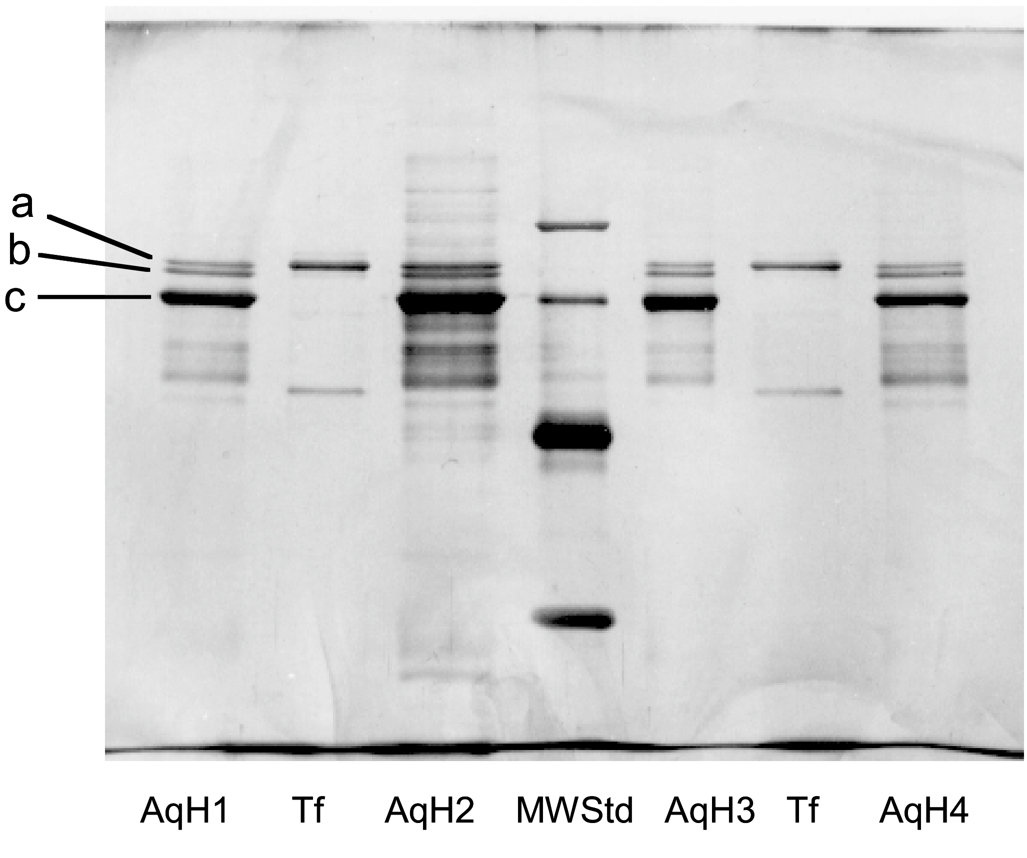
Supplemental Material

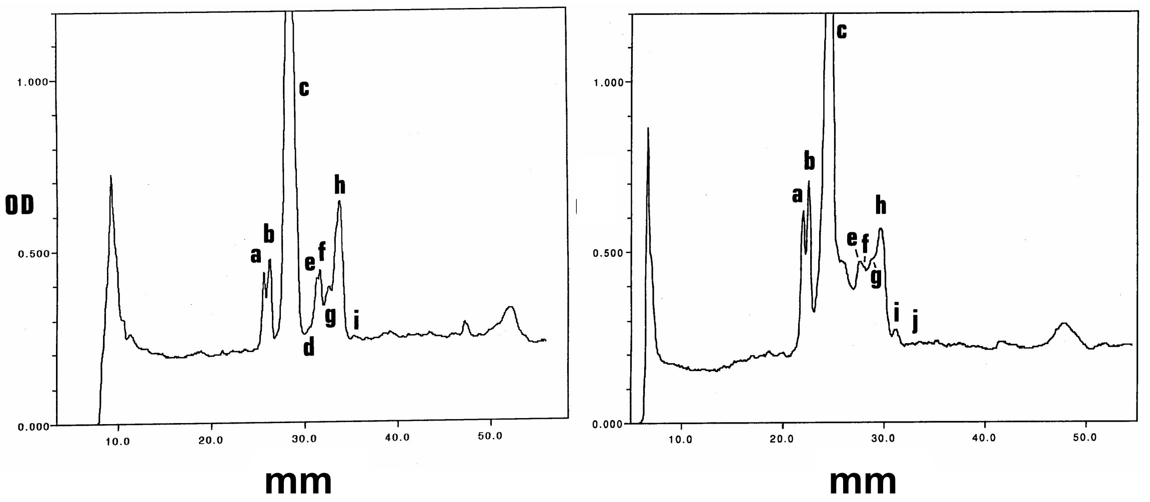
The following account is based on the presentation made at the ARVO Meeting of 1997 "Preliminary screening of aqueous humor proteins in human senile cataracts", by Cortés-Velázquez, A. and García-Castiñeiras, S. (Invest. Ophthalmol. Vis. Sci. 38, S294). The text was specifically written in 1999 for the Preliminary Results Section of a Grant Proposal (#131770) submitted to the NEI, entitled "Iron Metabolism in Age-Related Cataracts".

This study included 29 patients (mean age ± S.D. = 63.9 ± 11.5 years old) undergoing cataract surgery. Eight of these patients were diabetic. All patients signed an informed consent approved by the Institutional Review Committee of the Medical Sciences Campus of the University of Puerto Rico. Slit lamp photographs of the lenses to be removed were taken preoperatorily and used later to classify patients in three groups of increasing cataract severity, following a simple scheme based in LOCS II[1]. Group **I** represented LOCS II NS II cataracts (n=13); group **II** represented LOCS II NS III cataracts (n=8); and group **III** consisted of mature cataracts (n=8).

Samples of aqueous humor from those patients were taken intraoperatorily using a tuberculin syringe with a 27G needle. The samples were immediately transferred to small tubes and frozen at –70°C until electrophoresed at 40 mA in 10% SDS-polyacrylamide minigels (Hoefer Mighty Small II chamber) together with standards of known MW. The equivalent to 2.5 μl of aqueous humor was applied to the gels. Protein bands were visualized using a silver staining procedure and were afterwards submitted to densitometry (Bio-Rad GS-670 densitometer, Molecular Analyst software 2.1). There was an excellent linear relationship between the amount of protein applied to the gel and the area under the densitometric peaks, both for the standard proteins used and the aqueous humor bands. From the densitometer scans, the total area (total protein equivalent) in each lane was obtained as well as the relative individual contribution of each band in that lane. The mean of the total areas of the population was 2.71 ± 1.8 (S.D.) arbitrary units. The use of relative contributions permits a direct comparison of equivalent bands from sample to sample since the influence of differences in total protein in the samples is minimized.

