Baseline Levels of Retinol-Binding Protein 4 and Vitamin A in Healthy Subjects, Stargardt Disease, and Geographic Atrophy Patients

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Keywords

Retinal diseases · Retinol-binding protein 4 · Stargardt disease · Age-related macular degeneration · Visual cycle

Abstract

Introduction: The accumulation of lipofuscin is a hallmark in the pathogenesis of Stargardt disease type 1 (STGD1) and geographic atrophy (GA) secondary to age-related macular degeneration. Limiting lipofuscin accumulation by inhibiting the retinol-binding protein 4 (RBP4) is being explored as a potential treatment target for those diseases. In this study, we aimed to establish the concentration of RBP4 in the systemic circulation in different age cohorts of healthy individuals and to check if patients with STGD1 or GA may show abnormal RBP4 levels. **Methods:** Forty healthy subjects of various age-groups, 15 Stargardt patients, and 15 GA patients were included in the study. We measured RBP4 levels, serum retinol (SR) levels, complete blood count, and blood chemistry including liver function tests. **Results:** Mean RBP4 for all cohorts was $26,911.40 \pm 6,198.61$ ng/mL, and mean SR 1.75 ± 0.36 μ mol/L. Age was not found to significantly impact levels neither of RBP4 and SR nor of the RBP4-to-SR ratio. Also, the 2 patient groups showed similar blood levels to their age-matched controls. **Conclu-**

sion: Serum RBP4 and SR do not appear to be affected by age in healthy individuals and remain within normal limits in both STGD1 and GA.

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Introduction

Lipofuscin is a complex aggregate, including the cytotoxic N-retinylidene-N-retinylethanolamine (A2E) that accumulates in postmitotic metabolically highly active cells such as specific neurons, cardiac myocytes, and the retinal pigment epithelium (RPE) over time [1]. In the eye, the lipofuscin complex is amassed in an age- and light-dependent manner by the incomplete digestion of photoreceptors' outer segments [2]. This accumulation is thought to interfere with the cell function of the RPE and the photoreceptors, leading to cell loss and atrophy [2–4].

Stargardt disease type 1 (STGD1) is an autosomal recessively inherited retinal dystrophy and the most common juvenile macular dystrophy caused by mutations in the ABCA4 gene [5, 6]. ABCA4 is an ATP-binding cassette transporter and acts as a flippase within the rim of the photoreceptor's outer segment disc. The ABCA4 transporter accelerates all-trans-retinal clearance from

Table 1. Summary of the subjects' demographics and measurements over all groups

	Overall
<i>n</i>	70
Sex = M, <i>n</i> (%)	24 (34.3)
Age (mean [SD]), years	52.79 (21.53)
Age (median [range]), years	50.50 [19.00, 90.00]
Blood pressure diastolic (mean [SD]), mm Hg	122.33 (11.76)
Blood pressure diastolic (median [range]), mm Hg	122.50 [92.00, 145.00]
Blood pressure systolic (mean [SD]), mm Hg	79.54 (8.20)
Blood pressure systolic (median [range]), mm Hg	80.00 [60.00, 103.00]
Weight (mean [SD]), kg	68.06 (11.64)
Weight (median [range]), kg	65.50 [50.00, 102.00]
BMI (mean [SD]), kg/m ²	23.63 (2.94)
BMI (median [range]), kg/m ²	23.31 [18.04, 31.98]
SR (mean [SD]), μmol/L	1.75 (0.36)
SR (median [range]), μmol/L	1.77 [0.81, 2.48]
RBP4 (mean [SD]), ng/mL	26,911.40 (6,198.61)
RBP4 (median [range]), ng/mL	27,203.02 [10,820.76, 44,872.24]
Serum RBP4-to-retinol ratio (mean [SD]), g/mol	15,481.07 (2,319.79)
Serum RBP4-to-retinol ratio (median [range]), g/mol	15,179.13 [11,298.41, 25,929.34]

the outer segments by facilitating the transfer of the molecule to the cytoplasmic space for the subsequent reduction to all-trans-retinol [7]. The dysfunction of the ABCA4 transporter leads to the increased accumulation of toxic byproducts like A2E in the RPE and photoreceptors' outer segment [8]. The relevance of lipofuscin accumulation in the pathogenesis of age-related macular degeneration (AMD) is still a matter of debate as despite earlier studies indicating increased lipofuscin content in the RPE with age [3, 4, 9], newer data suggest a slowed visual cycle in AMD [10] and either normal or reduced quantitative autofluorescence [11].

