Cognitive impairment and MRI-findings in patients with HIV on antiretroviral treatment
Su, T.

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WHITE MATTER STRUCTURE ALTERATIONS IN HIV-INFECTED MEN WITH SUSTAINED SUPPRESSION OF VIREMIA ON TREATMENT

T. Su
M. W. A. Caan
F. W. N. M. Wit
J. Schouten
G. J. Geurtsen
J. H. Cole
D. J. Sharp
F. M. Vos
M. Prins
P. Portegies
P. Reiss
C. B. Majoie

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ABSTRACT

Objective: Cognitive impairment is highly prevalent in HIV-1-infected (HIV+) patients, despite adequate suppression of viral replication by combination antiretroviral therapy (cART). Cerebral white matter structure (WM) alterations are often associated with cognitive impairment and have commonly been reported in the natural course of HIV infection. However, the existence of these alterations in adequately treated HIV+ patients remains unknown, as well as its possible association with cognitive impairment.

Design: We used diffusion tensor imaging (DTI) to investigate whether WM structure alterations exist in HIV+ patients with sustained suppressed viral replication on cART, and if such alterations are related to HIV-associated cognitive deficits.

Methods: We compared 100 aviremic HIV+ men on cART with 70 HIV-uninfected, otherwise comparable men. Clinical and neuropsychological assessments were performed. From DTI data, WM fractional anisotropy (FA) and mean diffusion (MD) were calculated. Subsequently, tract-based spatial statistics (TBSS) was performed, with and without masking out WM lesions.

Results: HIV+ patients showed diffuse WM structure alterations as compared to HIV-uninfected controls, observed as widespread decreased FA and an increased MD. These WM structure alterations were associated with the number of years spent with a CD4-count below 500 cells/mm³, but not with HIV-associated cognitive deficits.

Conclusions: Cerebral WM structure alterations are found in middle-aged HIV+ men with sustained suppression of viremia on cART, and may result from periods with immune deficiency when viral toxicity and host-inflammatory responses were at their peak. These WM structure alterations were not associated with the observed subtle HIV-associated cognitive deficits.

Video abstract: https://youtu.be/Dg3AOLNxVVo

Keywords: aging, antiretroviral therapy, cerebral white matter, diffusion tensor imaging (DTI), HIV-1-infection, HIV-associated neurocognitive disorders (HAND), neuropsychological assessment
INTRODUCTION

Although treatment of HIV-1-infected (HIV+) patients with combination antiretroviral therapy (cART) prevents severe HIV-related complications,[1] cognitive impairment is often seen even when the infection appears adequately suppressed.[2] The reason for this observation is unclear, but might be explained by cerebral white matter (WM) structure alterations. Cerebral WM structure is particularly susceptible to direct cytopathic effects of viral replication and persistent immune activation and inflammation.[3] Although cART potently suppresses viral replication and restores immune function, increased pro-inflammatory cytokine expression may persist.[3,4] This is supported by elevated cerebrospinal fluid markers of immune-activation in patients on prolonged effective cART.[5] Moreover, toxicity of some cART-regimen may affect WM structure.[6] HIV+ patients are at increased risk of developing aging-associated pathologies such as cardiovascular disease, [6–9] which may be accentuated by certain prevalent lifestyle factors (e.g. smoking, alcohol and recreational drug use), possibly impacting the WM structure indirectly. As HIV+ patients age, all these factors may interact affecting tissue structure and eventually leading to cognitive deterioration.[10]

WM structure alterations can be captured by diffusion tensor imaging (DTI), which measures molecular diffusion of water, allowing WM structure to be studied non-invasively.[11] DTI provides information on the degree and localization of WM damage and type of tissue disruption.[12,13]

Studies reporting DTI results in HIV-infection have thus far been inconclusive, with some studies showing WM structure alterations in HIV+ patients,[14–36] where others did not.[37–39] Furthermore, WM structure alterations have been found to be variably related to the duration of HIV-infection, CD4 cell counts and cognitive impairment.[21–37,39,40] Within today’s context of HIV-treatment, these studies show a high variability in detectable viral load. Also, not all HIV-patients were adequately treated on cART. Finally, included HIV-uninfected controls were not always matched in terms of lifestyle and comorbid disease. Many of these studies have also used a region of interest approach to examine WM structure, which in the context of a diffuse pathological process is prone to miss abnormalities.[24,31,32] As it remains unclear to what extent WM structure alterations exist in chronic adequately-treated HIV-infection, and whether these alterations are related to cognitive impairment, a comprehensive whole brain approach is warranted.[26,28]

