. 1B, while calculation of the mean elution value indicated that only ESA was present.

4. Discussion

The presented method makes it possible to estimate the production site of EPO by measuring the interaction strength between the lectin WGA and the carbohydrate structures of EPO. EPO is rich in core fucosylated tetraantennary oligosaccharides [24], and WGA is expected to bind to the terminal parts of the carbohydrate structures, especially the protruding polylactosamine structures [25]. Such structures are much more common in rhEPO than in endogenous EPO [24]. The stronger WGA interaction of rhEPO is in accordance with our results, as rhEPO required higher elution volume than endogenous EPO to pass the WGA column. The broad distribution profile after WGA chromatography for endogenous EPO is most probably due to the presence of a large number of glycosylated EPO forms with minor differences in their WGA affinity, in accordance with the 20-30 forms found when separating the isoforms due to charge [17]. EPO from different production sites have different proportions of WGA reacting isoforms, which can be measured as a shift in the median elution volume. In this study, several of the patient samples contained both weaker and stronger WGA reacting EPO, compared to the normal endogenous form, and calculation of the median elution volume may then result in a false classification. By calculating the weak-to-medium and strong-to-medium WGA reacting ratio for the EPO distribution, it was

possible to correctly classify samples with three different populations of EPO as well. The obtained values were compared to the values from healthy individuals, and WWRE-ratio values above 97.8% CL classified the patient samples as containing deviating endogenous EPO while SWRE-ratios above 97.8% CL classified the patient samples as containing epoetin or darbepoetin.
It is well established that there is a remaining minor production in the liver in adulthood while most of the EPO, under normal conditions and in anaemia, is produced in the kidneys. The deviating weak WGA reacting endogenous EPO is most probably produced in the liver, which is the dominant non-renal production site. Also, the main source of EPO in the human fetus comes from the liver [9], and EPO in umbilical cord serum has been found to have a different glycosylation, with more basic charge, compared to adult EPO [17,26]. Two of the urine specimens from patients with deviating weak EPO forms, patient 5 and 15, showed also more basic EPO forms with an isoelectric point even higher than for rhEPO alpha and beta [26]. Substantial EPO production from non-renal sources has also been shown in anephric subjects, both animals and humans [9,12,13]. The assumption that the deviating EPO is produced in the liver is supported by reports of an increased non-renal EPO production during proliferative repair of the liver [12,13], and high levels of EPO mRNA in the liver during hypoxia [11]. We therefore conclude that the deviating, weak reacting, non-renal endogenous EPO form distinguished in this study emanates from the liver.
Liver EPO was found in 53% (8/15) of the tested patient samples, measured as deviatingly high weak-to-medium WGA reacting EPO ratio. The low percentage, below 10%, of liver-produced EPO expected in samples from healthy individuals has not been possible to distinguish from kidney EPO in the desorption profile (Fig. 1). The 8 patient samples had a higher percentage of liver EPO than the normal samples, and the main reason that it was possible to recognise liver EPO in the patient samples is due to their lack-, or low level, of kidney EPO. The 7 tested patients with severe renal impairment with maintenance haemodialysis were all producing liver EPO. It should be noted that only urine samples with a value above 7.5 ng EPO/L were selected for WGA chromatography, and for the 26 concentration-tested HD patients samples 27% were above 7.5 ng/L while 54% were below 3 ng/L. Among the 8 chromatography-tested haematological patients, only one showed presence of liver EPO.
Four samples contained 90-100% of liver EPO and in the range 8 to10 ng/L, which means that the liver EPO production was comparable to the kidney EPO production, with a median concentration of 8.5 ng/L, found in the urine from healthy individuals [22]. This indicates that the liver production can show more than a 20-fold increase, assuming 5 % liver EPO in the normal samples.
It seems that not all patients with renal failure or other anaemia can increase the non-renal EPO production, as 54% of the HD patients had EPO concentrations below 3 ng/L, compared to 7.5%

of the samples from healthy individuals [22]. However, none of our patients had an EPO level in their urine that was adequate for the degree of anaemia (a severe anaemia without renal disease may increase EPO levels a 1000-fold), indicating a limitation in the ability to induce liver EPO production.

In 5 of the 15 patient samples of EPO, no exogenous EPO was detected, even though the patients had therapy with epoetin or darbepoetin. The reason for this is that the clearance of exogenous EPO is very rapid and samples were not taken immediately after injection.

5. Conclusion

This study shows that some patients with end-stage renal insufficiency can increase liver EPO production up to a total EPO concentration within the reference range for normal individuals, although this concentration is not adequate for the degree of anaemia.

By the use of WGA based EPO-specific glycoform analytical methods, it seems, for the first time, to be possible to measure physiological and pathological shifts in EPO production sites. Such measurements can give answer to the question “Why not the liver instead of the kidney?” recently actualized in Blood [27]. Pharmacological treatment to enhance EPO production is a potential alternative to long term rhEPO treatment, and this method makes it possible to distinguish the enhanced EPO production rate for the liver and for the kidney.