Retinol-binding protein 4 (RBP4) is a protein that has a significant role in the visual cycle as the primary transporter of retinol to the RPE [7, 8]. In the circulation, RBP4 binds retinol and transthyretin (TTR) in a complex and delivers vitamin A to the eye and other organs [12]. Interestingly, the eye shows a higher dependency on RBP4-delivered retinol than other organ systems as RBP4 knockout mice and humans with RBP4 mutations only develop an ocular phenotype [13, 14]. Thus, the inhibition of RBP4 is being considered as a relatively safe way to limit the delivery of vitamin A to the retina in GA and STGD1 [15–17].

However, the concentration of RBP4 in the systemic circulation in different age cohorts of healthy individuals have not been established yet, and it remains unknown if patients with STGD1 or GA have similar or abnormal RBP4 levels. This knowledge is crucial as a better understanding of the normal values of RBP4 will be important

for the development of drugs targeting RBP4 in the treatment of those diseases. Therefore, we investigated RBP4 levels in different populations, including healthy individuals of various age-groups, patients with GA, and patients with STGD1.

Methods

The main objective of this single-center, open-label, noninterventional, nondrug study was to evaluate the blood levels of RBP4 and vitamin A in healthy subjects of different age cohorts and STGD1 and GA patients. This study was approved by the local Ethics Committee (Ethikkommission Nordwest-Schweiz) and was conducted in accordance with the tenets of the Declaration of Helsinki. All subjects have given written consent.

Healthy subjects and STGD1 patients older than 18 years and GA patients older than 60 were included in this study. Patients with BMI lower than 18 or higher than 31 years, with a history of vitamin A deficiency, vitamin A supplementation, history of significant cardiovascular disease, diabetes, hepatitis or other significant liver diseases, pancreatitis, uncontrolled thyroid disease, women if pregnant or lactating, history of clinically significant abnormal lab results at screening, any history of significant eye disorders (including retinal disorders), or visual disturbances other than STGD1 or GA were excluded from the study. The protocol of the study was to draw blood to test the following: RBP4 levels, vitamin A (serum retinol [SR]) levels, complete blood count, and blood chemistry including liver function tests.

After subjects were deemed eligible for the study, vital signs were taken (pulse, blood pressure, respiratory rate, and temperature) prior to a blood draw. Blood samples to test for the above-mentioned protocol were taken after at least 8 h of overnight fast. Blood was sent to three laboratories for analysis. Complete blood count and blood chemistry were done in the in-house laboratory

of the University Hospital Basel (Universitätsspital Basel, USB), and vitamin A levels were determined at Viollier, Basel, Switzerland (nephelometry) and RBP4 at 360biolabs, Melbourne, VIC, Australia (electrochemiluminescence). No further blood sampling was performed.

Associations of blood levels and patient characteristics were measured by Pearson correlation coefficients (corresponding to point biserial correlation coefficients for the binary variable sex). The effect of patient characteristics and diseases on blood levels was analyzed by linear models. More precisely, the effects of age-group and age (continuous) in healthy subjects were estimated by ANOVA and linear regression, respectively. The effects of STGD1 and GA (both compared to healthy subjects) were studied by ANOVA as well as analysis of covariance when adjusting for age (continuous). Finally, the effects of sex in all subjects were evaluated by ANOVA.

Results

A total number of 70 subjects were included in the study, distributed into healthy subjects of different age cohorts, STGD1, age ≥ 18 years, and GA disease; age ≥ 60 years. The data are summarized in Tables 1–3 (there are no missing values) and represented graphically in Figures 1–3. Correlations of blood levels with patient characteristics are listed in Table 4. There were no missing values, and no patients needed to be excluded from the study. Serum retinol levels are shown in Tables 1–3 and Figures 1 and 4.