Due to the evolving nature of the HIV/AIDS epidemic, it is likely that previous findings from studies of younger groups with high levels of viral load do not generalize to the majority of the HIV-infected population in the cART era. It is essential that experimental groups are as representative of the HIV population as possible, hence our aim was to study a large group of middle-aged HIV+ males with sustained suppressed viral load on cART and include an HIV-uninfected male control group that shared socio-demographic background and had similar life style and risk factors. Within this adequately treated and well-defined cohort, we aimed to identify the degree of WM structure alterations and associations with cognitive deficits.
CHAPTER 5

METHODS

Study design
Eligible participants from the main AGE_HIV Cohort were consecutively invited to participate in a nested neuroimaging sub-study. The AGE_HIV Cohort Study is an ongoing study on prevalence, incidence and risk factors of aging-associated comorbidities and organ dysfunction among HIV+ patients and highly comparable HIV-uninfected controls ≥45 years of age (i.e. same geographic region with similar socio-demographic and behavioural (risk) factors).[8] Inclusion criteria specific to this neuroimaging sub-study were as follows: male gender, and for the HIV+ patients sustained suppression of HIV viremia (plasma HIV-RNA <40 copies/mL) for ≥12 months. The presence of viral ‘blips’ (transient low-level viremia) was not an exclusion-criterion. Specific exclusion criteria were: current or past significant neurological disorders (i.e. traumatic brain injury with loss of consciousness >30 minutes, stroke, seizure disorders, multiple sclerosis and dementia), central nervous system infections and tumours. Current significant psychiatric disorders, injecting drug use, daily use of non-injection illicit drugs (with the exception of cannabis), excessive alcohol consumption (>48 units of alcohol/week), insufficient command of the Dutch language, intellectual disability and MRI contraindications were reasons for exclusion as well.

Standard protocol approval, registration and patient consent
The protocol of the AGE_HIV Cohort Study (including the abovementioned neuroimaging study) was approved by the institutional review board of the Academic Medical Center (AMC) and has been registered at www.clinicaltrials.gov (identifier: NCT01466582). Written informed consent was obtained separately for the cohort and neuroimaging studies, from all participants.

Life style, comorbidities and risk factors and HIV/ART-related factors
All subjects completed an extensive standardized questionnaire concerning a wide range of information on demographics, medical characteristics and life style factors. In addition, all subjects underwent standardized screening for age-associated comorbidity and organ dysfunction including a wide range of physical and functional medical measurements. Blood and urine samples were obtained for extensive laboratory testing and detailed information concerning HIV and ART history was obtained. Further details are provided in a previous publication.[8] To examine the effect of biologically possible confounders on changes in diffusion properties, confounders of the following categories were selected from the cohort database: Intoxicants, comorbidities and risk factors, biomarkers of immune activation and HIV/ART-related factors (see Table 1 and 2 for an overview).

<table>
<thead>
<tr>
<th>Table 1: Demographics, Neuroimaging and HIV/ART-related factors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>MSM&lt;sup&gt;1&lt;/sup&gt; (%)</td>
</tr>
<tr>
<td>Premorbid intelligence&lt;sup&gt;2&lt;/sup&gt; (IQ)</td>
</tr>
<tr>
<td>Neuroimaging</td>
</tr>
<tr>
<td>------------------------------</td>
</tr>
<tr>
<td>Philips Intera (%)</td>
</tr>
<tr>
<td>Philips Ingenia (%)</td>
</tr>
<tr>
<td>Brain volume^4</td>
</tr>
<tr>
<td>WBWM FA^5</td>
</tr>
<tr>
<td>WBWM MD^6 (·10^-3 mm^2/s)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIV/ART-related factors</th>
<th>HIV-infected patients (n=100)</th>
<th>HIV-uninfected controls (n=70)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time since diagnosis HIV-1 infection (years)</td>
<td>13.4 (7.5-17.1)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>Diagnosed with HIV-1 infection before 1996 (%)</td>
<td>35</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>CD4-count at enrolment (cells/mm^3)</td>
<td>620 (475-787)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>Nadir CD4-count (cells/mm^3)</td>
<td>170 (60-248)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>Known duration of CD4 &lt;500 cells/mm^3 (years)</td>
<td>3.9 (1.7-7.0)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>Duration of undetectable plasma viral load^6 (years)</td>
<td>10.5 (4.4-13.4)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>Time since start of first ART (years)</td>
<td>11.4 (4.9-14.9)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>Naive at start of cART^7 (%)</td>
<td>79</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>Prior clinical AIDS^8 (%)</td>
<td>34</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>CPE-score of current cART regimen^9</td>
<td>7</td>
<td>n.a.</td>
<td>-</td>
</tr>
</tbody>
</table>

