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# **Transthyretin levels in the vitreous correlate with change in visual acuity after vitrectomy**

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# ABSTRACT

**Background/aim:** Little is known about biochemical markers related to change in visual acuity after vitrectomy. We investigated the potential use of transthyretin (TTR), a carrier of the retinol/retinol-binding protein, as a biochemical marker protein.

**Methods:** We measured TTR using immunonephelometry in a group of patients (n=77) in longstanding (> 1 week) retinal detachment (n=29), fresh (< 1 week) retinal detachment (n=17), macular holes (n=20), or diabetic retinopathy (n=11). Vitreous samples were taken at the start of every vitrectomy procedure. For reference values, cadaver specimens (n=73) were used.

**Results:** Reference values for vitreous TTR (median 18 mg/l; IQR 4-24 mg/l) comprised 2.2% of reference values for vitreous protein levels (median 538 mg/l; IQR 269-987 mg/l). Vitreous TTR values of patients were comparable in all disorders. Vitreous TTR values were higher in phakic (median 22.5 mg/l; IQR 10-27 mg/l) than in pseudophakic patients (median 12 mg/l; IQR 8-19 mg/l)(p=0.06). Postoperative change in visual acuity correlated well with vitreous TTR values found peroperatively ( $r_s=0.408$ ;  $p=0.012$ ). Both change in visual acuity and lens status were the only variables which proved to explain the variance of TTR (multiple correlation coefficient: 0.494; phakic status:  $t=2.767$ ;  $p=0.0084$ ; and change in visual acuity  $t=2.924$ ;  $p=0.0056$ ).

**Conclusion:** Vitreous fluid concentrations of TTR can be regarded as a biochemical marker for retinal function.

# INTRODUCTION

TTR is a 55 kDa homotetrameric protein with a dimer configuration that is synthesized in the liver, choroid plexus, visceral yolk sac and retinal pigment epithelium. Each monomer is a 127 residue polypeptide rich in beta sheet structure. Association of two monomers forms an extended beta sandwich. Further association of another identical set of monomers produces the homotetrameric structure. In serum, it functions as a carrier for thyroxin and retinol-binding protein (RBP). It has two thyroxin binding sites per tetramer at the interface between the latter set of dimers, and two binding sites for RBP on the outer surface. As a carrier protein for thyroxin in serum, it acts in concert with thyroxin binding globulin and albumin. Retinol is transported bound to RBP, which itself is bound to TTR.<sup>1</sup> RBP has higher affinity for TTR when it is bound to a retinol ester.<sup>2</sup> The transport of protein-bound ligands across lipid-water interfaces, such as cell membrane surfaces, is thought to involve obligatory dissociation of protein-bound ligand into the free state before transmembrane transport.<sup>3</sup> The retinol-RBP-TTR complex cannot freely pass through cell membranes. STRA6 is a multitransmembrane domain protein functioning as the high-affinity tissue receptor for plasma RBP and mediating cellular uptake of vitamin A from the vitamin A-RBP complex.<sup>4</sup> It has been suggested that formation of the complex between TTR and single, or multiple, RBP molecules, prevents renal clearance of the retinol/RBP complex.<sup>5</sup>

In the eye, TTR is bound to retinol and RBP in various ocular tissues. In contrast to retinol, which is derived solely from the serum, TTR is synthesized locally. Plasma TTR does not cross Bruch's membrane into the retina. Ocular TTR is synthesized in the retinal pigment epithelium (RPE) and in the pigmented epithelium of the ciliary

body, the corneal endothelium and the optic nerve fibre layer of the retina and is transported to specific locations within the eye.<sup>6-8</sup> Although the physiological role of TTR in the eye is unknown, it is possible that it participates in retinol cycling.

TTR is able to deposit as amyloid fibrils in senile systemic amyloidosis (SSA) and familial amyloid polyneuropathy (FAP). At least 20 TTR mutations are associated with amyloidosis.<sup>9</sup> Vitreous amyloid fibrils are the result of local synthesis of mutated TTR. Nuclear cataract is associated with low protein intake and low serum levels of TTR.<sup>10</sup> Immunohistochemical analysis of drusen in patients with age-related macular degeneration showed the presence of TTR.<sup>11</sup> Comparison of aqueous humour between myopic and non-myopic patients showed a higher TTR level in the former.<sup>12</sup>

There exist no biochemical markers to differentiate between patients with excellent and poorer visual acuity after vitrectomy. Little is known about the TTR concentrations in normal and pathological human vitreous fluid. As there are no known reports of humans lacking TTR, the importance of TTR to vision has not been described. In view of the presumed key role of TTR in the vitamin A cycle, we wanted to explore the use of TTR in human vitreous fluid as a biochemical marker protein for retinal function.

