Liver Fatty Acid-binding Protein as a Sensitive Serum Marker of Acute Hepatocellular Damage in Liver Transplant Recipients, *Maurice M.A.L. Pelsers,[1,2] Alireza Morovat,[3] Graeme J.M. Alexander,[4] Wim T. Hermens,[2] Andrew K. Trull,[3] and Jan F.C. Glatz[1,2*]* ([1] Department of Physiology, [2] Cardiovascular Research Institute Maastricht, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands; Departments of [3] Clinical Biochemistry and [4] Medicine, Addenbrooke's Hospital, Cambridge CB2 2QQ, United Kingdom; * author for correspondence: fax 31-43-3884166, e-mail glatz@fys.unimaas.nl)

Serum tests of acute hepatocellular injury are commonly used to investigate the presence and monitor the progress of liver disease *(1, 2)*. The release of cytoplasmic proteins from damaged hepatocytes into the vascular system follows tissue necrosis caused by, e.g., acetaminophen intoxication, ischemia and reperfusion injury, or rejection after liver transplantation. Although the hepatocytes are in direct contact with the vasculature and no interstitial barrier is between the two, smaller proteins appear earlier in the circulation than do larger ones *(1)* and would therefore increase earlier in serum above their upper reference value after an acute hepatocellular injury. α-Glutathione *S*-transferase (α-GST; 26 kDa) is a more sensitive and specific marker of hepatocellular damage *(3, 4)* than either alanine transaminase (ALT; 96 kDa) or aspartate transaminase. α-GST is present in liver, kidney, and intestine; is released rapidly from damaged hepatocytes; and has a relatively short in vivo plasma half-life *(3, 4)*. The use of α-GST for the detection of hepatocellular injury secondary to acute rejection after liver transplantation improved the biochemical monitoring of patients and decreased mortality and morbidity *(3)*. In search of even smaller and more specific cytoplasmic proteins for the detection of liver injury, we have studied the liver-type fatty acid-binding protein (L-FABP). FABPs are a family of 15-kDa proteins that are involved in the intracellular transport of long-chain fatty acids *(5)*. To date, nine different FABPs have been identified and named according to the tissues in which they were first identified *(5)*. L-FABP occurs mainly in the liver but, in small quantities, also in kidney and small intestine. In the hepatic lobule, L-FABP is expressed in hepatocytes in a declining portal-to-central gradient *(5, 6)*. Extensive studies on heart-type FABP (H-FABP) have shown that this protein is released rapidly after myocardial injury and, in view of its low circulating concentrations in healthy individuals *(7, 8)*, is a sensitive marker of myocardial infarction *(9, 10)*, as is the intestinal FABP for intestinal injury *(11)*. Because all FABP types have a comparable mass *(5)*, we expected L-FABP to have a similar diagnostic capacity for acute liver damage.

The aim of our study was to compare the behavior of serum concentrations of L-FABP with ALT and α-GST in association with episodes of acute hepatocellular damage. The population of patients chosen was a group of 21 liver transplant recipients (15 males and 6 females) who had episodes of acute hepatocellular rejection during their posttransplantation stay in the hospital. Rejection episodes were diagnosed either histologically or, when biopsy was contraindicated (n = 3), by previously published clinical criteria based on deteriorating liver function tests, increased prothrombin time, and an increase in the peripheral blood eosinophil count *(12, 13)*. Immunosuppressive therapy consisted of treatment with steroids, azathioprine, and either tacrolimus or cyclosporine. After rejection, augmented immunosuppressive therapy was given in the form of three daily doses of 1 g of intravenous methylprednisolone. Blood samples were taken daily (usually in the early morning) up to 3 months after transplantation. The present study retrospectively considered the period from 4 days before to 3 days after either a biopsy-confirmed episode or the start of treatment for the episode. An increase in serum concentration or activity of at least 50% in relation to the previous daily value was taken to be significant. We chose the 50% value to exclude the influence of analytical variation on the interpretation of the changes in the daily values. Because L-FABP and α-GST are excreted by the kidneys, we measured plasma creatinine to evaluate kidney function *(14, 15)*.