Data presented as median (IQR) or percentage as appropriate, p = p-value, bold indicates statistical significance
Test type used: ^a Mann-Whitney U test, ^b Chi-square test, ^c Students t-test
1 The term “MSM” (Men having Sex with Men) applied to male subjects who self-reported to feel mostly or exclusively sexually attracted to men.
2 Premorbid intelligence quotient (IQ) was estimated using the Dutch Adult Reading Test (DART).[56]
3 Scans were performed on either a Philips 3T Intera scanner or 3T Philips Ingenia system (Philips Healthcare, Best, the Netherlands), using the exact same DTI-protocol.
4 Relative total brain volume consist of the ratio of total brain volume (sum of total grey and white matter volume) over intracranial volume (the sum of grey matter, white matter and cerebrospinal fluid) by SPM8[49], to account for inter-subject variability in brain volume by (age-related) neurodegenerative processes. Results displayed are adjusted for age and scanner system.
5 For each individual subject, its Fractional Anistropy (FA) and Mean Diffusion (MD) maps were projected on a skeletonised population-based FA-map. This FA and MD maps were averaged into white matter (WM) summary statistics. Results displayed are adjusted for age, brain volume and scanner system.
6 Duration of undetectable plasma viral load was defined as: number of years since last plasma viral load >200 copies/ml.
7 The term “cART” was used for a combination of ≥3 antiretroviral drugs, other than ritonavir used as a pharmacologic booster.
8 The term “prior clinical AIDS” was used in case of a previous AIDS-defining condition according to the United States Centers for Disease Control and Prevention (CDC) classification.
9 Central nervous system penetration effectiveness (CPE) score of the cART regimen of each HIV-infected participant was calculated using the algorithm as proposed by Letendre et al. in 2010.[57]
Neuropsychological assessment

All subjects underwent a detailed neuropsychological assessment, covering fluency, attention, processing speed, memory, executive function and fine motor function. Using normative standards, test scores were converted to age and education corrected scores. Multivariate normative comparison (MNC) was performed to detect cognitive impairment.[41] Our previous work demonstrated that the MNC method can detect cognitive impairment more reliably as compared to the Frascati criteria for HIV-associated neurocognitive disorders (HAND).[42]

MRI data acquisition

All subjects underwent a MRI examination at the AMC and scanning was performed on a 3T Intera and continued on a 3T Ingenia system (Philips Healthcare, Best, the Netherlands) due to a scanner upgrade. This upgrade was statistically accounted for in all analyses and the distribution of subjects according to HIV serostatus per scanner system is provided in Table 1. The diffusion weighted MRI scanning parameters were: TE/TR=92/7081-9665 ms; 55 to 64 continuous slices depending on head size; data matrix 112x112; voxel size=2x2x2 mm³; diffusion weighting of b=1000 s/mm² along 64 directions and four averages with b=0 s/mm². For volumetric analyses a sagittal magnetization prepared rapid gradient echo (MPRAGE) scan was acquired (TE/TR=3.1/6.6 ms; 270x270 mm² FOV, 170 sagittal slices of 1.2 mm thickness, 1.1x1.1 mm² in-plane resolution) and to define WM lesions of presumed vascular origin a 3-dimensional fluid attenuated inversion recovery (3D-FLAIR) scan was performed (TE/TR=355/4800 ms; TI=1650 ms; FOV 250x250 mm²; 321 sagittal slices of 0.56 mm thickness; 1.1x1.1 mm² in-plane resolution).

Table 2: Lifestyle, comorbidities, risk factors and biomarkers

<table>
<thead>
<tr>
<th></th>
<th>HIV-infected patients (n=100)</th>
<th>HIV-uninfected controls (n=70)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intoxications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily to monthly use of cannabis (%)</td>
<td>15</td>
<td>14</td>
<td>1.00a</td>
</tr>
<tr>
<td>Weekly to monthly use of cocaine (%)</td>
<td>4</td>
<td>4</td>
<td>0.90b</td>
</tr>
<tr>
<td>Weekly to monthly use of ecstasy (%)</td>
<td>2</td>
<td>11</td>
<td>0.02a</td>
</tr>
<tr>
<td>Alcohol intake (units per week)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2-14)</td>
<td>6</td>
<td>5</td>
<td>0.74d</td>
</tr>
<tr>
<td><strong>Comorbidities and co-infections</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes Mellitus, type 2 (%)</td>
<td>6</td>
<td>4</td>
<td>0.74a</td>
</tr>
<tr>
<td>Renal disease² (%)</td>
<td>2</td>
<td>4</td>
<td>0.07a</td>
</tr>
<tr>
<td>Hypertension³ (%)</td>
<td>40</td>
<td>36</td>
<td>0.57a</td>
</tr>
<tr>
<td>Cardiovascular disease⁴ (%)</td>
<td>8</td>
<td>6</td>
<td>0.50b</td>
</tr>
<tr>
<td>Prior history of non-AIDS cancer (%)</td>
<td>7</td>
<td>7</td>
<td>1.00a</td>
</tr>
<tr>
<td>Current chronic or active HBV-infection (%)</td>
<td>2</td>
<td>0</td>
<td>0.52a</td>
</tr>
<tr>
<td>Current chronic or active HCV-infection (%)</td>
<td>1</td>
<td>0</td>
<td>0.41b</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use of antihypertensive medication (%)</td>
<td>18</td>
<td>14</td>
<td>0.57c</td>
</tr>
<tr>
<td>Use of lipid-lowering medication (%)</td>
<td>12</td>
<td>11</td>
<td>0.69b</td>
</tr>
<tr>
<td>Use of psychotropic medication⁵ (%)</td>
<td>15</td>
<td>14</td>
<td>0.90b</td>
</tr>
</tbody>
</table>
White matter alterations in treated HIV