There was a trend of SR being lower in STGD1 ($p = 0.051$), but it can be explained by age alone ($p = 0.179$ when adjusting for age). A difference between GA and healthy subjects was not detected ($p = 0.755$ and $p = 0.795$, when adjusting for age). Finally, there was some evidence for an effect of sex on the SR levels in all cohorts, with females showing lower levels ($p = 0.053$). RBP4 levels are shown in Tables 1–3 and Figures 2 and 5.

There was some evidence for a difference between STGD1 patients and healthy subjects ($p = 0.078$), but it can also be explained by age alone ($p = 0.323$ when adjusting for age). A difference between GA and healthy subjects was not detected ($p = 0.101$ and $p = 0.697$, when adjusting for age). Finally, there is no clear evidence for an effect of sex ($p = 0.408$). The serum RBP4-to-retinol ratio is shown in Tables 1–3 and Figures 3 and 6.

The SR and RBP4 levels were highly correlated: the Pearson correlation coefficient was 0.78, with a 95% confidence interval of 0.67 and 0.86. There was no evidence for an effect of age-group or age (linear) in healthy subjects ($p = 0.307$ and $p = 0.493$, respectively). Also, there was no evidence for a difference between STGD1 and healthy subjects ($p = 0.842$ and $p = 0.883$, when adjusting

Table 2. Summary of the subjects' demographics and measurements in groups 1–4

	Healthy 18–35	Healthy 36–50	Healthy 51–65	Healthy ≥ 66
N	10	10	10	10
Sex = M, n (%)	4 (40.0)	4 (40.0)	3 (30.0)	3 (30.0)
Age (mean [SD]), years	27.60 (4.33)	44.00 (4.32)	58.30 (3.83)	72.60 (3.89)
Age (median [range]), years	28.00 [19.00, 33.00]	45.50 [38.00, 50.00]	58.50 [51.00, 64.00]	74.00 [66.00, 77.00]
Blood pressure diastolic (mean [SD]), mm Hg	119.80 (6.25)	118.90 (10.62)	120.80 (12.35)	124.30 (12.07)
Blood pressure diastolic (median [range]), mm Hg	120.50 [111.00, 130.00]	117.50 [104.00, 134.00]	120.50 [92.00, 134.00]	126.50 [106.00, 138.00]
Blood pressure systolic (mean [SD]), mm Hg	78.40 (5.02)	81.30 (6.09)	81.10 (8.88)	75.80 (8.22)
Blood pressure systolic (median [range]), mm Hg	78.00 [71.00, 87.00]	82.50 [72.00, 88.00]	82.00 [62.00, 92.00]	75.50 [60.00, 86.00]
Weight (mean [SD]), kg	70.50 (11.98)	69.94 (15.53)	68.60 (14.95)	63.36 (7.46)
Weight (median [range]), kg	70.00 [54.00, 89.00]	70.70 [50.00, 90.00]	67.00 [52.00, 102.00]	63.50 [52.60, 80.00]
BMI (mean [SD]), kg/m ²	23.59 (2.89)	24.87 (3.22)	23.76 (3.16)	22.59 (2.62)
BMI (median [range]), kg/m ²	23.33 [18.83, 28.34]	25.57 [20.31, 30.42]	22.67 [21.22, 30.46]	22.08 [18.20, 26.44]
Serum retinol (mean [SD]), $\mu\text{mol/L}$	1.72 (0.37)	1.81 (0.36)	1.84 (0.17)	1.78 (0.31)
Serum retinol (median [range]), $\mu\text{mol/L}$	1.67 [1.00, 2.32]	1.77 [1.28, 2.48]	1.86 [1.48, 2.12]	1.77 [1.23, 2.31]
RBP4 (mean [SD]), ng/mL	26.077.45 (5.233.72)	25.430.44 (3.673.34)	27.757.47 (5.953.18)	28.660.44 (6.862.62)
RBP4 (median [range]), ng/mL	26.487.38 [17.500.12, 33.367.34]	26.491.78 [17.821.06, 30.339.41]	28.403.82 [17.212.45, 35.904.91]	27.524.62 [18.281.40, 44.872.24]
Serum RBP4-to-retinol ratio (mean [SD]), g/mol	15.304.81 (1.400.48)	14.247.80 (1.772.90)	15.058.98 (2.832.45)	16.149.20 (2.552.82)
Serum RBP4-to-retinol ratio (median [range]), g/mol	15.059.16 [13.349.10, 17.500.12]	13.844.37 [12.233.63, 17.601.23]	15.451.44 [11.298.41, 19.300.77]	15.521.44 [13.271.33, 20.377.19]

Fig. 1. Strip-chart boxplots of the subjects' SR levels by the group (left panel) and sex (right panel).

