Inflammatory and Neuronal Biomarkers Associated With Retinal Thinning in Pediatric HIV

Charlotte Blokhuis,1 Susanne Doeleman,1 Sophie Cohen,1 Nazli Demirkaya,2 Henriëtte J. Scherpbier,1 Neeltje A. Kootstra,3 Jens Kuhle,4 Charlotte E. Teunissen,5 Frank D. Verbraak,2,6,7 and Dasja Pajkrt1

1Department of Hematology, Immunology and Infectious Diseases, Emma Children’s Hospital/Academic Medical Center, University of Amsterdam, The Netherlands
2Department of Ophthalmology, Academic Medical Center, University of Amsterdam, The Netherlands
3Department of Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands
4Neurology, Departments of Medicine, Biomedicine and Clinical Research, University Hospital Basel, Basel, Switzerland
5Neurochemistry Laboratory and Biobank, Department of Clinical Chemistry, VU University Medical Center and Neurocampus Amsterdam, The Netherlands
6Department of Biomedical Engineering and Physics, Academic Medical Center, University of Amsterdam, The Netherlands
7Department of Ophthalmology, VU University Medical Center, Amsterdam, The Netherlands

Correspondence: Charlotte Blokhuis, Department of Pediatric Hematology, Immunology, and Infectious Diseases, Emma Children’s Hospital/Academic Medical Center, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands; c.blokhuis@amc.nl.

CB and SD contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: May 19, 2017
Accepted: September 28, 2017

**Purpose.** The pathophysiology of neuroretinal thinning in children with human immunodeficiency virus (HIV) is poorly understood. The current study aimed to assess whether neuroretinal thinning in clinically stable perinatally HIV-infected children was associated with biomarkers of immune activation, inflammation, and neuronal damage.

**Methods.** Inflammation-associated and neuronal damage markers were measured in blood and cerebrospinal fluid (CSF) of HIV-infected children aged 8 to 18 years. Using mixed-effects regression analyses, we assessed associations between these biomarkers and neuroretinal layer thickness, as measured with spectral-domain optical coherence tomography.

**Results.** Thirty-two HIV-infected children (median age 13.6 years, 50% male) were included. Blood plasma levels of interleukin-6, monocyte chemoattractant protein-1, and soluble intercellular adhesion molecule-1 were inversely correlated with foveal inner plexiform layer thickness (coef = −4.40, P < 0.001; coef = −9.67, P = 0.047; coef = −10.48, P = 0.042, respectively). Plasma interleukin-6 was inversely correlated with foveal ganglion cell layer thickness (coef = −2.49, P = 0.010). Total Tau levels in CSF were inversely correlated with outer nuclear layer and inner segments thickness (foveal: coef = −19.3, P = 0.029; pericentral: coef = −18.09, P = 0.006) and pericentral total retinal thickness (coef = −28.2, P = 0.017).

**Conclusions.** Neuroretinal thinning was associated with inflammation-associated and neuronal injury biomarkers in a cohort of antiretroviral therapy-treated perinatally HIV-infected children. These findings suggest that ongoing immune activation, inflammation, and neuronal injury occur in parallel with retinal thinning in pediatric HIV.

Keywords: perinatally HIV-infected children, retina, SD-OCT, neuronal injury, immune activation, inflammation

Human immunodeficiency virus (HIV) infection has been associated with retinal structural abnormalities and subtle visual impairments in perinatally infected children, despite adequate viral suppression and absence of ocular opportunistic infections (Fig.).1–5 As the retina contains neuronal tissue, the pathogenesis of retinal structural damage in HIV infection may have similarities with central nervous system (CNS) damage in HIV. This hypothesis is supported by the previously detected association between retinal thinning and white matter microstructural injury in HIV patients.5

The pathogenesis of retinal and cerebral abnormalities in pediatric HIV is poorly understood, and may not only involve direct injury by HIV and/or antiretroviral therapy (cART), but also chronic immune activation, inflammation, and microvascularopathy.5,6 Several biomarkers associated with systemic inflammation are increased in plasma of HIV-infected children, including C-reactive protein (CRP), monocyte chemoattractant protein (MCP-1), soluble CD14 (sCD14), soluble intercellular cell adhesion molecule-1 (sICAM-1), and soluble vascular cell adhesion molecule-1 (sVCAM-1).7–11 Studies in HIV-infected adults report that immune activation and inflammation correlate with biochemical and neuroimaging markers of neuronal injury, including elevated cerebrospinal fluid (CSF) levels of neurofilament light chain (NFL), poorer white matter microstructural integrity, and altered magnetic resonance spectroscopy neurometabolites.12–14 Further, inflammation-associated markers MCP-1 and interleukin (IL)-6 in plasma have previously been shown to impair blood-retinal barrier (BBR) function, leading to increased vulnerability of the retina to potential insults by HIV and/or inflammation.15
Increased levels of neuronal cytoskeleton components, such as neurofilaments and Tau protein, have been associated with HIV-associated neurocognitive disorders (HANDs) in adults. Release and aggregation of these proteins have also been linked to several forms of non-HIV-associated retinal pathology, prompting us to study these markers in the context of HIV-related retinal thinning.