**Table 2:**

<table>
<thead>
<tr>
<th>Lifestyle, comorbidities, risk factors and biomarkers</th>
<th>HIV-infected patients (n=100)</th>
<th>HIV-uninfected controls (n=70)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vascular risk factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (IFCC - mmol)</td>
<td>35 (32-39)</td>
<td>37 (35-41)</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aortic pulse wave velocity&lt;sup&gt;a&lt;/sup&gt; (m/s)</td>
<td>7.87 (7.18-8.75)</td>
<td>7.75 (7.17-8.87)</td>
<td>0.60&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tobacco smoking (packyears)</td>
<td>20 (7.7-35)</td>
<td>8 (2.4-19.5)</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24 (22-26)</td>
<td>26 (24-28)</td>
<td>0.002&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Waist-to-hip ratio ≥ 0.9 (central obesity) (%)</td>
<td>86</td>
<td>69</td>
<td>0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.28 (1.02-1.53)</td>
<td>1.32 (1.01-1.56)</td>
<td>0.81&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.21 (2.34-3.86)</td>
<td>3.36 (2.86-3.93)</td>
<td>0.13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.39 (4.49-6.24)</td>
<td>5.39 (5.07-6.16)</td>
<td>0.32&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.85 (1.19-2.80)</td>
<td>1.61 (1.08-2.45)</td>
<td>0.31&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lipoprotein(a) (mg/L)</td>
<td>0.75 (0.62-0.87)</td>
<td>0.68 (0.58-0.86)</td>
<td>0.31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>D-dimer (mg/L)</td>
<td>0.21 (0.20-0.33)</td>
<td>0.26 (0.20-0.40)</td>
<td>0.14&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Biomarkers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-reactive protein &gt;5 mg/L (%)</td>
<td>14</td>
<td>6</td>
<td>0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soluble CD14 (ng/mL)</td>
<td>1528 (1313-2031)</td>
<td>1196 (992-1509)</td>
<td>&lt;0.001&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soluble CD163 (ng/mL)</td>
<td>276 (204-418)</td>
<td>242 (182-356)</td>
<td>0.12&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4/CD8-ratio</td>
<td>0.74 (0.53-1.01)</td>
<td>1.72 (1.29-2.25)</td>
<td>&lt;0.001&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data presented as median (IQR) or percentage as appropriate, p = p-value, bold indicates statistical significance

Test type used: * Fisher’s exact test, † Chi-square test, ‡ Students t-test, ‡ Mann-Whitney U test.

1 Diabetes mellitus type 2 was considered present if HbA1c (IFCC) ≥48 mmol and/or elevated blood glucose (non-fasting ≥11.1 mmol/L or fasting ≥7.0 mmol/L), or if on antidiabetic medication.

2 Hypertension was considered present if diastolic blood pressure ≥90 mmHg and/or systolic blood pressure ≥140 mmHg in all three measurements (Omron 705IT) with a one-minute interval, or if on antihypertensive medication.

3 The variable past cardiovascular disease included angina pectoris, myocardial infarction, and/or peripheral arterial disease.

4 Psychotropic medication included: antidepressants, benzodiazepines, and/or methylphenidate.

5 Higher aortic pulse wave velocity is indicative for arterial stiffness and was measured by Arteriograph® system.

6 The waist-to-hip ratio was considered higher than normal if it was ≥0.9.

**Image processing**

All data were anonymized prior to analysis. Preprocessing of DTI data was performed with software developed in-house (Matlab, MathWorks, Natick, MA), using the HPCN-UvA Neuroscience Gateway and using resources of the Dutch e-Science Grid. Head motion...
and deformations induced by eddy currents were corrected for by an affine registration of the
diffusion weighted images (DWI) to the non-diffusion weighted image. The gradient directions
were corrected by the rotation component of the transformation.[44] Rician noise in the DWI
was reduced by an adaptive noise filtering method.[45] Diffusion tensors were calculated
using a non-linear least squares estimation. Subsequently, fractional anisotropy (FA) and mean
diffusion (MD) maps were computed for each subject. All subjects-data were then aligned into
a common space using the nonlinear registration tool FNIRT.[46]