To determine the biological variation of circulating L-FABP on the reference interval, plasma samples were obtained, as described previously *(7, 8)*, from 80 healthy individuals (40 males and 40 females) to study the influence of age and gender and from another 12 healthy individuals (6 males and 6 females) to study the influence of circadian rhythm. L-FABP was measured with a sandwich ELISA developed in collaboration with HyCult Biotechnology (Uden, The Netherlands). No influence of different matrices (plasma or serum) or cross-reactivity with other types of FABP was noticed. The detection limit of the assay was 0.1 μg/L. With calibrators containing 2 and 20 μg/L L-FABP, the intra- and interassay CVs were <5% and <15%, respectively. α-GST concentrations were measured by an enzyme immunometric assay obtained from Biotrin International. The CVs were 14% and 5.5% at α-GST concentrations of 7.5 and 25 μg/L, respectively. ALT activity was measured on a DuPont Dimension multichannel analyzer according to the manufacturer's instructions. The CV of the ALT method was <12%. Creatinine concentrations were measured by the Jaffe rate method and also on a DuPont Dimension multichannel analyzer.

The Mann–Whitney test for unpaired values was used to asses the influence of age, gender, and circadian rhythm on the values from the healthy donor groups, with $P < 0.05$ regarded as significantly different. A paired Wilcoxon signed-rank test was used to assess any difference between the increases in serum L-FABP, ALT, and α-GST. All data are expressed as medians, with the 25th–75th percentiles.

Healthy individuals had a median plasma L-FABP concentration of 9.46 μg/L, and the 99th percentile was 17.5 μg/L. In contrast to H-FABP *(5)*, there was no significant influence of age or gender on L-FABP concentrations in healthy individuals between 21 and 70 years of

Table 1. L-FABP plasma concentrations.[a]

Time	Plasma FABP, μg/L
0930–1700 (day)	6.11 (5.00–7.11)
1700–0100 (evening)	5.88 (4.80–6.60)
0100–0930 (night)	10.16 (8.0–12.78)[b]

[a] L-FABP plasma concentrations in healthy individuals (six males and six females, ages, 19–27 years) during different time blocks. Values are expressed as median (25th–75th percentile) for n = 12.
[b] $P < 0.01$.

age: females, 8.54 (7.14–11.06) μg/L; males, 9.87 (8.9–12.13) μg/L. However, similar to H-FABP *(5, 14)*, there was a significant increase ($P < 0.01$) in plasma L-FABP in healthy individuals during the night (Table 1). This may be explained by a reduced glomerular filtration rate at night, which decreases the clearance of L-FABP from the circulation. The presence of a circadian variation in serum L-FABP would not have influenced our results significantly, because daily blood samples were routinely collected during a narrow time window in the morning.

In the patient population, until the day of rejection, a significant (>50%) increase in serum L-FABP was observed in association with all 21 acute rejection episodes studied, whereas α-GST increased in association with 19 (91%) and ALT in association with only 9 (42%) episodes. L-FABP increased by a median of 2.0 days (range, −1 to 4 days) earlier than the day that acute rejection was diagnosed, whereas α-GST increased 1.5 days (range, −2 to 3 days) and ALT 0 days (range, −3 to 1 day) earlier. Notably, serum L-FABP increased earlier ($P = 0.038$) than α-GST. The cumulative number of patients with a significant (>50%) increase in serum marker protein concentrations or activities relative to the day of diagnosis of rejection is shown in Fig 1. (Figures showing the L-FABP, α-GST, and ALT concentrations in the intervals just before rejection or initiation of treatment are available with the online version of this Technical Brief at http://www.clinchem.org/content/vol48/issue11.)

