Supplementary Material – Investigation of the Antibody Specificity

Materials and Methods
Gel filtration (GF) was performed to analyze the specificity of anti-IGFBP-1 antibodies used in immunoblotting. Amniotic fluid, which is known to have IGFBP-1 as the only IGFBP, served as a sample. Amniotic fluid (10 μL, IGFBP-1 concentration was 5.4 μmol/L) diluted to 1 mL with 50 mM phosphate buffered saline (PBS) pH 7.5 was incubated with 5 × 10^5 cpm ^125^I-IGF-I (1 pmol) at 4°C overnight and chromatographed on the sephadex G-100 column (1.8 × 60 cm). Proteins were eluted with PBS buffer and the radioactivity of the collected fractions (2 mL) was measured. In the 2nd experiment, unlabeled IGF-I (100 pmol) was added to the incubation mixture. In the 3rd experiment, a sample of amniotic fluid was incubated with anti-IGFBP-1 antibodies (2.5 nmol IgG) at 4°C overnight and the complexes formed between IGFBP-1 and the corresponding antibodies were sedimented using immobilized protein A (Prowse et al. Immunocytochemistry 1978; 15: 429-436). The supernate left after centrifugation was incubated with ^125^I-IGF-I in a standard manner and chromatographed. Molecular mass markers (IgG, 150 kDa and ovalbumin, 45 kDa) were from Pharmacia Biotech (Uppsala, Sweden).

Results
Using GF it was shown that ^125^I-IGF-I interacted with proteins in amniotic fluid forming 2 types of complexes, the major fraction corresponded to the binary ^125^I-IGF-I/IGFBP-1 complex (approximately 40 kDa) and the minor had the molecular mass ≥ 150 kDa (eluted in Vo). Their relative abundance was 9:1. When unlabeled IGF-I was added in the incubation mixture, it completely inhibited binding of ^125^I-IGF-I in the binary complex, while ^125^I-IGF-I still interacted to some extent with proteins of the higher molecular mass. The same effect was achieved when IGFBP-1 was depleted from the amniotic fluid by immunoprecipitation (Fig. 7).

![Fig. 7](image_url) Evaluation of the specificity of anti-IGFBP-1 antibodies by GF of 3 samples: 1) amniotic fluid incubated with ^125^I-IGF-I, 2) amniotic fluid incubated with ^125^I-IGF-I/IGF-I, and 3) IGFBP-1 depleted amniotic fluid incubated with ^125^I-IGF-I.
Supplementary Material – Additional Information for the Materials and Methods Section (Chromatographic Details)

Metal affinity chromatography (MAC)
Serum sample (0.1 mL), diluted to 1 mL with 50 mM MES (2-(N-morpholino)ethanesulfonic acid)-buffered saline pH 5.5 was applied to the iminodiacetic acid (IDA)-agarose column (Sigma-Aldrich, Steinheim, Germany), 0.9 × 2.0 cm, saturated with Fe³⁺ ions (1 mL of 0.1 M FeCl₃ in distilled water, followed by 2 mL of distilled water, 1 mL 0.1% acetic acid and 3 mL of MES buffer), and circulated at room temperature for 1 h (Machida et al. FEBS J 2007; 274: 1576–1587). Unbound material was washed away with the forementioned MES buffer (5 mL). Bound molecules were eluted with 0.2 M phosphate buffer pH 8.0 (8 mL, 1 mL fraction). Affinity matrix was regenerated with 0.1 M borate buffer pH 9.0 (10 mL, conditions recommended by the supplier) and saturated with metal ions before the next sample was applied. The second specifically eluted fraction had the highest protein content, so it was further analyzed by electrophoresis, together with the unbound material. Serum samples (0 h) from 10 persons in each study group were subjected to MAC randomly. Collected fractions were kept at −20°C until electrophoresis and immunoblotting was performed.

Affinity chromatography with immobilized antibodies (IgY-C)
Affinity chromatography (Nedić and Masnikosa, J Chromatogr 2009; 877: 743–746) was performed using Proteomelab IgY-12 High Capacity Proteome Partitioning kit (Beckman Coulter, Fullerton, USA). The partitioning was done using immobilized avian antibodies (IgY) raised against the 12 most abundant proteins from human plasma: albumin, IgG, IgA, IgM, α₁-antitrypsin, transferrin, haptoglobin, α₁-acid glycoprotein, α₁M, HDL (apo A-I and apo A-II) and fibrinogen. According to the producer, the resin is highly specific for all mentioned proteins, with partitioning efficiency ranging from 90–99%. 20 μL of serum was diluted to 0.5 mL with equilibrating 10 mM TRIS (tris-hydroxymethyl aminomethane)-HCl buffered-saline pH 7.4, loaded onto IgY-12 spin column (1.2 mL of microbeads) and incubated for 15 min at room temperature using rotator. The unbound proteins were separated by centrifugation for 30 s at 2000 g and the column was washed 3 times with the forementioned TRIS buffer (0.5 mL each). The bound proteins were eluted with 0.5 mL of 0.1 M Glycine-HCl buffer pH 2.5, for 3 min at room temperature using rotator, separated by centrifugation and immediately neutralized with 50 μL of 1 M TRIS-HCl buffer pH 8.0. IgY-12 spin column was washed 3 times with the elution buffer to remove the bound proteins completely, neutralized and washed with the equilibrating buffer prior to the next chromatographic cycle. The first fraction specifically eluted from the column was further analyzed by electrophoresis, together with the unbound material. Serum samples (0 h) from 10 persons in each study group were chromatographed on IgY randomly (the same samples that underwent MAC). The collected fractions were kept at −20°C until electrophoresis and immunoblotting was performed.

Lectin affinity chromatography (LAC)
Serum sample (0.1 mL) diluted to 1 mL with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) buffered saline pH 7.5 (HBS) was applied to the SNA (Sambucus nigra agglutinin)-agarose column (Vector Laboratories, Burlingame, CA, USA), 0.9 × 2.0 cm and circulated at room temperature for 1 h (Masnikosa et al., Biochimie 2010; 92: 97–101). SNA is a lectin that specifically interacts with terminal sialic acid residues present in serum glycoproteins. Unbound material was washed away with the HBS buffer (20 mL). The elution of the bound glycoproteins was performed in 2 steps using 0.5 M lactose in (i) HBS buffer (15 mL) and (ii) 0.1 M acetic acid pH 3.0 (10 mL). Eluted fractions (1 mL) at pH 3.0 were immediately neutralized with 1 M TRIS-HCl buffer pH 8.0 (0.5 mL). The second specifically eluted fraction in both steps had the highest protein content, it was dialysed against distilled water for 6 h and further analyzed by electrophoresis. Pooled serum sample, obtained by mixing sera from 10 persons (0 h, 10 μL each) was subjected to LAC.