The current study aimed to assess whether increased immune activation, inflammation, and neuronal injury is associated with retinal thinning in clinically stable perinatally HIV-infected children. To investigate this, we looked for potential correlations between spectral-domain optical coherence tomography (SD-OCT)-measured retinal thickness (RT), and systemic and intrathecal biomarkers of immune activation, inflammation, and neuronal injury in a cohort of perinatally HIV-infected children, of whom most were on long-time suppressive cART.

**METHODS**

This study is part of the NOVICE study, an interdisciplinary observational cross-sectional case-control study; assessing neurological, cognitive, and ophthalmic performance in perinatally HIV-infected children as compared to uninfected controls matched for age, sex, ethnicity, and socioeconomic status (SES) in The Netherlands (Dutch Trial Registry ID NTR4074). This study adhered to the tenets of the Declaration of Helsinki and informed consent was obtained from all parents and from children aged 12 and older. The research was approved by the investigational review board at the Academic Medical Center in Amsterdam.

**Study Participants**

We included participants from the NOVICE cohort, consisting of perinatally HIV-1-infected children between 8 and 18 years old, recruited from the Amsterdam Medical Center in Amsterdam between December 2012 and January 2014. Exclusion criteria for participation in this study were traumatic brain injury, (history of) intracerebral neoplasms, chronic HIV-unrelated neurological disease, psychological disorders, and absence of biomarker data. Additional ophthalmic exclusion criteria were visual acuity below 0.1 on the logMAR chart, intraocular pressure above 21 mm Hg, high refractive errors (spherical equivalent [SE] exceeding >5.5 or <8.5 D), significant media opacities, and a history of ocular surgery or disease.

Of the 36 HIV-infected children included in this cohort, we selected those with paired OCT and laboratory data available. This led to exclusion of three children for whom OCT data were unavailable (no consent, n = 2; history of bilateral cytomegalovirus retinitis, n = 1). We additionally excluded OCT data of eyes affected with retinal abnormalities (uveitis, n = 1; congenital toxoplasmosis lesions, n = 1); for these participants, we excluded measurements from the left (affected) eye, and used measurements from the right eye. Finally, we excluded one participant with missing laboratory data.

**SD-OCT and Retinal Layer Segmentation**

Thickness of individual retinal layers was assessed using SD-OCT (Topcon 3D OCT-100; Topcon, Inc., Paramus, NJ, USA) as described previously. In short, OCT images were obtained using 3D macular and disc volume scan protocols. Low-quality
TABLE 1. Participant Characteristics

<table>
<thead>
<tr>
<th>HIV-Infected Children, N = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic characteristics</td>
</tr>
<tr>
<td>Sex, male</td>
</tr>
<tr>
<td>Age at OCT, y</td>
</tr>
<tr>
<td>Ethnicity</td>
</tr>
<tr>
<td>Black</td>
</tr>
<tr>
<td>Mixed race</td>
</tr>
<tr>
<td>Other</td>
</tr>
</tbody>
</table>

CD4+ T-cell counts and HIV viral load

| Peak HIV viral load, log copies/mL* | 5.58 (5.06 to 5.96) |
| Nadir CD4+ T-cell count*            | 445 (270 to 570)    |
| Z score                            | −0.7 (−1.4 to 0.4)  |
| Viral suppression at study inclusion| 26 (81)             |
| CD4+ T-cell count at study inclusion| 770 (580 to 970)    |
| Z score                            | −0.1 (−0.3 to 0.2)  |
| Time with CD4+ T-cell count <500 10^6/L, mo| 1.1 (0.0 to 37.6) |

**cART**

| Use at inclusion                  | 27 (84) |
| Naive                            | 1 (3)   |
| Age at cART initiation, y        | 2.6 (1.2 to 6.2) |
| Duration cART use, y             | 10.7 (5.3 to 15.7) |

Demographic, HIV- and cART-related characteristics of 32 included participants, displayed as median (IQR) or N (%). CDC, Centers for Disease Control and Prevention stage (N/A, no or minimal symptoms; B, moderate symptoms; C, severe symptoms or acquired immunodeficiency syndrome).