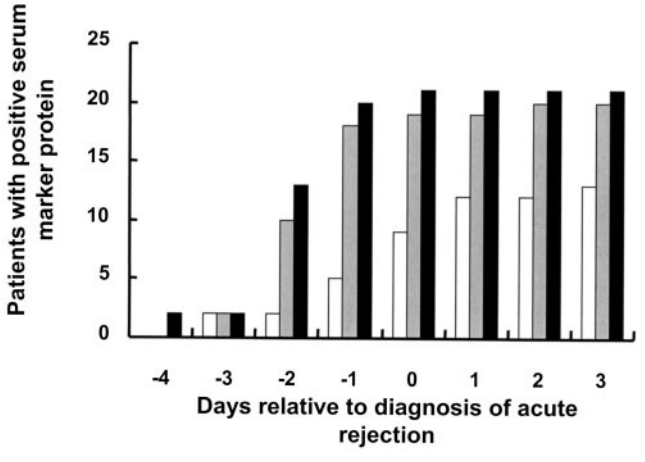

Fig. 1. Cumulative number of patients with a significant (>50%) increase in serum marker protein concentration or activity relative to the day of diagnosis of rejection.
□, ALT; ▨, α-GST; ■, L-FABP.

A significant impairment of renal function leads to an increased half-life of L-FABP in the circulation *(14)*. In our study, 13 transplant recipients had mild to marked impairment of renal function (creatine clearance range, 23.5–68.7 mL/min; median, 55.9 mL/min). Although this did not appear to hamper the ability of L-FABP to detect acute hepatocellular damage by serial sampling in these patients, care should be exercised in the interpretation of L-FABP values in patients with renal impairment.

This is the first study showing that L-FABP is a promising biochemical marker for the early detection of hepatocellular injury and that, in this small group of liver transplant patients, L-FABP performs as well if not better than α-GST. We believe that a combination of the low molecular mass of L-FABP and its relative abundance in the liver (2.8 mg/g wet weight; unpublished data) are the contributing factors to the relatively large and rapid increases in circulating L-FABP in association with acute liver injury, thus making L-FABP a sensitive marker. More frequent blood sampling at the time of a suspected rejection may enhance this sensitivity even more. The development of biosensor *(16, 17)* and turbidimetric assays *(18)* for FABPs should simplify the use of L-FABP and encourage its future application in routine clinical practice.

We thank J.P. Chapelle for providing blood samples to establish reference values and D. van der Voort for graphical assistance. This study was supported by the Ministry of Economic Affairs of The Netherlands (Grant BTS 97.188).

References

1. Dufour DR, Lott JA, Nolte FS, Gretch DR, Koff RS, Seeff LB. Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests. Clin Chem 2000;46:2027–9.
2. Trull AK. The clinical validation of novel strategies for monitoring transplant recipients. Clin Biochem 2001;34:3–7.
3. Hughes VF, Trull AK, Gimson A, Friend PJ, Prevost T, Alexander GJ, et al. Randomized trial to evaluate the clinical benefits of serum α-glutathione *S*-transferase concentration monitoring after liver transplantation. Transplantation 1997;64:1446–52.
4. Platz KP, Mueller AR, Haller GW, Müller C, Wenig M, Neuhaus R, et al. Determination of α- and π-glutathione-*S*-transferase will improve monitoring after liver transplantation. Transplant Proc 1997;29:2827–9.
5. Glatz JF, van der Vusse GJ. Cellular fatty acid-binding proteins: their function and physiological signification [Review]. Prog Lipid Res 1996;3:243–82.
6. Bass NM, Barker ME, Manning JA, Jones AL, Ockner RK. Acinar heterogeneity of fatty acid-binding protein in the livers of male, female and clofibrate treated rats. Hepatology 1989;9:12–21.
7. Pelsers MM, Chapelle J-P, Knapen M, Vermeer C, Muijtjens A, Hermens WT, et al. Influence of age and sex and day-to-day and within-day biological variation on plasma concentrations of fatty acid-binding protein and myoglobin in healthy subjects [Letter]. Clin Chem 1999;45:441–3.
8. Wodzig KWH, Pelsers MM, Van der Vusse GJ, Roos W, Glatz JF. One-step enzyme-linked immunosorbent assay (ELISA) for plasma fatty acid-binding protein. Ann Clin Biochem 1997;34:263–8.
9. Van Nieuwenhoven FA, Kleine AH, Wodzig KWH, Hermens WT, Kragten HA, Maessen JG, et al. Discrimination between myocardial and skeletal muscle injury by assessment of the plasma ratio of myoglobin over fatty acid-binding protein. Circulation 1995;92:2848–54.
10. Ishii J, Wang JH, Naruse H, Taga S, Kinoshita M, Kurokawa H, et al. Serum concentrations of myoglobin vs human heart-type cytoplasmic fatty acid-binding protein in early detection of acute myocardial infarction. Clin Chem 1997;43:1372–8.