To reduce the risk of partial volume effects (PVE), we focused our analysis on the central parts
of the WM tracts, for which a population-based FA-map was created and skeletonized (FA was
thresholded at 0.2).[47] The resulting FA-based skeleton represents the center of all major tracts
in the population-based template. For each individual subject, its aligned FA and MD maps were
projected onto this FA skeleton (Figure 1).[47] Subsequently, each FA and MD maps were averaged
to a WM summary statistic or used for tract-based spatial statistics (TBSS) in FSL.[47][48]

Anatomical images were used for grey matter (GM), WM and cerebrospinal fluid (CSF)
segmentation by SPM8.[49] The intracranial volume (ICV) was computed by summing the GM,
WM and CSF volumes. The relative total brain volume was defined as the ratio of total brain
volume (summing the GM and WM) over ICV.

In addition to WM summary statistics for each individual DTI metric, normal appearing white
matter (NAWM) DTI metrics were derived in which WM lesion areas (hyperintense on 3D-FLAIR)
were masked out (Figure 1).[50] This involved training a ‘random-forest’ classification algorithm
on a manual annotation set of 20 individuals with varying lesion load, to detect the presence
of lesions.[51] This was then applied in the current dataset to identify regions of WM lesions in
HIV+ patients and controls.

Statistical Analysis

Group comparisons of subject characteristics between HIV+ patients and controls were
performed (Table 1 and 2).

Across all subjects, the effect of HIV serostatus on WM FA and MD measures were examined
by linear regression analyses, while adjusting for age, relative brain volume and scanner system.
To examine possible accelerated aging effects in HIV+ patients, interaction effects between age
and HIV serostatus on WM FA and MD measures were assessed.

To identify confounders and determinants (intoxicants, comorbidities and risk factors,
biomarkers of immune activation and HIV/ART-related factors) of WM FA and MD measures,
stepwise linear regression analyses were performed ($p<0.05$ probability to enter and $p>0.1$
probability to remove), while adjusting for age, brain volume and scanner system.

To examine associations of WM FA and MD measures with cognition, HIV+ patients were
classified as either cognitively impaired or unimpaired by MNC. Group comparison on cognitive
status was performed on the WM FA and MD measures, adjusted for age, brain volume and
scanner system. The test statistic of the MNC method was Hotelling’s $T^2$. To create a continuous
measure of cognitive function and prevent a bimodal distribution, each Hotelling’s $T^2$ statistic
was subtracted from the lowest Hotelling’s $T^2$ statistic and multiplied by the direction of
development (i.e. positive or negative deviation reflecting better or poorer cognition as compared
**Figure 1:** Methods overview. Fractional Anisotropy (FA) and Mean Diffusivity (MD) are measured in a series of axial slices (inset). White matter (WM) tissue degradation is hypothesized to lead to decreased FA and increased MD. FA and MD maps of HIV+ patients and controls were processed as illustrated. Mean WM FA and MD were first analyzed, after which Tract-based spatial statistics (TBSS) further localized effects within WM.
to the control group). Associations of this transformed statistic with WM FA and MD, were examined by linear regression, adjusting for age, brain volume and scanner system.

To provide spatial information on significant findings of WM DTI metrics, TBSS group comparison and correlational analyses were performed, while adjusting for age, brain volume and scanner system. Multiple comparisons were corrected for by using permutation tests. This was implemented using the Randomise software within FSL, employing the threshold free cluster enhancement (TFCE), in which \( p \)-values<0.05 were considered significant. All analyses were subsequently repeated with NAWM DTI metrics.

MNC was performed using R statistical software,[52] while remaining analyses were done in SPSS (version 20.0, IBM).

RESULTS

Demographic and clinical characteristics
Participants were enrolled to the neuroimaging study between December 2011 and August 2013. Neuroimaging data were available from 100 HIV+ patients and 70 controls. An overview of the demographics, neuroimaging and HIV/ART-related factors are shown in Table 1, while Table 2 provides an overview of intoxicants, comorbidities and risk factors and biomarkers of immune activation. The HIV+ patients (median age: 54 (IQR 49-61) years) were highly comparable to the controls (median age: 53 (IQR 49-59) years). Both groups were also similar in their substance use behaviour, except for ecstasy use, which was more common in the controls (11% vs. 2%, \( p=0.02 \)). Controls also had higher plasma concentrations of glycated haemoglobin (HbA1c, 37 vs. 35, \( p=0.01 \)) and body mass index (BMI, 26 vs. 24, \( p=0.002 \)). HIV+ patients fulfilled more often the criteria for central obesity (i.e. waist/hip ratio >0.9) and had greater lifetime tobacco exposure as measured by pack-years of smoking (\( p=0.03 \), \( p=0.01 \)). Levels of soluble CD14 were higher in HIV+ patients, whereas CD4/CD8 ratios were lower as compared to controls (\( p<0.001 \), \( p<0.001 \)). No group differences were found for the remaining factors.