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References

[1] G.L. Semenza, HIF-1 and mechanisms of hypoxia sensing. Curr Opin Cell Biol 13 (2001) 167-71.
[2] D. Lando, D.J. Peet, J.J. Gorman, D.A. Whelan, M.L. Whitelaw, and R.K. Bruick, FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 16 (2002) 1466-71.
[3] A.C. Epstein, J.M. Gleadle, L.A. McNeill, K.S. Hewitson, J. O'Rourke, D.R. Mole, M. Mukherji, E. Metzen, M.I. Wilson, A. Dhanda, Y.M. Tian, N. Masson, D.L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P.H. Maxwell, C.W. Pugh, C.J. Schofield, and P.J. Ratcliffe, C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107 (2001) 43-54.
[4] C. Dame, H. Fahnenstich, P. Freitag, D. Hofmann, T. Abdul-Nour, P. Bartmann, and J. Fandrey, Erythropoietin mRNA expression in human fetal and neonatal tissue. Blood 92 (1998) 3218-25.
[5] A. Weidemann, and R.S. Johnson, Nonrenal regulation of EPO synthesis. Kidney Int 75 (2009) 682-8.
[6] V.H. Haase, Hypoxic regulation of erythropoiesis and iron metabolism. Am J Physiol Renal Physiol 299 (2010) F1-13.
[7] W.M. Bernhardt, M.S. Wiesener, P. Scigalla, J. Chou, R.E. Schmieder, V. Gunzler, and K.U. Eckardt, Inhibition of prolyl hydroxylases increases erythropoietin production in ESRD. J Am Soc Nephrol 21 (2010) 2151-6.
[8] W. Querbes, R.L. Bogorad, J. Moslehi, J. Wong, A.Y. Chan, E. Bulgakova, S. Kuchimanchi, A. Akinc, K. Fitzgerald, V. Koteliansky, and W.G. Kaelin, Jr., Treatment of erythropoietin deficiency in mice with systemically administered siRNA. Blood 120 (2012) 1916-22.
[9] E.D. Zanjani, J.L. Ascensao, P.B. McGlave, M. Banisadre, and R.C. Ash, Studies on the liver to kidney switch of erythropoietin production. J Clin Invest 67 (1981) 1183-8.
[10] K.U. Eckardt, P.J. Ratcliffe, C.C. Tan, C. Bauer, and A. Kurtz, Age-dependent expression of the erythropoietin gene in rat liver and kidneys. J Clin Invest 89 (1992) 753-60.
[11] C.C. Tan, K.U. Eckardt, J.D. Firth, and P.J. Ratcliffe, Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. Am J Physiol Renal Physiol 263 (1992) F474-481.
[12] S. Brown, J. Caro, A.J. Erslev, and T.G. Murray, Spontaneus increase in erythropoietin and hematocrit value associated with transient liver enzyme abnormalities in an anephric patient undergoing hemodialysis. Am J Med 68 (1979) 280-284.
[13] M. Radovic, W. Jelkmann, L. Djukanovic, and V. Ostric, Serum erythropoietin and interleukin-6 levels in hemodialysis patients with hepatitis virus infection. J Interferon Cytokine Res 19 (1999) 369-73.
[14] H.J. Gabius, Biological information transfer beyond the genetic code: the sugar code. Die Naturwissenschaften 87 (2000) 108-21.
[15] A.J. Erslev, J. Caro, E. Kansu, O. Miller, and E. Cobbs, Plasma erythropoietin in polycythemia. Am J Med 66 (1979) 243-7.
[16] G. Birgegard, O. Miller, J. Caro, and A. Erslev, Serum erythropoietin levels by radioimmunoassay in polycythaemia. Scand J Haematol 29 (1982) 161-7.
[17] L. Wide, and C. Bengtsson, Molecular charge heterogeneity of human serum erythropoietin. Br. J. Haematol. 76 (1990) 121-7.
[18] L. Wide, C. Bengtsson, B. Berglund, and B. Ekblom, Detection in blood and urine of recombinant erythropoietin administered to healthy men. Medicine and Science in Sports and Exercise 27 (1995) 1569-76.
[19] L. Franco Fraguas, J. Carlsson, and M. Lönnberg, Lectin affinity chromatography as a tool to differentiate endogenous and recombinant erythropoietins. J. Chromatogr. 1212 (2008) 82-88.