11. Marks WH, Gollin G. Biochemical detection of small intestinal allograft rejection by elevated circulating levels of serum intestinal fatty acid binding protein. Surgery 1993;114:206–10.
12. Ascher N, Stock PG, Baumgarder GL, Payne WD, Najarian JS. Infection and rejection of primary hepatic transplant in 93 consecutive patients treated with triple immunosuppressive therapy. Surg Gynecol Obstet 1988;167: 474–84.
13. Hughes VF, Trull AK, Joshi O, Alexander GJM. Monitoring eosinophil activation and liver function following liver transplantation. Transplantation 1998; 65:1334–9.
14. Wodzig KW, Kragten JA, Hermens WT, Glatz JF, van Dieijen-Visser MP. Estimation of myocardial infarct size from plasma myoglobin or fatty acid-binding protein: influence of renal function. Eur J Clin Chem Biochem 1997;35:191–8.
15. Rees GW, Trull AK, Doyle S. Evaluation of an enzyme-immunometric assay for serum α-glutathione *S*-transferase. Ann Clin Biochem 1995;32:575–83.
16. Schreiber A, Feldbrugge R, Key G, Glatz JF, Spener F. An immunosensor based on disposable electrodes for rapid estimation of fatty acid-binding protein, an early marker of myocardial infarction. Biosens Bioelectron 1997;12:1131–7.
17. Key G, Schreiber A, Feldbrugge R, McNeil CJ, Jorgenson P, Pelsers MM, et al. Multicenter evaluation of an amperometric immunosensor for plasma fatty acid-binding protein: an early marker for acute myocardial infarction. Clin Biochem 1999;32:229–31.
18. Robers M, van der Hulst FF, Fischer M, Roos W, Salud CE, Eisenwiener HG, et al. Development of a rapid microparticle-enhanced turbidimetric immunoassay for fatty acid-binding protein in plasma, an early marker of acute myocardial infarction. Clin Chem 1998;44:1564–7.

Comparison of the Isoelectric Focusing Patterns of Darbepoetin Alfa, Recombinant Human Erythropoietin, and Endogenous Erythropoietin from Human Urine,

Don H. Catlin,[1,3] Andreas Breidbach,[1] Steve Elliott,[2] and John Glaspy[3]* ([1] UCLA Olympic Analytical Laboratory, Department of Molecular and Medical Pharmacology, and [3] Department of Medicine, University of California, Los Angeles, CA 90025; [2] Amgen Inc., Thousand Oaks, CA 91320-1799; * address correspondence to this author at: UCLA Olympic Analytical Laboratory, 2122 Granville Ave., Los Angeles, CA 90025; fax 310-206-9077, e-mail dcatlin@ucla.edu)