Table 3. Summary of the subjects' demographics and measurements in groups 5–6

	≥18 STGD1	≥60 GA
N	15	15
Sex = M, n (%)	5 (33.3)	5 (33.3)
Age (mean [SD]), years	31.20 (10.16)	80.13 (5.24)
Age (median [range]), years	28.00 [19.00, 50.00]	78.00 [72.00, 90.00]
Blood pressure diastolic (mean [SD]), mm Hg	115.27 (12.24)	133.07 (6.90)
Blood pressure diastolic (median [range]), mm Hg	114.00 [96.00, 138.00]	130.00 [123.00, 145.00]
Blood pressure systolic (mean [SD]), mm Hg	77.73 (11.14)	82.40 (6.81)
Blood pressure systolic (median [range]), mm Hg	76.00 [61.00, 103.00]	85.00 [69.00, 90.00]
Weight (mean [SD]), kg	69.13 (10.97)	66.87 (9.72)
Weight (median [range]), kg	68.00 [54.00, 91.00]	66.00 [55.00, 86.00]
BMI (mean [SD]), kg/m ²	22.94 (2.88)	24.15 (2.95)
BMI (median [range]), kg/m ²	23.39 [18.04, 28.33]	23.74 [20.96, 31.98]
Serum retinol (mean [SD]), µmol/L	1.58 (0.44)	1.82 (0.39)
Serum retinol (median [range]), µmol/L	1.49 [0.81, 2.38]	1.82 [1.04, 2.34]
RBP4 (mean [SD]), ng/mL	23,782.91 (6,859.91)	29,853.09 (6,200.88)
RBP4 (median [range]), ng/mL	22,493.63 [10,820.76, 36,784.73]	30,786.58 [17,312.95, 41,650.85]
Serum RBP4-to-retinol ratio (mean [SD]), g/mol	15,066.10 (1,407.81)	16,671.68 (2,941.85)
Serum RBP4-to-retinol ratio (median [range]), g/mol	15,014.28 [13,059.52, 17,677.78]	16,381.41 [13,646.83, 25,929.34]

for age). There was a trend indicating that GA patients may have a higher RBP4-to-SR ratio than healthy subjects ($p = 0.0503$), but it can be explained by age alone ($p = 0.273$ when adjusting for age). Finally, there was no evidence for an effect of sex ($p = 0.377$).

Discussion

This single-center observational study allowed characterizing the blood levels of RBP4 in healthy subjects as well as in patients with STGD1 or GA secondary to AMD.

Fig. 2. Strip-chart boxplots of the subjects' RBP4 levels by the group (left panel) and sex (right panel).

Fig. 3. Strip-chart boxplots of the subjects' serum RBP4-to-retinol ratio levels by the group (left panel) and sex (right panel).

Fig. 4. Scatter plot of the subjects' SR levels over age.

Table 4. Pearson correlation coefficients with 95% confidence intervals

	Serum retinol	RBP4	Serum RBP4 to retinol ratio
Age	0.21 [−0.02, 0.43]	0.33 [0.11, 0.53]	0.23 [−0.01, 0.44]
Sex	0.23 [−0.00, 0.44]	0.10 [−0.14, 0.33]	−0.11 [−0.33, 0.13]
Blood pressure diastolic	0.19 [−0.05, 0.41]	0.26 [0.02, 0.46]	0.19 [−0.05, 0.41]
Blood pressure systolic	0.08 [−0.15, 0.31]	0.01 [−0.22, 0.25]	−0.03 [−0.27, 0.20]
Weight	0.06 [−0.18, 0.29]	0.00 [−0.23, 0.24]	−0.04 [−0.27, 0.20]
BMI	0.01 [−0.23, 0.24]	−0.02 [−0.25, 0.22]	−0.00 [−0.24, 0.23]