HIV+ patients had been treated with antiretroviral therapy for a median duration of 11.4 (IQR 4.9-14.9) years) and showed substantial immune recovery. Their median nadir CD4-count was 170 (IQR 60-248), with a current CD4-count of 620 (IQR 475-787) cells/mm³.

Group comparisons on white matter diffusion properties
Across the WM there were significant differences between the HIV+ patients and controls. HIV+ patients showed significantly lower WM FA (\( p=0.03 \)) and significantly higher WM MD (\( p=0.02 \)). See Table 1 for further details. Interaction effects were assessed and no interaction effect of age and HIV serostatus was found for these WM DTI-metrics (FA: \( p=0.59 \), MD: \( p=0.58 \)).

Voxel-wise comparison by TBSS showed a widespread pattern of lower FA and higher MD in HIV+ patients compared to controls (Figure 2). Patterns of reduced FA were seen in projection- and thalamic fibers (i.e. cortical spinal tract and anterior thalamic radiation), all major association fibers (i.e. superior longitudinal fasciculus, inferior longitudinal fasciculus, inferior-fronto-occipital fasciculus and uncinated fasciculus), limbic system fibers (i.e. cingulum) and callosal fibers (i.e. forceps minor and forceps major). Differences in MD were less pronounced.
White matter alterations in treated HIV

Figure 2: Group comparison and correlation analysis by tract-based spatial statistics (TBSS)
Tract-based spatial statistics (TBSS) showed (A) significantly decreased fractional anisotropy (FA) and (B) increased mean diffusion (MD) in cerebral white matter structure in HIV-infected patients (n=100) compared to highly comparable HIV-uninfected controls (n=70). TBSS showed (C) cerebral white matter MD was significantly correlated with the duration in years spent with immune deficiency (i.e. CD4 count below 500 cells/mm³) in HIV-infected patients (n=100). T-statistics are color-coded and shown in voxels where \( p < 0.05 \), corrected by threshold free cluster enhancement (TFCE).

and mainly localized in the left hemisphere, although lowering the statistical threshold also showed contralateral effects (data not shown).

Determinants and confounders of altered white matter diffusion properties
Joint analysis of HIV+ patients and controls showed that HIV serostatus, age, lower brain volume and the number of antihypertensive medications used were significantly associated with lower WM FA and higher WM MD (Table 3: Model 1). Remaining possible confounding variables examined (see Table 1 and 2 for a complete overview) did not contribute sufficiently to the WM DTI-metrics and were therefore not selected for the final model.

When restricting the analysis to the HIV+ group, the number of antihypertensive medications used remained significantly associated. Additionally, higher LDL-cholesterol and duration spent with CD4 cell count below 500 cells/mm², were significantly associated with
higher WM MD (Table 3: Model 2). Patients who had been treated with mono- or dual therapy with nucleoside reverse transcriptase inhibitors before the start of cART showed significantly higher WM MD ($p=0.04$). However, this effect was confounded by duration spent with CD4 cell count below 500 cells/mm$^3$ and did not remain significant. No collinearity was found between age or volume and the determinants.

Voxel-wise correlation analyses by TBSS showed a diffuse pattern of significantly increased MD with longer duration spent with a CD4 cell count below 500 cells/mm$^3$ (Figure 2). This relation was found in projection and thalamic fibers, all major association fibers, limbic system fibers and callosal fibers.