[20] M. Lonnberg, M. Andren, G. Birgegard, M. Drevin, M. Garle, and J. Carlsson, Rapid detection of erythropoiesis-stimulating agents in urine and serum. Anal. Biochem. 420 (2012) 101-14.
[21] M. Lonnberg, U. Bondesson, F. Cormant, P. Garcia, Y. Bonnaire, J. Carlsson, M.A. Popot, N. Rollborn, K. Rasbo, and L. Bailly-Chouriberry, Detection of recombinant human EPO administered to horses using MAIIA lateral flow isoform test. Anal. Bioanal. Chem. 403 (2012) 1619-28.
[22] M. Lönnberg, M. Drevin, and J. Carlsson, Ultra-sensitive immunochromatographic assay for quantitative determination of erythropoietin. J. Immunol. Methods 339 (2008) 236-244.
[23] M. Lonnberg, Y. Dehnes, M. Drevin, M. Garle, S. Lamon, N. Leuenberger, T. Quach, and J. Carlsson, Rapid affinity purification of erythropoietin from biological samples using disposable monoliths. J Chromatogr A 1217 (2010) 7031-7.
[24] M. Takeuchi, S. Takasaki, H. Miyazaki, T. Kato, S. Hoshi, N. Kochibe, and A. Kobata, Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. J Biol Chem 263 (1988) 3657-63.
[25] T. Osawa, and T. Tsuji, Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. Annu. Rev. Biochem. 56 (1987) 21-42.
[26] Y. Dehnes, M. Lönnberg, M. Borgen, and P. Hemmersbach, Shifted isoelectric profiles of endogenous EPO possible due to different physiological origin. Recent Advances in Doping Analysis 16 (2008) 359-362.
[27] J. Fandrey, Why not the liver instead of the kidney? Blood 120 (2012) 1760-1.

Fig. 1. EPO desorption profiles for WGA-Sepharose chromatography.
Panel A shows the distribution profiles of endogenous EPO (n=9), rhEPO (n=5, epoetin α and β), and the EPO analogue darbepoetin (n=2). The recombinant forms required a higher concentration of the WGA competing sugar derivative GlcNAc for desorption compared to endogenous EPO. Panel B shows the mean result for the individual analysis of urine from three patients (pat 5, 12 and 14) having a weak WGA reacting EPO isoform distribution, clearly distinguished from both the normal endogenous urine EPO as well as from the recombinant EPO forms. For patient 2, treated with darbepoetin, both the expected darbepoetin and the deviating EPO form was found. The arrows in panel B indicate the fractions that have been used to calculate ratios between weak (9.5 mL), medium (13.3 mL), and strong WGA (19.6 mL) interaction.

Fig. 2. Identification of deviating EPO by the median EPO elution volume.
Urine EPO from healthy individuals had a median elution volume at 13.48 ± 0.23 mL and the 95% confidence interval for this group is drawn in the figure. The prepared samples with darbepoetin and epoetin α and β were desorbed from the column at higher elution volumes due to their stronger affinity for the WGA ligand. 60% of the patients treated with darbepoetin (○) or epoetins (△) showed values indicating the presence of ESA. A very interesting group (27%) among the patients showed a weak WGA reacting form of EPO, different from both normal endogenous EPO and recombinant EPO, with a median elution volume at 11.3 ± 0.12 mL. However, patient 2 having a distribution profile revealing presence of both weak and strong WGA reacting EPO forms, showed a misleading median elution volume of 14.86 mL.

Fig. 3.

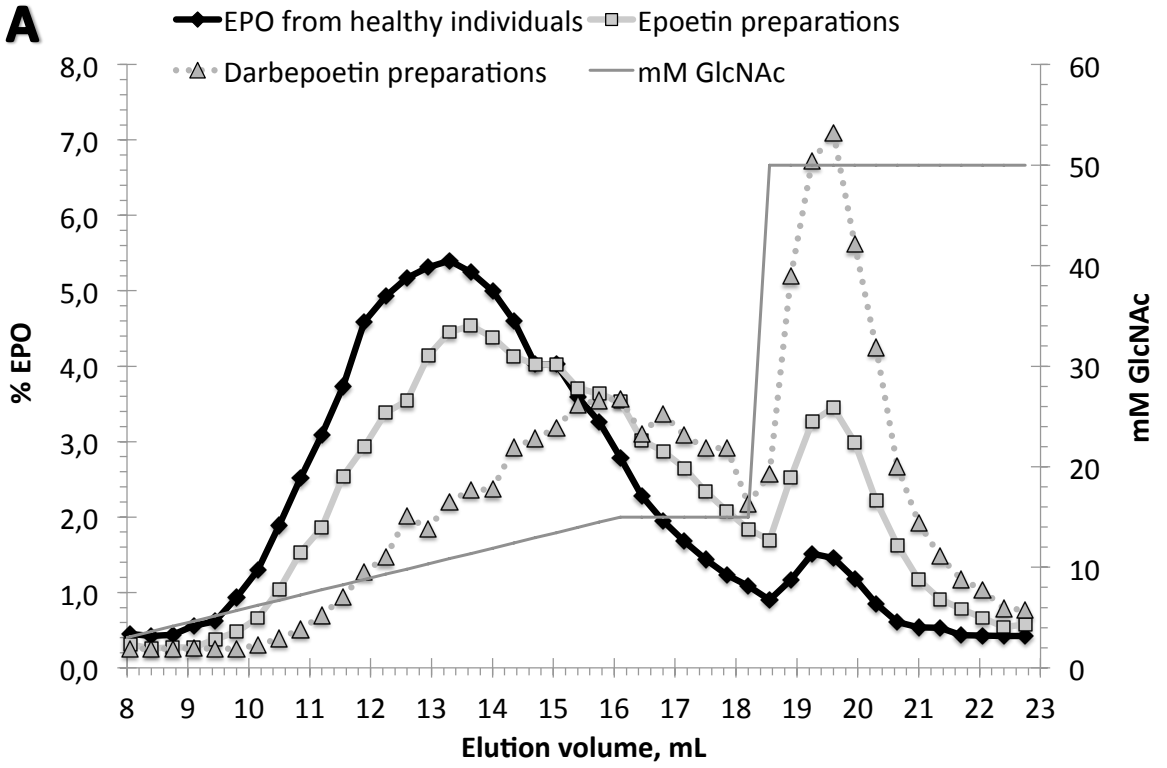
Identification of samples containing three EPO populations was made by calculating specific ratios.