* Historical data were incomplete for peak HIV viral load (n = 28; 4 missing), nadir CD4 T-cell counts (n = 30; 2 missing), and cART initiation and duration (n = 29; 3 missing).

images with a Topcon image quality factor (QF) <60 were excluded. Individual neuroretinal layers were automatically segmented from 3D macular volumes using the Iowa Reference Algorithm21 that enables computations of individual retinal layer thickness for each of the nine macular regions as defined by the Early Treatment of Diabetic Retinopathy Study (EDTRS) (see Supplementary Fig.). For the current study, we included the total foveal and perifoveal RT, as well as the thickness of retinal layers that contain neuronal tissue, most of which were affected by thinning in our cohort5: the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), and outer nuclear layer + inner segments (ONL-IS).

**Biomarker Analysis**

On the same day as SD-OCT, samples for biomarker analysis were obtained. Blood samples were collected from all participants using venipuncture. Lumbar puncture to obtain CSF was performed in a random subset of HIV-infected children (n = 24) to study potential neurological and neurocognitive dysfunction in HIV-infected children compared to matched healthy controls.22 Samples were centrifuged within 2 hours at 1700g for 10 minutes, after which the supernatant was transferred into a polypropylene tube (Sarstedt, Numbrecht, Germany) and stored at −80°C until biomarker analysis.

We selected the following biomarkers representing HIV-associated immune activation, inflammation, and vascular endothelial activation that were previously found to be upregulated in our cohort10,11 and/or in other HIV-infected populations7–9,23: CRP, IL-6, IL-15, interferon-gamma (IFN-γ), interferon-gamma inducible protein 10 (IP-10), MCP-1, sCD14, sICAM-1, and sVCAM-1. These inflammation-associated biomarkers were quantified in blood plasma and CSF using Meso Scale Discovery, a highly sensitive electrochemiluminescence-based immunoassay, according to the manufacturer’s instructions,24 except sCD14, which was analyzed using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA).

Neuronal damage marker NFL was quantified in CSF using an ELISA (Uman Diagnostics, Umeå, Sweden) and in serum using an in-house developed test for the Meso Scale Discovery platform, using the same antibodies as in the Uman ELISA.25 CSF neurofilament heavy chain (NFH) was measured using an in-house developed Luminex assay,26 and CSF total Tau (tTau) was measured using the Innotest (Fujirebio, Gent, Belgium).27

**Statistical Analysis**

Statistical analyses were performed using Stata Statistical Software, release 13 (StataCorp LP, College Station, TX, USA). We assessed associations between inflammation-associated and neuronal damage biomarkers (independent parameters) and thickness of selected retinal layers (dependent parameters) using mixed-effects multivariable linear regression. This regression model was chosen to take correlation between right and left eyes within participants into account. Biomarker levels that were reported as below or above the range of quantification were imputed using the lower or upper limit of quantification, respectively. Biomarkers were transformed using base-10 log transformation. Variables that did not approach a normal distribution were dichotomized using median splits. All analyses were adjusted for age, sex, and SE. Analyses were not corrected for OCT QF, because median QF was high in both groups (median QF: HIV-infected = 85.07; controls = 85.17; P = 0.80). The threshold for statistical significance was set at a P-value of <0.05. In line with the explorative nature of this study, we did not perform power calculations or adjust for multiple comparisons, and results should be interpreted accordingly.

**RESULTS**

**Participants**

A total of 32 clinically stable HIV-infected children (50% male, median age 13.6 [interquartile range (IQR) 11.8–15.9] years) were included in the current study. Demographical and clinical characteristics of included participants are presented in Table 1. At the time of study assessment, 27 (84%) participants had been using cART for a median duration of 10.7 (IQR 5.3–13.7) years, of whom all but one (96%) were virologically suppressed using median splits. All analyses were adjusted for age, sex, and SE. Analyses were not corrected for OCT QF, because median QF was high in both groups (median QF: HIV-infected = 85.07; controls = 85.17; P = 0.80). The threshold for statistical significance was set at a P-value of <0.05. In line with the explorative nature of this study, we did not perform power calculations or adjust for multiple comparisons, and results should be interpreted accordingly.