### Table 3: Models of determinants of white matter diffusion tensor imaging metrics

<table>
<thead>
<tr>
<th></th>
<th>WM FA</th>
<th>WM MD</th>
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<tbody>
<tr>
<td></td>
<td>$\beta$</td>
<td>$p$</td>
</tr>
<tr>
<td><strong>Model 1: Multiple linear regression model of effects of determinants (HIV+ patients and controls combined)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>$-0.346$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Brain Volume$^1$</td>
<td>$0.285$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Scanner$^2$ (1/2)</td>
<td>$-0.091$</td>
<td>0.13</td>
</tr>
<tr>
<td>HIV serostatus$^3$ (0/1)</td>
<td>$-0.142$</td>
<td>0.02</td>
</tr>
<tr>
<td>Antihypertensives (n)</td>
<td>$-0.210$</td>
<td>$0.001$</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>$-0.086$</td>
<td>0.15</td>
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<table>
<thead>
<tr>
<th></th>
<th>WM FA</th>
<th>WM MD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$</td>
<td>$p$</td>
</tr>
<tr>
<td><strong>Model 2: Multiple linear regression model of effects of HIV/ART-related factors (HIV+ patients only)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>$-0.340$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Brain Volume$^1$</td>
<td>$0.270$</td>
<td>0.002</td>
</tr>
<tr>
<td>Scanner$^2$ (1/2)</td>
<td>$-0.173$</td>
<td>0.04</td>
</tr>
<tr>
<td>Antihypertensives (n)</td>
<td>$-0.208$</td>
<td>0.01</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>$-0.063$</td>
<td>0.43</td>
</tr>
<tr>
<td>CD4 count &lt;500 cells/mm$^3$ (years)</td>
<td>$-0.119$</td>
<td>0.16</td>
</tr>
</tbody>
</table>

$\beta$=standardized beta coefficients; $\eta^2$=partial eta squared; $p$=p-value, bold indicates statistical significance

**Abbreviations:** WM=white matter, FA=fractional anisotropy, MD=mean diffusion

$^1$Relative total brain volume consist of the ratio of total brain volume (sum of total grey and white matter volume) over intracranial volume (the sum of grey matter, white matter and cerebrospinal fluid) by SPM8[49], to account for inter-subject variability in brain volume by (age-related) neurodegenerative processes.

$^2$Scans were performed on either a 3T Intera (1) or 3T Ingenia (2) system (Philips Healthcare, Best, the Netherlands), using the exact same DTI-protocol.

$^3$Participants of this study were either HIV negative (0) or HIV seropositive (1).

### Altered white matter diffusion properties and its association with cognitive performance

Sixteen percent of the HIV+ patients were classified as cognitively impaired by MNC (alpha was 5%, one-tailed, assuming a specificity of 95%, which was previously verified).[42] Comparing
cognitively impaired and cognitively unimpaired HIV+ patients, no significant differences in WM FA ($p=0.82$) or MD ($p=0.91$) were found. Overall poorer cognitive performance was not associated with WM FA ($\beta=0.007$, $p=0.82$, $\eta^2<0.001$) or MD ($\beta=-0.051$, $p=0.61$, $\eta^2=0.003$).

Repeating the analyses on the normal appearing white matter

All findings on WM FA and MD alterations persisted after excluding WM lesion areas from the analyses (including effects of HIV serostatus, the number of antihypertensive medications used, LDL-cholesterol and duration spent with CD4 cell count below 500 cells/mm$^3$). See supplementary Results and Tables 1 and 2.

DISCUSSION

Key findings

In this study, cerebral WM structure was assessed in 100 middle-aged HIV+ males with well-suppressed viral load on cART and compared to 70 HIV-uninfected, but otherwise highly comparable, controls. We found significant WM structure alterations in HIV+ patients, which consisted of lower FA and higher MD, as assessed by DTI. TBSS showed that these effects were widespread throughout the brain. Additionally, deleterious effects of hypertension, dyslipidaemia and duration of past immune deficiency were found. HIV-associated cognitive deficits were not found to be associated with WM structure alterations.

Interpretation of findings

The diffuse pattern of altered diffusion properties found in HIV+ patients relative to highly comparable HIV-uninfected controls may indicate subtle but widespread WM injury. Consistent findings of alterations in FA and MD have been reported previously in middle-aged and older HIV+ patients compared to healthy controls.[14,16,19,22,23,26,27,29,30,33,34,36] The origin of the subtlety of our findings compared to previous studies may be two-fold. First, all HIV-patients in our cohort were adequately treated on cART. Second, healthy controls were carefully matched on lifestyle and comorbid disease. Comparing to previous work, one might infer that improved treatment diminishes HIV-induced WM structure alterations. The fact that two comparable studies which exclusively included aviremic HIV+ patients did not report WM structure alterations might be due to smaller sample sizes as compared to our study.[35,39] Furthermore, one of the studies also excluded all possible comorbidities[39] and therefore may have excluded HIV effects, since HIV itself has been reported to be independently associated with cardiovascular disease and many other comorbidities.[10]

In addition to the effects of HIV serostatus and aging, we found independent associations of hypertension and dyslipidemia with WM structure alterations. Effects of hypertension were particularly consistent, which might be partly HIV-mediated, as increased cardiovascular risk has been frequently reported in HIV.[10] Evidence for possible accelerated CNS aging could not be derived from this cross-sectional analysis. Follow-up measurements are currently underway.
and may provide more insight into a possible interaction effect of age and HIV serostatus on WM structure alterations in adequately treated HIV+ patients.