## **MATERIALS AND METHODS**

### **Subjects**

Vitreous samples obtained from human cadavers (n=73), obtained through vitreous tap 4 mm from the limbus in the temporal upper or lower quadrant, were used for establishing references values for TTR. In table 1, the age, sex, post-mortem interval, and possible pre-existing diseases are provided. The median post mortem interval

was 1.5 days (IQR 0.9-2.4 days). In addition, the cause, mechanism and manner of death of each individual are specified. Furthermore, we analyzed 77 freshly collected vitreous fluid samples submitted to our laboratory. Samples were collected by two vitreoretinal surgeons (MV in the Rotterdam Eye Hospital, the Netherlands, and EVA in Ghent University Hospital, Belgium). A vitreous tap of 0.5 ml was performed at the start of every vitrectomy procedure via a syringe connected to the collecting canula of the vitreous cutter. All samples were immediately stored at 4°C. Informed consent was obtained from each patient. According to diagnosis, the patient could be divided in longstanding retinal detachment (n=29), fresh retinal detachment (n=17), macular holes (n = 20), or advanced proliferative diabetic retinopathy (n=11). As definitions we used: “longstanding retinal detachment” for retinal detachments of patients with typical symptoms for a retinal detachment for at least 1 week before surgical intervention, “fresh retinal detachment” for retinal detachments of patients with typical symptoms for a retinal detachment for less than 1 week before surgical intervention, “macular hole” for stage 3 (>400 μ) macular holes characterized by OCT existing more than 6 months, and “advanced proliferative diabetic retinopathy” for patients with a fundus partially obscured by vitreous haemorrhage according to ETDRS standards.<sup>13</sup> Pre- and postoperative visual acuity are measured at Snellen charts at 6 meters.

### **Biochemical investigations**

TTR was measured immunonephelometrically on undiluted vitreous fluid samples with use of commercially available Behring antibodies on a Behring Nephelometer II analyzer (Dade Behring) standardized against the widely accepted WHO/College of American Pathologists Certified Reference Material 470. Total protein in vitreous fluid

was measured by a pyrogallol red method (n = 129) with Standard Reference Material 917a as a standard and commercially available reagents (Instruchemie, Delfzijl, The Netherlands) on a Modular P system (Roche).<sup>14</sup> The 2.5 and 97.5 percentiles of the human cadaver specimens were used for calculating the reference interval. All samples were kept frozen at -20°C until analysis. Between run coefficient of variation was 5.86 % and the detection limit was 3.35 mg/l.

### **Clinical investigations**

In the patient group, best corrected visual acuity (Snellen) was evaluated before and 3-4 months after vitrectomy. The diagnosis of all preoperative conditions was made by funduscopy by a trained vitreoretinal surgeon. Additionally, an Optical Coherence Tomography (OCT) was performed for patients presenting with macular hole. Patients that had yet undergone cataract surgery before were defined “pseudophakic”. Patients that had not yet undergone cataract surgery before or had cataract surgery simultaneously with the vitrectomy were defined “phakic”. Characteristics of patients are found in Table 2.

### **Statistics**

*P* values <0.05 were considered significant. Agreement between data was evaluated by Spearman rank analysis. The lower limit of detection was calculated as the mean value - 3 SD for a blank sample. Diagnostic accuracy was assessed by ROC analysis using commercially available statistical software (MedCalc<sup>®</sup>, SPSS). The variance of TTR was studied using a stepwise backward elimination logistic regression model, starting with all covariates in the model followed by a stepwise removal of the term associated with the highest p-value.

# RESULTS

## Reference values

In the reference group (n = 73, median age: 46 years; IQR 35-58 years), median total protein concentration was 538 mg/l (IQR 269-987 mg/l). Considering the causes of death and the pre-existing pathologies, our control cases were a varied group and thus represent the normal population, including a few diabetics (n = 10; see Table 1). Distribution of total protein was skewed. Median TTR concentration was 18 mg/l (IQR 4-24 mg/l) (Table 2). The concentration of TTR was correlated with total protein concentration of the vitreous fluid (correlation coefficient: 0.459;  $p < 0.01$ ). TTR concentrations were not significantly affected by the post-mortem interval.