Within the study group, the majority of children (26/32) were on cART and 25/32 were suppressed, with a median duration of 10.7 (IQR 5.3–13.7) years. Of the remaining six children not on cART, three were naive, two had interrupted/ nonadherence, and one had nonadherence. The majority of children had been using cART for a median duration of 10.7 (IQR 5.3–13.7) years, of whom all but one (96%) were virologically suppressed using median splits. All analyses were adjusted for age, sex, and SE. Analyses were not corrected for OCT QF, because median QF was high in both groups (median QF: HIV-infected = 85.07; controls = 85.17; P = 0.80). The threshold for statistical significance was set at a P-value of <0.05. In line with the explorative nature of this study, we did not perform power calculations or adjust for multiple comparisons, and results should be interpreted accordingly.
Biomarkers of Retinal Thinning in Pediatric HIV

DISCUSSION

In this study, we explored whether biomarkers of immune activation, inflammation, and neuronal injury were associated with retinal thinning in perinatally HIV-infected children. Systemic levels of inflammation-associated biomarkers IL-6, MCP-1, and sICAM-1, and of CSF neuronal damage marker tTau, were inversely correlated with RT. These findings could indicate that immune activation, inflammation, vascular endothelial activation, and neuronal injury occur in parallel with retinal thinning. These markers may be relevant for further research to unravel the pathogenesis of retinal structural changes in pediatric (and adult) HIV.

A possible mechanism by which increased peripheral blood levels of inflammation-associated markers IL-6, MCP-1, and sICAM-1 could relate to retinal thinning is impairment of retinal barrier function. Both MCP-1 and IL-6 have been shown to impair tight junctions in the BRB, which may render the retina more vulnerable to viral particles and proinflammatory cytokines.15 Barrier dysfunction has also been described in human brain microvascular endothelial cells of the BBB, where HIV has been shown to upregulate IL-6, MCP-1, and sICAM-1, facilitating adhesion and migration of monocytes into the brain.28,29

We found no associations between retinal thinning and CSF inflammation-associated biomarkers. We hypothesize that this may be explained by the relatively short half-life of most cytokines and the larger distance between the retina and CSF as compared to blood.56 Additionally, immune responses may differ between the two compartments, which is supported by the limited concordance between systemic and intrathecal levels of these markers in our cohort.19 With the sparsity of pediatric data on CSF biomarkers, further studies are needed to confirm our findings, and to elucidate how plasma and CSF immune activation and inflammation relate to each other and to HIV-associated neuroretinal injury over time. We previously found that the pathogenesis of neuroretinal thinning may share common features with that of microstructural white matter injury in HIV-infected children.4 Thus, investigating systemic and intrathecal inflammation-associated biomarkers in relation to white matter microstructure may contribute to our understanding of the pathogenesis of both cerebral and retinal injury in pediatric HIV.

Levels of tTau in CSF inversely correlated with thickness of the foveal and pericentral ONL+IS, and with pericentral total RT. Tau is a neuronal cytoskeletal protein that, when distorted, causes neurodegeneration via various pathways, for instance by accumulation in oligomers and tangles in hyperphosphorylated form.31 In HIV-infected adults, elevated tTau levels were associated with HAND severity, indicating a potential role in HIV-related neuronal injury.72,53 Studies evaluating Tau in the retina are scarce in the field of HIV, but tauopathy has been shown to contribute to retinal injury in various other ocular and neurodegenerative diseases, including glaucoma and Alzheimer’s disease.19,51 Additionally, in a mouse model of retinal degeneration, Tau deposits were associated with degeneration of photoreceptors, with a concomitant reduction in thickness of the inner and outer nuclear layers, the latter of which contains photoreceptor cell bodies.54 As the ONL+IS showed the most prominent thinning in our cohort,3 it is valuable to further investigate the localization and different forms of Tau proteins to clarify their exact role in HIV-associated retinal pathology.

Associations between CSF neuronal damage marker NFH and retinal thinning were not statistically significant, possibly due to our study being underpowered. Studies specifically addressing the relevance of NFH in retinal injury associated with HIV or other neurodegenerative diseases are scarce, hindering comparison of our results with other findings. It may be still a marker worth future investigation, as it is thought to reflect neuronal injury in HIV infection. A study in untreated HIV-infected adults showed that higher CSF NFH levels were associated with increased monocyte activation and impaired processing speed.55 Additionally, a recent study showed that NFH measured in the vitreous body of the eye was increased for up to 2 years after non-HIV-related retinal pathology (such as retinal detachment), confirming that NFH reflects neuronal degeneration in the retina.56 This technique could be useful to study the potential role of NFH in HIV, although ethical considerations considerably limit its use.

Our study does not provide evidence that NFL plays a role in neuroretinal injury in pediatric HIV, as no associations were found between retinal thinning and increased blood or CSF levels of NFL. While CSF NFL has been consistently associated with severity of HIV disease and HAND in adults,56,57 the relevance of NFL in pediatric HIV-associated CNS injury remains less clear due to lack of CSF data in pediatric populations.