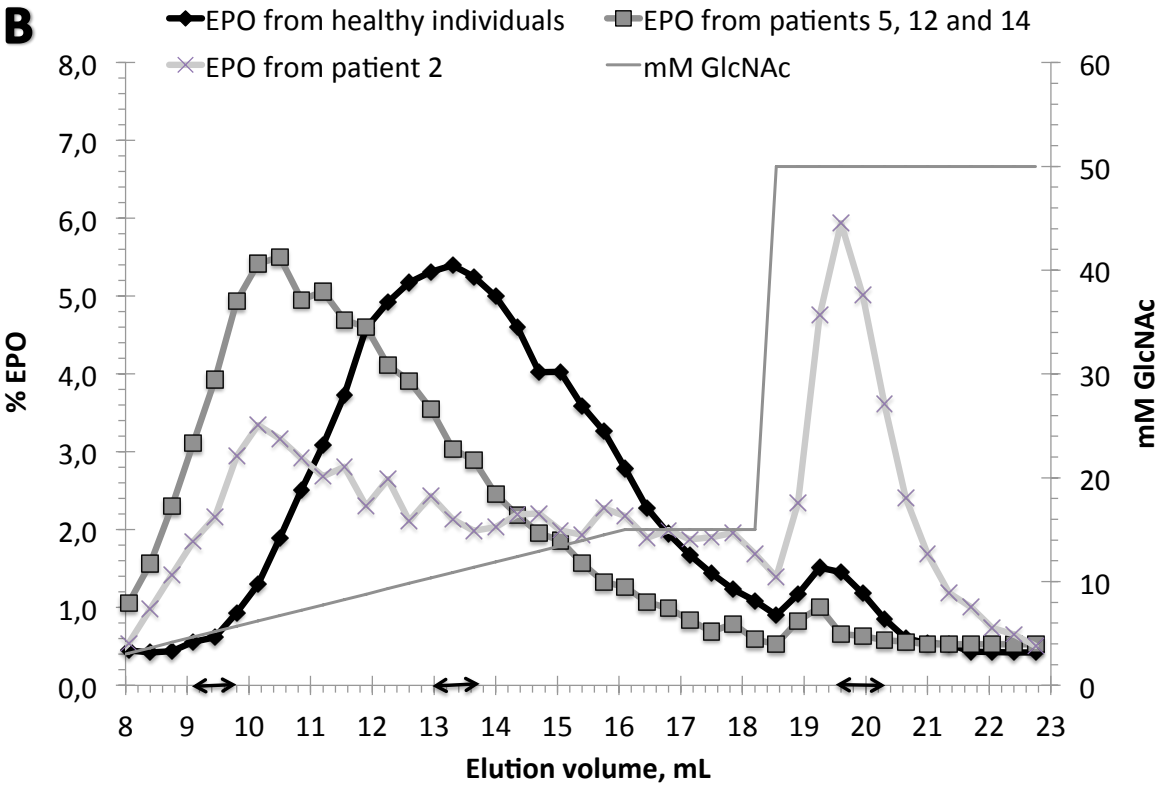
The results for normal endogenous EPO is found in field 3, delimited with the upper 97.8% CL shown as dotted lines. Field 1 and 2 show eight samples containing deviating endogenous EPO with weak WGA interaction. Field 2 and 4 show samples containing epoetin or darbepoetin, and only samples containing darbepoetin have a SWRE-ratio above 1. Field 2 shows the results for four ESA treated patients having both recombinant forms and deviating endogenous EPO in their urine. Patient 2 is in this group, for which only the presence of darbepoetin was shown when calculating the median elution volume.