## Values in various pathological conditions

Preoperative and postoperative visual acuity are given in Table 3. Total protein levels in patients with fresh or longstanding retinal detachment and in patients with proliferative diabetic retinopathy were higher than total protein reference levels. Among the patient groups, highest TTR values were recorded in the patients presenting with proliferative diabetic retinopathy and lowest in patients presenting with macular hole stage 3 (Table 4). Median TTR values in any pathological condition did not differ statistically with reference TTR values. TTR values in patients who were phakic prior to the vitrectomy procedure (median 22.5 mg/l) were higher than in pseudophakic patients (median 12 mg/l) (fig 1). TTR/total protein ratios were calculated, as this ratio corrects for the vascular permeability changes. TTR/total protein ratios in any disease did not differ with reference TTR/total protein values. Peroperative vitreous TTR values correlated well with the visual outcome following

treatment (fig 2). In a logistic regression model, both change in visual acuity and lens status were the only variables which proved to explain the variance of TTR (multiple correlation coefficient: 0.494; phakic status:  $t= 2.767$ ;  $p=0.0084$ ; and change in visual acuity  $t=2.924$ :  $p= 0.0056$ )

## DISCUSSION

This is the first report to the authors' knowledge about a biochemical marker related to change in visual acuity after vitrectomy and for measuring TTR in vitreous samples by immunonephelometry. In the present study, high concentrations of TTR (mean: 19 mg/l) were found in vitreous fluid of cadaver eyes. The observed values are comparable to the ones observed in cerebrospinal fluid (mean: 17 mg/l). Median serum TTR levels are 300 mg/l (IQR 250-350 mg/l). Total protein levels in vitreous fluid are 538 mg/l and are comparable with total protein levels in serum and in CSF (150-500 mg/l). Total protein levels in serum are much higher ( $64 \cdot 10^3 - 83 \cdot 10^3$  mg/l). Reference TTR/total protein ratios in the vitreous (2.2%) largely exceed the TTR/total protein levels in serum (0.4%-0.5%). The reference TTR values correlated with total protein levels in the vitreous. The origin of vitreous proteins includes the tissues surrounding the vitreous, the hyalocytes of the vitreous, or the surrounding vasculature. The much higher TTR/total protein ratios found in the vitreous as compared to serum values implicates that simple diffusion from the serum cannot be the source of vitreous TTR. We conclude that the physiological level of TTR found in the vitreous of cadaver eyes is derived from surrounding tissues as RPE cells.

We found a significant correlation between postoperative change in visual acuity and high TTR concentrations in the vitreous preoperatively. Ramakrishnan et al. found low TTR levels in the vitreous of diabetic patients.<sup>15</sup> TTR levels in the former study

however were not measured by immunonephelometry but calculated semi quantitatively by densitometry after electrophoresis. Our results show that TTR levels in the vitreous are not related to diagnosis but to RPE function. TTR expression is a marker of differentiation of RPE cells in vitro.<sup>8</sup> We presume a higher metabolic state of RPE cells in patients with high TTR expression, and thus a better regeneration after a vitrectomy procedure.

The function of TTR in the vitreous remains to be elucidated. RPE of the eye and choroid plexus of the brain are developmental and functional homologues, sharing many of the characteristics of transporting epithelia: the cells are joined by tight apical membrane junctions and they regulate the transport of fluids and serum proteins into their respective humours. The secretion of TTR by the choroid plexus of the brain provides the mechanism for the regulation of T4 (thyroxin) levels in the brain. TTR in the CSF is involved in the delivery of T4 to stem cells and progenitor cells within the brain, which require T4 for regulation of the cell cycle.<sup>16-17</sup> The abundance of TTR mRNA in RPE is, save for the choroid plexus in the brain, unmatched by any other tissue of the body.<sup>8</sup> TTR protein is secreted predominantly in an apical direction by RPE cells in vitro.<sup>18</sup> The fact that the retinol metabolism is altered in TTR-null mice suggests that TTR plays a major role in the retinol metabolism in the interphotoreceptor matrix (IPM).<sup>19</sup> Interphotoreceptor retinoid-binding protein (IRBP), a non-selective scavenger and stabilizer of retinoids released from photoreceptors and pigment epithelial cells, is the most abundant soluble protein component of the IPM. IRBP plays a direct role in the release of all-trans retinol from the rods.<sup>20</sup> All-trans retinol generated in rod photoreceptors upon the bleaching of rhodopsin is known to move from the rods to the RPE, where it is enzymatically converted to 11-cis retinal in the retinoid visual cycle. Experiments