Reduction of RBP4-binding capacity is a potential target to reduce vitamin A delivery to the retina and therefore slowing down the visual cycle. This aims to decrease the amount of damaging toxins produced as a byproduct in the visual cycle, such as A2E, which has been shown to play an important role in retinal disease [18–20]. As a consequence, it is hypothesized that the slowing or inhibition of the A2E accumulation should potentially slow down the progression of diseases associated with lipofuscin accumulation [15–17, 21, 22]. The new class of pharmacological agents targeting the visual cycle are called visual cycle modulators. There exist several sites of action for slowing down A2E formation, including reducing levels of serum RBP, inhibiting RPE65-mediated isomerization of all-trans-retinal, or the dimerization of A2E directly with deuterated vitamin A [8, 22].

Vitamin A, a fat-soluble group of vitamins that include retinol and its metabolites, is essential for humans. This

hydrophobic nature limits the diffusion in aqueous environments and presents a barrier for vitamin A being transported to the target sites including the eye and between cell membranes [23]. Therefore, retinol that is liberated from its hepatic storage site forms a complex with RBP4, a lipocalin. This complex of RBP4 and retinol is called holo-RBP4 in its loaded form and apo-RBP4 when retinol-free [7]. The complex forms an eight-stranded beta-barrel, and the retinol-binding site is orientating all-trans-retinol with its hydroxyl group toward the opening, i.e., to the solvent. This is sequestering retinol reversibly away from the watery environment and allows for the transport through the plasma [24]. Additionally, RBP4 forms a complex with TTR, which acts as a combined carrier for thyroxine and RBP. The formation of this complex dramatically increases the molecular weight and prevents RBP-TTR from being metabolized and excreted in the urine, thus increasing its half-life time [12]. Com-

Fig. 5. Scatter plot of the subjects' RBP4 levels over age.

Fig. 6. Scatter plot of the subjects' serum RBP4-to-retinol ratio over age.

pared to other tissues, the eye is highly dependent on acquiring retinol from the retinol-RBP complex and is not able to meet its need to support vision through uptake of dietary retinol alone. This is also suggested as RBP-deficient mice predominantly develop an ocular phenotype [14]. In the eye, 11-cis retinaldehyde is crucial in the visual cycle by building the light-sensitive chromophore of rhodopsin [25]. Deficiency in vitamin A leads to night

blindness or full blindness and is in fact a leading cause of blindness in the world [26, 27]. Especially, malnutrition during pregnancy can lead to vitamin A deficiency which leads to visual problems in the newborn [28]. Accordingly, patients with a malfunctioning RBP4 gene show a "xerophthalmic fundus" phenotype, resembling vitamin A deficiency in the eye. The two sisters reported by Seeliger et al. [13] with a compound heterozygous missense

mutation in RBP4 indeed show a progressive RPE atrophy, a prolonged dark adaption and reduced mixed responses in the electroretinogram.

Despite its high potential as a novel therapeutic target, baseline levels for RBP4 in the most common lipofuscin-associated ophthalmic diseases, namely AMD and STGD1, have not been established yet. Serum RBP is strictly regulated and maintained in spite of variations in vitamin A uptake and is generally considered to be around 2–3 μM in humans [29, 30], which is in line with the results reported in this study. This range is also true for different age-groups and patients with STGD1 or GA. The results are not surprising as serum RBP4 levels are mainly regulated by liver and kidney function. The hepatocytes show the highest expression of RBP4 in the body, which also correlates with the highest concentration of vitamin A in any organ. RBP4 and SR have been shown to be good indicators of liver function [31]. Excretion of RBP4 is mainly performed by the kidney after its dissociation from TTR and the release of retinol. Reduced kidney function is associated with higher RBP4 levels. Further, besides the hepatocytes, RBP is also secreted by the adipocytes, and it has been shown that it is increased in states of impaired insulin sensitivity, metabolic syndrome, and cardiovascular disease [23, 32, 33].