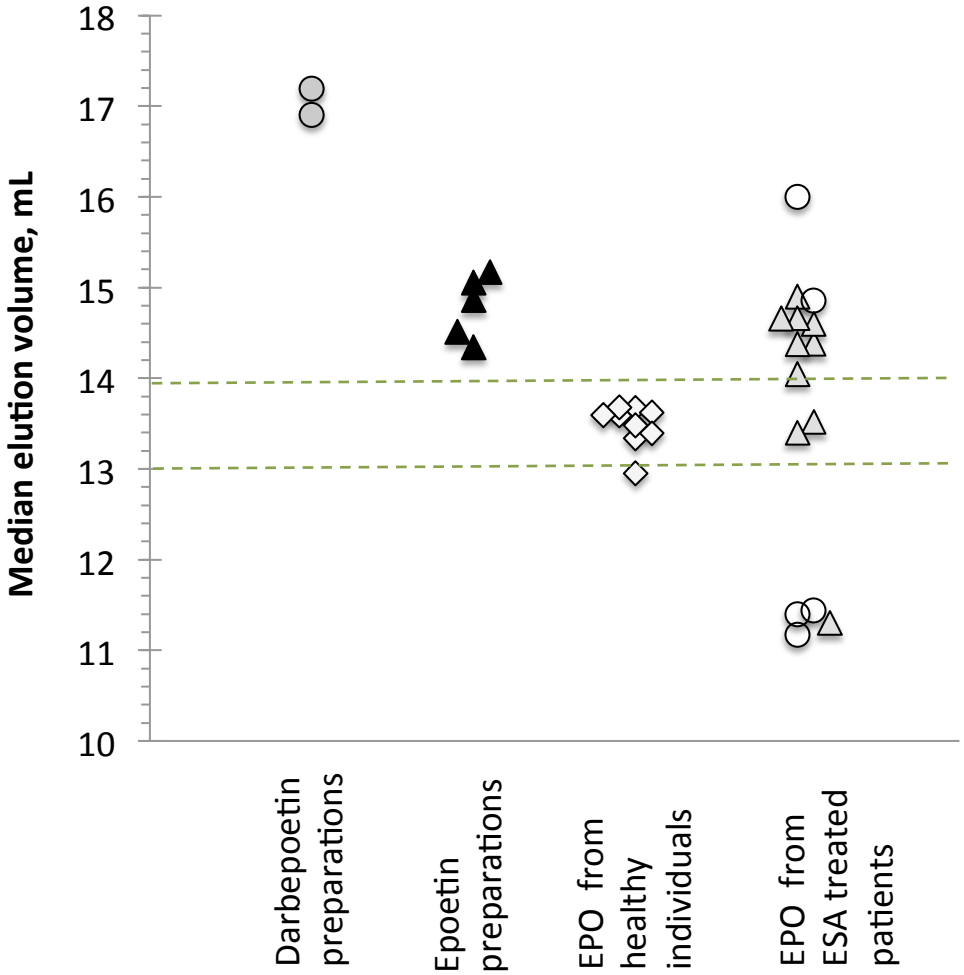
Table 1

Data and results for the 15 patient samples.

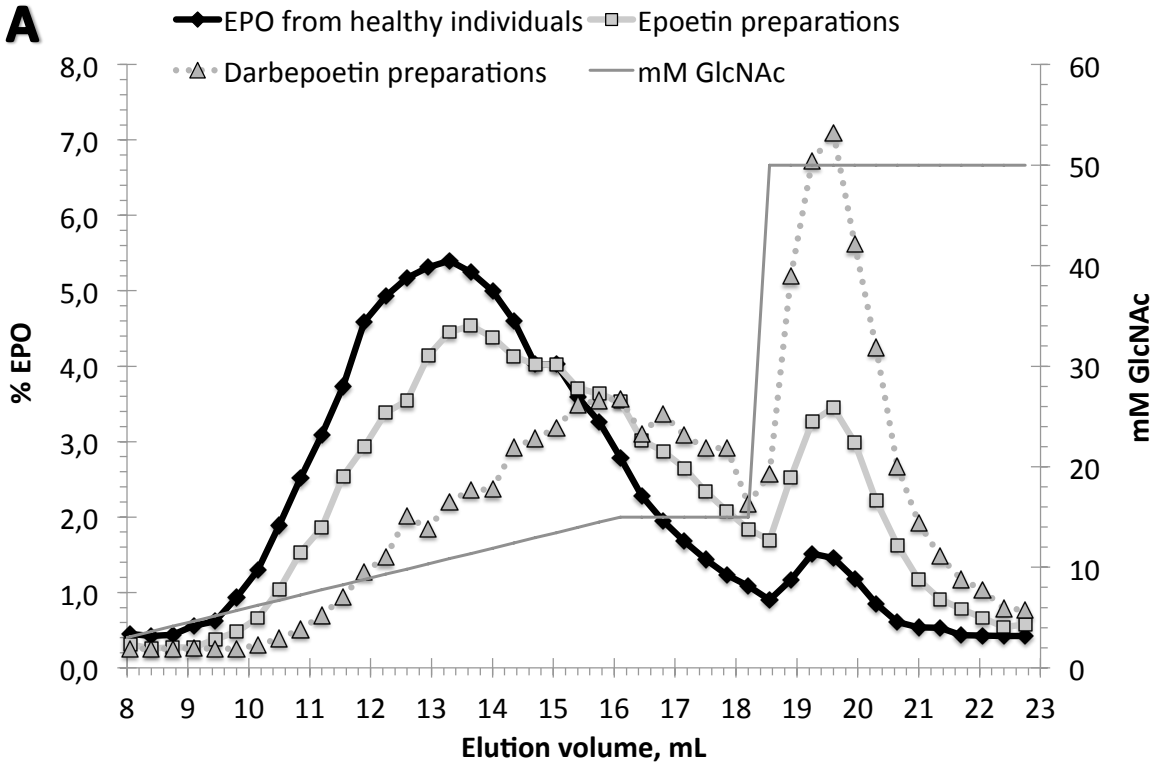
The calculation from the WGA-Sepharose chromatography, showing the presence of different EPO populations, is found to the right in the table. Presence of epoetin and darbepoetin, which are strong WGA reacting EPOs, were found in only 67% (10/15) of the patient samples ,due to the rapid clearance of exogenous forms. A deviating endogenous EPO form, interacting more weakly with WGA than the normal endogenous EPO form, was found in 53% (8/15) of the patient samples. This EPO form is likely to emanate from the liver and was found in all the haemodialysis (HD) patients. The percentage (%) of the EPO population with stronger or weaker WGA reactivity, compared to normal endogenous EPO, was estimated from the distribution profile. Four of the patient samples showed more than 90% of the liver EPO population, and in a quite normal total EPO concentration.