conducted on toad RPE-eyecups, mammalian RPE cells and isolated RPE membranes indicate that IRBP specifically promotes the release of 11-*cis* retinal from the RPE apical surface, a step necessary for the movement of 11-*cis* retinal to the rods.<sup>21</sup> IRBP thereby directly supports the regeneration of rhodopsin in the visual cycle.<sup>22</sup> An emerging concept is that the central role of retinoid-binding proteins in the visual cycle is to achieve tight control of retinoid concentration while protecting the retinal cells from the toxicity of free retinoids. In analogy with the CSF where thyroxin concentrations are titrated by binding to TTR, a spatial gradient of free IRBP molecules and IRBP/retinoid complexes might as well be regulated by TTR in the IPM.

We found higher TTR concentration in phakic patients than in pseudophakic patients. The vitreous proteome is found to be different in pseudophakic patients.<sup>23</sup> Lens cortical fibre debris for example is leaking into the vitreous during cataract extraction. Cataract surgery might thus account for the easier passage of TTR from the posterior chamber to the anterior chamber, resulting in a higher outflow via the trabecular meshwork.

Total protein levels in the vitreous of diabetic patients were 4.3 x higher than reference total protein levels. Electrophoresis of vitreous fluid proteins in diabetics revealed a plasma-like protein pattern suggesting the increased protein concentration is due to a major diffusion from the pathological vessels. Increased vascular permeability and pathological neovascularization are major aspects of diabetic retinopathy. Reduced integrity of adherens junctions and tight junctions of human diabetic retina have been reported.<sup>24-25</sup> TTR values in the vitreous of diabetic patients were fairly similar to reference TTR values, as can be deduced from the following example. A vitreous cavity with a 4 ml content is filled with blood corresponds to an

influx of only 12  $\mu\text{g}$  TTR from the serum into the vitreous cavity (3 mg/l TTR x 4 ml).

The amount of TTR that has originated from the serum into the vitreous cavity cannot cause a significant rise in the TTR concentration already present in the vitreous.

In conclusion, vitreous TTR concentrations strongly exceed the values obtained in serum. Therefore, their concentrations in vitreous fluid reflect local production by the RPE. The present study suggests that TTR can be used as a biochemical marker for assessing retinal function.

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**Table 2 Characteristics of patients.**

	<b>Retinal detachment &gt; 1 week</b>	<b>Retinal detachment &lt; 1 week</b>	<b>Advanced proliferative diabetic retinopathy</b>	<b>Macular hole Stage 3</b>
<b>Number</b>	<b>29</b>	<b>17</b>	<b>11</b>	<b>20</b>
<b>Age (range)</b>	<b>71 (61-79)</b>	<b>57 (51-69)</b>	<b>56 (50-59)</b>	<b>71 (61-74)</b>
<b>Phakic</b>	<b>15 (52%)</b>	<b>6 (35%)</b>	<b>6 (55%)</b>	<b>14 (70%)</b>
<b>Men</b>	<b>13 (45%)</b>	<b>8 (47%)</b>	<b>7 (64%)</b>	<b>7 (35%)</b>

**Table 3. Preoperative visual acuity, postoperative visual acuity and change in visual acuity for all patients individually in Snellen (6 meters). Visual acuity of 0.017 (1/60) equals fingers counting at 1 metre, visual acuity of 0.003 (1/300) equals hand movement at 1 metre. Mean values +/- SD for each patient group are given below. The Kruskal-Wallis one-way analysis of variance by ranks did not show a statistically significant difference between the patient groups (p>0.05).**