Serum RBP4 and SR show a high level of correlation in our study population. In some disease states, the serum RBP4-to-retinol ratio has been shown to be a better indicator of disease than serum RBP4 or SR alone. In children, an increased RBP4-to-SR ratio is more strongly associated with metabolic syndromes than RBP4 [34]. Also, an increased RBP4-to-SR ratio has been shown to be better indicative of DMII than RBP4, even in the absence of elevated RBP4 levels [35]. Patients with diseases that are known to affect the vitamin A metabolism were excluded from participating in the study, and thus, in our healthy population, there was a low correlation with body weight, BMI, blood pressure, RBP4, SR, or the RBP4-to-SR ratio. This was also true for patients with STGD and GA.

Fenretinide (N-(4-hydroxyphenyl)retinamide) is a synthetic derivative of vitamin A and has been extensively used in rheumatologic diseases and in cancer treatment. Fenretinide competes with retinol on binding to RBP. The binding of fenretinide to RBP4 reduces its molecular weight and reduces its half-life by allowing for a rapid elimination in the urine compared to holo-RBP4. The effect is fully reversible after cessation of the drug and is dose-dependent. Due to the high dependency of the eye on RBP4 bound retinol compared to other tissues, fenretinide preferentially reduces ocular retinol concentra-

tion, thus reducing the levels of available chromophores in the visual cycle [36]. A1120 is another compound that inhibits the interaction of RBP4 with TTR [17]. In the ABCA4 $-/-$ mouse, those compounds dramatically slow, in a dose-dependent manner, the production of A2E [17, 36]. Importantly, fenretinide showed a trend toward slowing lesion growth in GA patients, especially in those with serum RBP4 <2 mg/dL [15]. As an expected side effect of the inhibition of the visual cycle, patients experience a delay in dark adaption.

In this study for the first time, the blood RBP4 levels were established in different age-groups of healthy subjects as well as in patients with STGD1 and geographic AMD. Age and the two retinal/macular diseases appear not to affect RBP4 levels, so the authors do not suggest analyzing them routinely in an ophthalmic setting. Nevertheless, such normative values are an essential prerequisite for treatment strategies, aiming at slowing progression by lowering RBP4 levels. To date, there is no effective treatment available for neither STGD1 nor GA, and so this still presents a major unmet medical need in ophthalmology.

Statement of Ethics

This research project was conducted in accordance with the protocol, the Declaration of Helsinki, the principles of Good Clinical Practice, the Human Research Act, and the Human Research Ordinance as well as other locally relevant regulations. This study protocol was reviewed and approved by Ethikkommission Nordwest-Schweiz, approval number 2020-00579. All participants have given their written consent.

Conflict of Interest Statement

Dr. Hendrik Scholl is supported by the Swiss National Science Foundation, National Center of Competence in Research Molecular Systems Engineering “Molecular Systems Engineering,” project funding in biology and medicine (“Developing novel outcomes for clinical trials in Stargardt disease using structure/function relationship and deep learning” #310030_201,165), the Wellcome Trust (PINNACLE study), the Translational Research Acceleration Program Award by the Foundation Fighting Blindness (“Cone-based optogenetics for vision restoration” #TA-NMT-0621-0805-TRAP), and the Foundation Fighting Blindness Clinical Research Institute (ProgStar study). Dr. Scholl is member of the scientific advisory board of Apellis Switzerland GmbH; ARCTOS medical AG; Astellas Pharma Global Development, Inc./Astellas Institute for Regenerative Medicine; Biogen MA Inc.; Boehringer Ingelheim Pharma GmbH & Co; Gyroscope Therapeutics Ltd.; Janssen Research & Development, LLC (Johnson & Johnson); Novartis Pharma AG (CORE); Okuvision GmbH; Pharma Research & Early Development (pRED) of F. Hoffmann-La Roche Ltd; reVision Therapeutics, Inc.; Stargazer Pharmaceuticals, Inc.; and Third

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Author Contributions

Lucas Janeschitz-Kriegl: data acquisition and manuscript writing; Marco Cattaneo: statistical analysis and manuscript writing; and Hendrik Scholl: project leader and manuscript writing.

Data Availability Statement

The data will be publicly available through the cloud service <https://zenodo.org>.

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