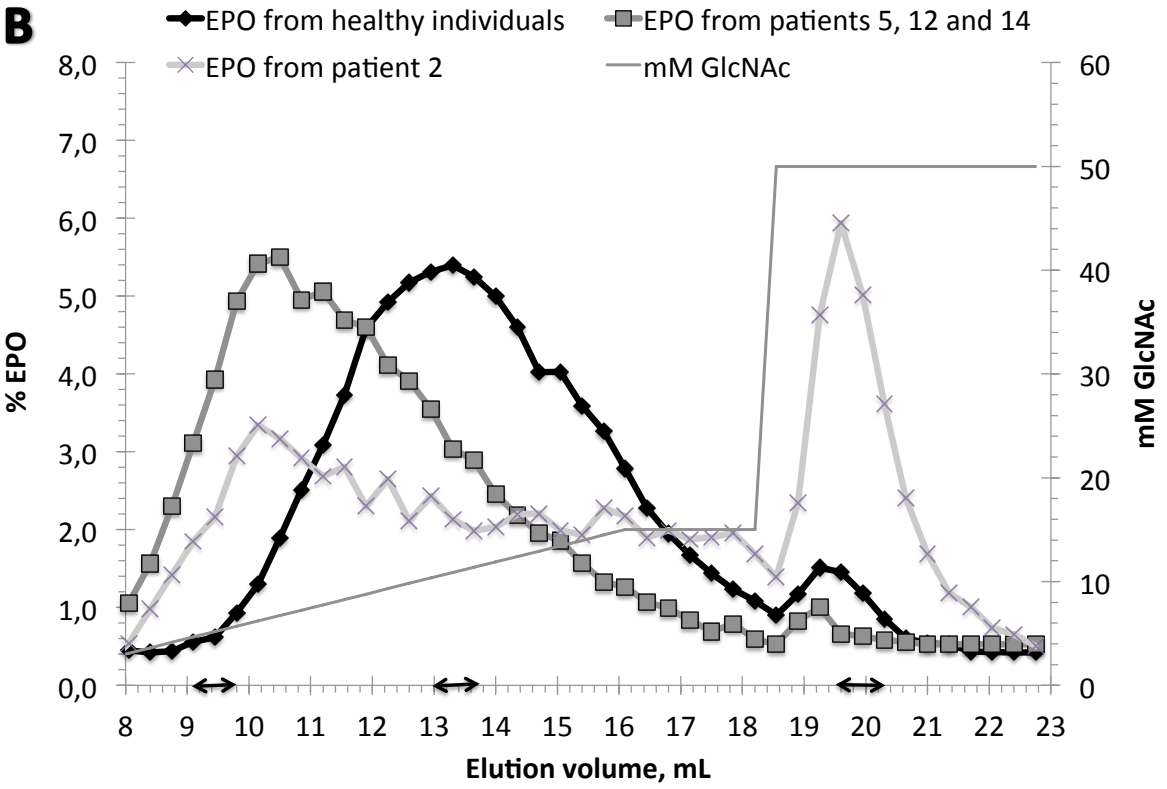

A
EPO from healthy individuals
Epoetin preparations
Darbepoetin preparations
mM GlcNAc
% EPO
mM GlcNAc
Elution volume, mL