	Visual acuity preoperatively	Visual acuity postoperatively	Change in visual acuity
<b>Retinal detachment&gt;1week (n=29)</b>	0.1	0.9	0.8
	0.7	1.0	0.3
	0.2	0.7	0.5
	0.003	0.017	0.014
	0.16	0.3	0.14
	0.1	0.1	0
	0.05	0.9	0.85
	0.05	0.7	0.65
	0.003	0.4	0.397
	0.02	0.6	0.58
	0.003	0.02	0.017
	0.003	0.2	0.197
	0.7	0.7	0
	0.003	0.2	0.197
	0.1	0.16	0.06
	1.0	0.7	-0.3
	0.05	0.2	0.15
	0.003	0.017	0.004
	0.017	0.3	0.283
	0.9	0.8	-0.1
	0.1	0.16	0.06
	0.4	0.4	0
	0.2	0.16	-0.04
	0.02	0.02	0
	0.003	0.2	0.197
	0.003	0.15	0.147
	0.003	0.6	0.597
	0.017	0.16	0.143
	0.2	0.16	-0.04
<b>mean±SD</b>	<b>0.2±0.28</b>	<b>0.4±0.3</b>	<b>0.2±0.3</b>
<b>Retinal detachment&lt;1week (n=17)</b>	0.003	0.4	0.397
	0.8	1	0.2
	1.0	0.7	-0.3
	0.003	0.6	0.597
	0.003	0.9	0.897
	0.017	0.9	0.883
	0.003	0.5	0.497

	1.0	0.8	-0.2
	0.003	0.5	0.497
	0.7	0.6	-0.1
	0.8	0.8	0
	0.017	0.3	0.283
	0.6	0.6	0
	0.003	0.5	0.497
	0.003	0.5	0.497
	0.3	0.3	0
	0.003	0.2	0.197
<b>mean±SD</b>	<b>0.3±0.4</b>	<b>0.6±0.2</b>	<b>0.3±0.3</b>
<b>Advanced proliferative diabetic retinopathy (n=11)</b>	0.1	0.3	0.2
	0.017	0.2	0.183
	0.2	0.3	0.1
	0.4	0.4	0
	0.4	0.4	0
	0.003	0.003	0
	0.6	0.6	0
	0.2	0.3	0.1
	0.3	0.3	0
	0.6	0.5	-0.1
	0.16	0.4	0.24
<b>mean±SD</b>	<b>0.3±0.2</b>	<b>0.3±0.2</b>	<b>0.1±0.1</b>
<b>Macular hole stage 3 (n=20)</b>	0.2	0.2	0
	0.3	0.3	0
	0.16	0.2	0.04
	0.1	0.4	0.3
	0.3	0.3	0
	0.4	0.5	0.1
	0.15	0.4	0.25
	0.2	0.2	0
	0.3	0.3	0
	0.3	0.3	0
	0.3	0.3	0
	0.2	0.4	0.2
	0.02	0.02	0
	0.1	0.3	0.2
	0.4	0.7	0.3
	0.2	0.2	0
	0.3	0.3	0
	0.2	0.3	0.1
	0.5	0.7	0.2
	0.6	0.6	0
<b>mean±SD</b>	<b>0.3±0.1</b>	<b>0.3±0.2</b>	<b>0.1±0.1</b>

**Table 4 Reference values and values for total protein and TTR in vitreous fluid of the various patient groups. The within- and between-run values for total protein and TTR are summarized.**

Retinal condition	Total protein (mg/l)		TTR (mg/l)		TTR/total protein (%)	
	median	IQR	median	IQR	median	IQR
reference	538	269-987	18	4-24	2.2	0.9-3.9
RDD>1 week old	781*	460-1881	19	12-24	1.6	0.6-3.3
RDD<1 week old	816*	427-1527	14	7-25	1.8	1.7-3.1
Diabetes mellitus	2326*	1880-2818	20	10-24	1.3	1.2-1.4
Macular hole	701	241-1456	13	9-25	1.5	1.1-3.2

\*: P<0.05 vs reference values. Mann-Whitney U-test.

## Legends of figures.

**Figure 1 Box-Whisker plots for TTR values in vitreous samples of patients.**

**Comparison between TTR values of pseudophakic and phakic patients.**

**$p=0.064$  Mann-Whitney U test.**

**Figure 2 Correlation between vitreous TTR concentration in patients and change in visual acuity postoperatively.**

Agreement was fair between the TTR concentration ( $x$ ) and the sight measurements ( $y$ ). The following regression equation was obtained:  $y$  (Snellen) =  $0.0462 + 0.0042 \log X$  (mg/L); rho Spearman = 0.408;  $p = 0.012$ ; 95% confidence interval 0.106-0.641.