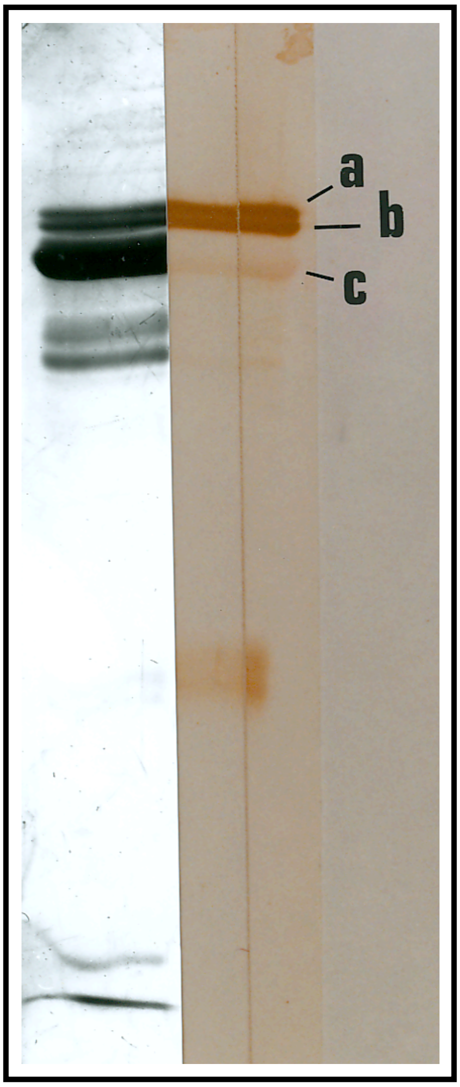


Suppl Figure 1. Aqueous humor proteins. A typical 10% SDS-PAGE slab gel of four aqueous humor samples (AqH1-4). Tf are apotransferrin standards. Band **a** is 77kD Tf; band **b** is 75kD Tf; band **c** is albumin. See also **Suppl Figure 3**. The Tf of lower MW (75kD) corresponds to the tau fraction (ß$_2$Tf) of the cerebrospinal fluid (CSF)[5,6], a Tf isoform once considered typical of the CSF. The faster electrophoretic behaviour of this special Tf under denaturing conditions appears to be due to the loss of several or all of the sialic acid residues (sialic acid MW ≈ 300D) present in the glycoprotein and perhaps to a simultaneous decrease in the affinity for the polyacrylamide support secondary to the reduced size of the carbohydrate part of Tf. Standards of known MW (MWStd) are from top to bottom: phosphorylase b (97kD), serum albumin (66kD), ovalbumin (45kD), and carbonic anhydrase (29kD)



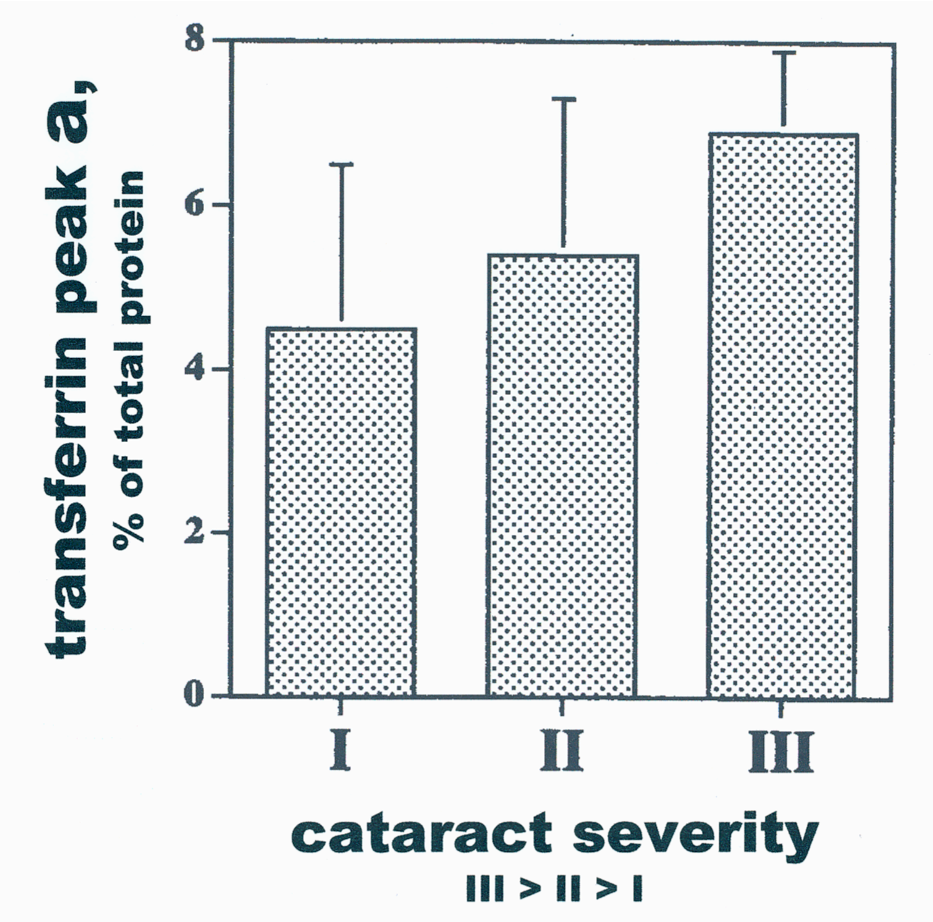
Suppl Figure 2. Representative densitometric profiles of 10% SDS-PAGE slab gels of aqueous humor samples. Peak **a** is 77kD Tf; peak **b** is 75kD Tf; peak **c** is albumin. Other proteins have not been identified. The mean total area under the profile of all the aqueous humor samples was 2.7 ± 1.8 (S.D.) arbitrary units. The corresponding values for the four individual samples of aqueous humor in **Suppl Figure 1** are: 1.28 (AqH1), 3.54 (AqH2), 1.45 (AqH3) and 1.6 (AqH4).