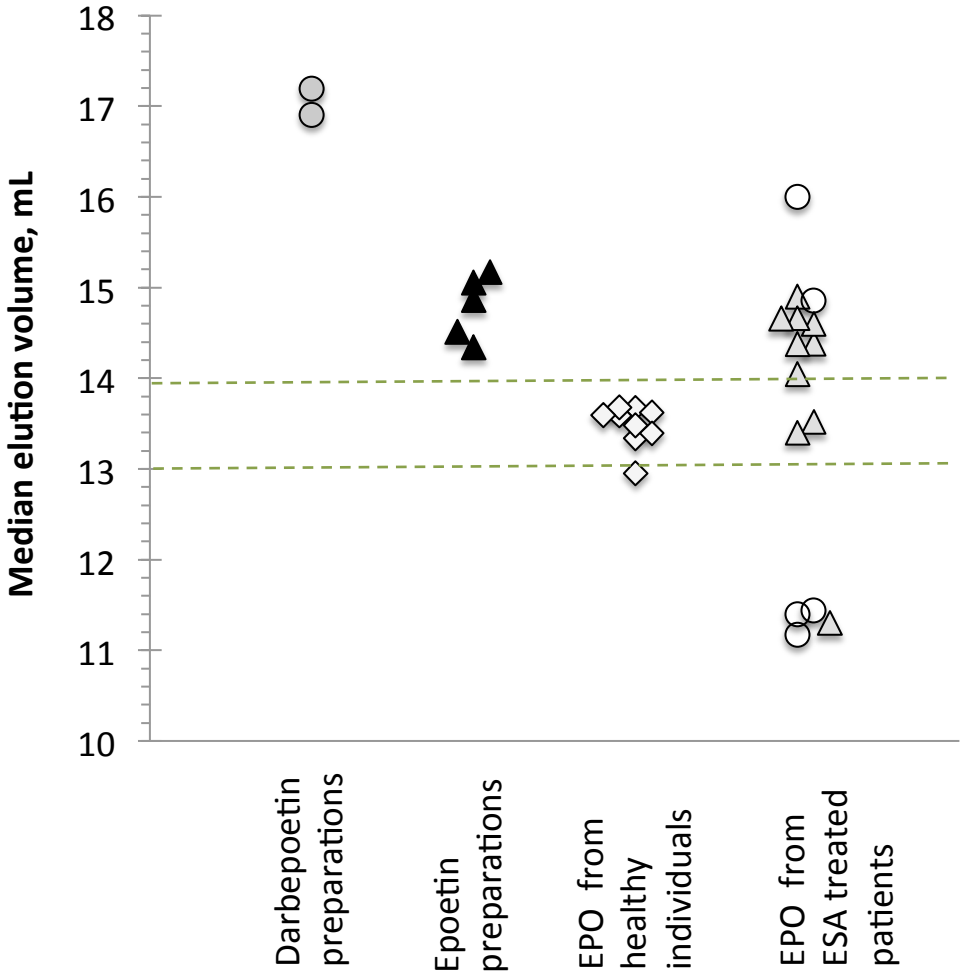

B
EPO from healthy individuals
EPO from patients 5, 12 and 14
EPO from patient 2
mM GlcNAc
% EPO
mM GlcNAc
Elution volume, mL