Other studies evaluating retinal abnormalities in HIV-infected adults and children have reported both thinning and thickening of the macula and peripapillary retinal nerve fiber layer.58,59 It would be interesting to see how these markers of inflammation, immune activation, and neuronal damage relate to the RT in different age groups and disease stages, and whether this can explain some of the observed variation.

While we are the first to explore a potential relationship of immune activation, inflammation, and neuronal injury with retinal alterations in perinatally HIV-infected children on long-term cART, our study was subject to some limitations. Our small sample size for the regression analyses may have led to insufficient power to detect relevant correlations. While adjusting our analyses for age may in part account for variations in ocular growth and development, the lateral measurement areas were not adjusted for individual differences in axial
<table>
<thead>
<tr>
<th>Table 2. Correlations Between Inflammatory Markers and RT in HIV-Infected Children</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood plasma, ( n = 32 )</strong></td>
</tr>
<tr>
<td>CRP</td>
</tr>
<tr>
<td>IL-6</td>
</tr>
<tr>
<td>IL-15</td>
</tr>
<tr>
<td>IFN-( \gamma )</td>
</tr>
<tr>
<td>IP-10</td>
</tr>
<tr>
<td>MCP-1</td>
</tr>
<tr>
<td>sCD14</td>
</tr>
<tr>
<td>sVCAM-1</td>
</tr>
<tr>
<td>sICAM-1</td>
</tr>
</tbody>
</table>

This table shows the results of the mixed effects regression analyses, evaluating associations between retinal layers and inflammation-associated biomarkers. According to distribution, variables are log pg/mL, or dichotomized using a median split where specified. Data are presented as coefficient (\( P \)).

* Variable dichotomized using median split.
† \( P < 0.05 \).
‡ \( n = 24 \) for sCD14 and \( n = 22 \) for all other biomarkers.
<table>
<thead>
<tr>
<th>Biomarkers of Retinal Thinning in Pediatric HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TABLE 3. Correlations Between Neuronal Damage Markers and RT in HIV-Infected Children</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neuronal Damage Markers</th>
<th>Serum NFL/C0</th>
<th>CSF NFL/C0</th>
<th>tTau/C0</th>
<th>IL-6/C0</th>
<th>MCP-1/C0</th>
<th>sICAM-1/C0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum [NFL]</td>
<td>7.15 (14.2)</td>
<td>0.01 (&lt; 0.01)</td>
<td>9.16 (11.5)</td>
<td>37.37 (0.07)</td>
<td>18.63 (0.28)</td>
<td></td>
</tr>
<tr>
<td>CSF [NFL]</td>
<td>3.88 (14.7)</td>
<td>0.24 (0.3)</td>
<td>4.03 (1.5)</td>
<td>6.27 (0.07)</td>
<td>4.19 (0.46)</td>
<td></td>
</tr>
<tr>
<td>tTau [C0]</td>
<td>9.16 (11.5)</td>
<td>3.88 (0.3)</td>
<td>4.03 (1.5)</td>
<td>6.27 (0.07)</td>
<td>4.19 (0.46)</td>
<td></td>
</tr>
</tbody>
</table>

This table shows the results of the mixed effects regression analyses, evaluating associations between retinal layers and neuronal damage markers in serum and CSF. Data are presented as coefficient (P). *n = 20 for NFL and n = 22 for all other biomarkers.

Acknowledgments
The authors thank all participants and their legal guardians, A.M. Weijisenfeld and A. van der Plas for their help with recruiting participants.

Data were in part presented at the Netherlands Conference on HIV Pathogenesis, Epidemiology, Prevention and Treatment (NCHIV) on November 22, 2016, in Amsterdam, the Netherlands, and included in the respective conference abstract books.

Supported by Bayer Healthcare’s Global Ophthalmology Awards Program 2012, the Emma Foundation (Grant Number 11.001), Stichting Mititalto, ViV Healthcare, AIDSFonds, and Dr. C.J. Vaillantfonds. These funding bodies had no role in the design or conduct of the study, nor in the analysis or interpretation of the results.

Disclosure: C. Blokhuis, None; S. Doeleman, None; S. Cohen, None; N. Demirkaya, None; H.J. Scherpibler, None; N.A. Kootstra, None; J. Kuhle, ECTRIMS Research Fellowship Programme (F), University of Basel (F), Swiss MS Society (R, F), Swiss National Research Foundation (F), Bayer Schweiz (F), AG (C,
References