Aqueous humor proteins (Suppl. Figure 1, Suppl. Figure 2) from cataract patients showed a remarkably consistent qualitative pattern of bands, with most of them appearing between the 97kD and 45kD markers. The main band (65% of total protein) was identified as albumin (67kD) based on its electrophoretic mobility relative to that of standard serum albumin samples. Transferrin was the only quantitatively important protein component of MW higher than albumin. Tf appeared as a duplet (77kD and 75kD components), the 77kD band **a** exactly coinciding with human plasma apotransferrin. The identity of the second component (75kD band **b**) was ascertained by doing Western blots of the gels on nitrocellulose paper (Suppl. Figure 3). For this purpose goat anti-human transferrin was the primary antibody and biotinylated anti-goat IgG was the secondary antibody. The latter was reacted with extravidin peroxidase and the paper was finally incubated with diaminobenzidine. Both the 77 and 75kD bands were strongly positive for Tf. The rest of the gel consisted mainly of overlapping bands and its densitometric profile was sliced for analysis using some clear, reproducible landmarks in the profiles.



Suppl Figure 3. Western blot of an aqueous humor sample (peroxidase staining, right side of the figure; silver staining, left side of figure) to show the identity of bands **a** and **b** with transferrin. See also the legend to **Suppl Figure 1**.

When the percent contribution of each protein band or slice to the total area of the densitometric profile was plotted against the cataract severity groups, the 77kD Tf component significantly increased from group **I** (4.5%) to group **III** (6.9%) as revealed by variance analysis ($p<0.021$) (Suppl. Figure 4). The 75kD component showed also the same tendency.



Suppl Figure 4. Percent peak **a** (77kD transferrin) and cataract severity. The correlation was statistically significant ($p<0.021$), indicating that transferrin is specifically enriched among the proteins of the aqueous humor as cataract severity increases.

The effect of cataract severity on aqueous humor transferrin (77kD component) was also determined after adjusting for age and diabetes as potential confounding factors. A Poisson regression model was used[2,3]. For this analysis the cataract severity groups were those already described and the response or dependent variable was defined as the amount of 77kD Tf divided by total aqueous humor protein equivalent. Since the concentration of Tf is small relative to total protein the Poisson model was considered the most adequate to explain the proportion. The statistical package used was GLIM4[4].

The results showed that the adjusted relative risk for the relative concentration of Tf in group II cataract patients was 1.82 (95% CI: 1.05, 3.15) times higher than the proportion in group I. A similar pattern was observed in group III vs group I. Therefore we concluded, even with this small sample of 29 patients, that there is statistical evidence of a relationship between Tf relative concentration in the aqueous humor and cataract severity, validating the previously reached conclusion.

Regression analysis showed a tendency to the increase with age and with cataract severity of total aqueous humor protein equivalent, suggestive of a slow, progressive aperture of the blood aqueous humor barrier as a function of age and cataract development. This tendency, however, cannot explain the specific enrichment in aqueous humor transferrin described above.

Acknowledgements

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References

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