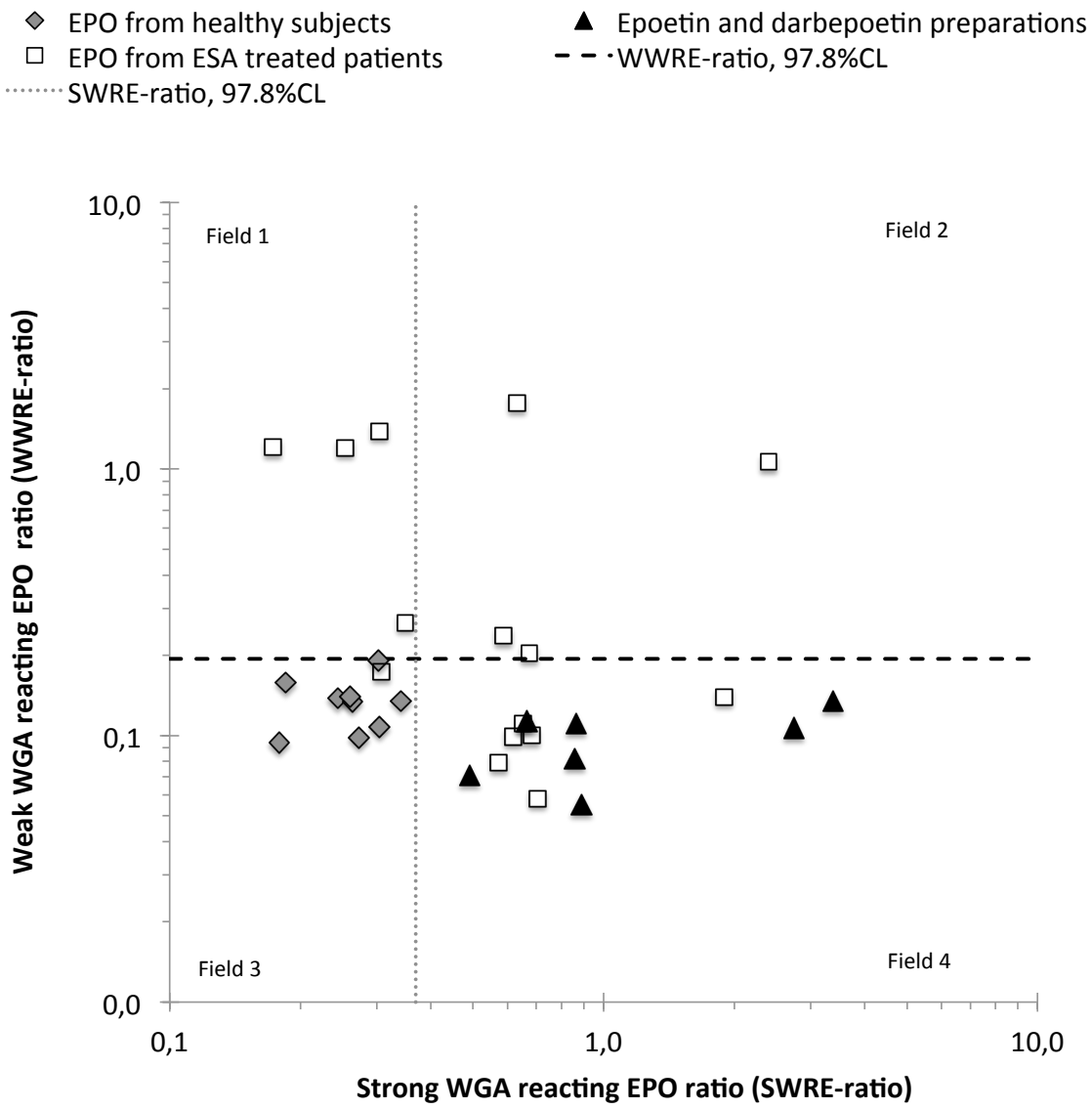

Median elution volume, mL
18
17
16
15
14
13
12
11
10
Darbepoetin preparations
Epoetin preparations
EPO from healthy individuals
EPO from ESA treated patients

Patients						WGA-Sepharose results					
						Strong WGA reacting EPOs			Weak WGA reacting EPOs		
				Days after	uEPO	Epoetin and Aranesp			Liver EPO?		
ID	Type	Drug	sc dose	last injection	ng/L	SWRE-ratio		%	WWRE-ratio		%
15	HD	Aranesp	20 µg/w		8	0,63		<20	1,77		90
14	HD	Aranesp	20 µg/2nd w		10	0,30	N	<20	1,39		98
5	HD	Neorecormon	3000 IU, 2x w		9	0,17	N	<20	1,21		100
12		Aranesp	150 µg/w	16	8	0,25	N	<20	1,20		98
2	HD	Aranesp	40-50 µg/5th day		37	2,40		50	1,06		50
1	HD	Neorecormon	5000 IU, 2x w		30	0,35	N	<20	0,27		20
3	HD	Neorecormon	4000 IU, 4x w		22	0,59		60	0,24		15
4	HD	Neorecormon	2000 IU/w		13	0,68		75	0,20		13
8		Neorecormon	10000 IU/3rd w	9	30	0,31	N	<20	0,17	N	
10		Aranesp	150 µg/w	4	152	1,90		75	0,14	N	
9		Eprex	10000 IU/w	7	798	0,65		75	0,11	N	
11		Neorecormon	10000 IU/w	4	25	0,68		75	0,10	N	
6		Neorecormon	30000 IU/day	5	63	0,62		60	0,10	N	
13		Neorecormon	30000 IU/w	1	310	0,57		*75*	0,08	N	
7		Neorecormon	20000 IU/day	0,25	208	0,71		80	0,06	<N	
Healthy reference group (n=9)					mean ± 2SD	0,15-0,37			0,07-0,19		
Epoetin preparations (n=5)					mean ± 2SD	0,41-1,10			0,04-0,14		
Darbepoetin preparations (n=2)					mean ± 2SD	2,17-3,96			0,08-0,16		
						N= normal ratio			N= normal ratio		


A
EPO from healthy individuals
Epoetin preparations
Darbepoetin preparations
mM GlcNAc
% EPO
mM GlcNAc
Elution volume, mL


B
EPO from healthy individuals
EPO from patients 5, 12 and 14
EPO from patient 2
mM GlcNAc
% EPO
mM GlcNAc
Elution volume, mL


Median elution volume, mL
18
17
16
15
14
13
12
11
10
Darbepoetin preparations
Epoetin preparations
EPO from healthy individuals
EPO from ESA treated patients


EPO from healthy subjects
EPO from ESA treated patients
SWRE-ratio, 97.8%CL
Epoetin and darbepoetin preparations
WWRE-ratio, 97.8%CL
Field 1
Field 2
Field 3
Field 4
10,0
1,0
0,1
0,0
0,1
1,0
10,0
Weak WGA reacting EPO ratio (WWRE-ratio)
Strong WGA reacting EPO ratio (SWRE-ratio)