Novel erythropoiesis-stimulating protein (Aranesp™; darbepoetin alfa) is a glycoprotein hormone with a longer serum half-life than recombinant human erythropoietin (rHuEPO) *(1)*. The polypeptide backbone of the human EPO molecule has an invariant amino acid sequence; however, the carbohydrate side chains exhibit microheterogeneity in sugar content and structure *(2–4)*. A negatively charged sialic acid molecule typically caps the end of each arm of a carbohydrate chain. As a consequence, the variable nature of the sialic acid content gives rise to EPO isoforms with differences in charge *(3)*. After purifying isoforms of rHuEPO, Egrie and coworkers *(5, 6)* discovered a direct correlation between the number of sialic acid groups on the carbohydrate part of rHuEPO and both its serum half-life and biological activity, as well as an inverse relationship with receptor binding. These data showed that pharmacokinetic factors have a greater influence on biological activity than receptor binding affinity. These principles explain the increased half-life and increased in vivo activity of darbepoetin alfa, which contains 5 N-linked carbohydrate chains and up to 22 sialic acids *(5, 7)*. In contrast, rHuEPO has 3 N-linked carbohydrate chains and a maximum of 14 sialic acids *(5, 7)*.

Similar clinical responses can be achieved by administering darbepoetin alfa once a week or rHuEPO three times a week *(8, 9)*. The efficacy of darbepoetin alfa in the treatment of anemia associated with chronic renal failure has been shown *(10)*, and in 2001 it was approved by the US Food and Drug Administration for that indication. Darbepoetin alfa is under investigation for the treatment of anemia in cancer patients *(11)* and other applications. Although darbepoetin alfa was approved only recently, we detected darbepoetin alfa in the urine of three athletes competing in the 2002 Winter Olympic Games in Salt Lake City. To date, it has not been reported in human urine.

The isoelectric focusing (IEF) patterns of standard rHuEPO, endogenous human EPO in urine extracts, and administered rHuEPO in urine extracts have been reported *(12)*. This report describes the IEF pattern observed after applying the same method to standard darbepoetin alfa and post-administration urine extracts.

The pooled urine of two healthy, drug-free males was used as the endogenous HuEPO control urine (QC1). The rHuEPO positive control urine (QCP) was pooled urine from healthy individuals (eight males and seven females) who received rHuEPO on nine visits over 19 days (50 IU/kg at each visit). Some, but not all, urines were included in the pool. A urine collected from a female cancer patient 1 week after a single dose (0.675 μg/kg) of darbepoetin alfa (Aranesp; Amgen Inc., Thousand Oaks, CA) was used as the darbepoetin alfa control urine. The participants gave written informed consent under applications approved by the UCLA Office of Human Subject Protection.

Aranesp (60 mg/L) containing human serum albumin was obtained from a pharmacy. EPO Biological Reference Preparation (BRP) was obtained from the European Directorate for the Quality of Medicines (Strasbourg, France). Tris base, phosphate-buffered saline tablets, glycine, 100 mL/L Tween 80R (low peroxide), dithiothreitol, sucrose, and bovine serum albumin (RIA grade) were purchased from Sigma. Protease inhibitor (Complete) was purchased from Roche Diagnostics. Urea, Ready-Mix IEF acrylamide/bisacrylamide (29:1 by weight), ammonium persulfate, and *N,N,N,N*-tetramethylethylenediamine were purchased from Amersham Biosciences, and the ampholytes Servalyt 2-4, 4-6, and 6-8 were purchased from Serva. Nonfat dry milk was purchased in a supermarket. The primary antibody (AE7A5; monoclonal mouse anti-hEPO) was obtained from R&D Diagnostics, and the secondary antibody conjugate [biotin–goat anti-mouse IgG (H+L)] and horseradish peroxidase–streptavidin conjugate (both Zymax grade) were obtained from Zymed Laboratories. The chemiluminescence substrate (ChemiGlow) was obtained from Alpha Innotech Corp. Phosphoric acid was obtained from Aldrich Chemicals, glacial acetic acid (HPLC grade) was from Mallinckrodt Chemical, and black ink (Tusche A) was from Pelikan.