The total duration of immune deficiency (i.e. the number of years spent with CD4 cell count lower than 500 cells/mm$^3$) was also strongly associated with WM structure alterations. This may reflect irreversible damage that has occurred during immune deficiency by both direct viral and host-derived proinflammatory factors. Previously reported persistent WM injury in HIV+ patients with partial immune reconstitution, provides evidence that such damage could be permanent in nature.[33] Moreover, the use of cART and higher current CD4 cell counts have been associated with higher FA values,[40] suggesting that prevention of immune deficiency may avert irreversible WM structure damage.

Among HIV+ patients in the cART-era, effects of cumulative exposure to immune deficiency are possibly insufficiently captured by the nadir CD4 cell count.[33–35,39,40] Subtle WM structure alterations may be better captured by a measure of cumulative exposure to immune deficiency, as used in this study. These findings provide additional support for the current HIV treatment guidelines that stress the importance of preventing immune deficiency and initiating anti-retroviral therapy in all patients irrespective of CD4 count.[53]

Pretreatment with nucleoside-analogue reverse transcriptase inhibitors (NRTI) before the start of cART was associated with WM structure injury, but this relationship seemed to be driven by the duration of past immune deficiency. This is compatible with HIV+ patients diagnosed in the pre-cART era to have been more likely to have experienced more prolonged periods of advanced immune deficiency.

Although associations between systemic markers of immune activation and inflammation (i.e. sCD14 and sCD16) with WM structure alterations were not observed, ongoing pro-inflammatory reactions within the CNS affecting white matter structure cannot be ruled out. Post-mortem studies have reported persistent levels of elevated markers of microglia/macrophage activation in cART treated HIV cases,[54] suggesting pro-inflammatory reactions may not be normalized in the context of cART and that the continued presence of neuro- and myelinotoxic cytokines may induce subtle WM alterations, such as those observed in the current study.

While several studies have previously reported WM structure alterations to be related to HIV-associated cognitive impairment,[24,27,28,31,34,37] others did not report such an relationship. [26,30,35,39] An association between WM structure alterations and cognitive deficits was not observed in the current study. The magnitude of the effect of HIV-serostatus on cognition and WM structure alterations in the current study was small; hence a possible relationship between the two would likely be subtle. Note that in absolute numbers, our group of 16 cognitively impaired patients is of comparable size to the CHARTER cohort, reporting 10 impaired patients [28]. The successful treatment of HIV+ patients and exclusion of otherwise confounding factors are in our opinion key to the subtlety of effects we report. If WM structure is to be used as a biomarker to predict cognitive impairment and subsequent deterioration in aging HIV+ patients, then larger, multi-center cohorts with hundreds of patients are needed. Also, sufficient time between follow-up measurements is required in future studies, since cognitive decline was not related to a significant increase of MD in HIV+ patients after one year of follow-up.[27]
The presence of WM lesions of presumed vascular origin is associated with findings of WM structure alterations. Greater microstructural alterations have been reported in HIV+ patients with WM lesions, compared to HIV+ patients with no lesions. While some studies have carefully reviewed the analysed regions for WM lesions, we studied the NAWM separately by subject-wise excluding lesion areas. All effects persisted in whole brain measures.

**Limitations**

Although controls enrolled in this study had very similar demographics and lifestyle, HIV+ patients reported more lifetime tobacco exposure and controls more ecstatic use. Moreover, although HIV+ patients had lower BMI, they fulfilled more often the criteria for central obesity, and showed increased monocyte activation (i.e. higher levels of soluble CD14) and lower CD4/CD8 ratio. Such vascular risk factors and increased immune activation are known complications of HIV-infection or its treatment. However, none of these parameters were related to WM structure alterations in the current study. About one-third of HIV+ patients and controls were scanned on a different scanning system due to a scanner replacement. We have adjusted for the scanner effects by factoring its effects in the statistical model. When analyzing the subset of subjects on a single machine, the effects within HIV+ patients are persistent, the relation between CD4 count < 500 cells/mm³ and MD remains significant (beta=0.26, p=0.005). When comparing groups, effects sizes are of equal sign but slightly smaller, and p-values thus higher (FA: beta=-0.010, p=0.19; MD: beta=0.11, p=0.09).

**Conclusion**

In this 3T DTI study, middle-aged HIV+ males with suppressed viremia on cART showed pronounced WM structure alterations as compared to highly comparable HIV-uninfected controls. The association with duration of exposure to immune deficiency suggests irreversible damage from previous periods of immune deficiency when host inflammatory and virus toxicity were at their peak. In addition, independent associations between vascular risk factors and WM structure abnormalities were found. Longitudinal follow-up studies are needed to determine the progression and synergistic effects of these risk factors.
REFERENCES


SUPPLEMENTARY MATERIALS

Supplementary results 1

Results on normal appearing white matter (NAWM)

White matter (WM) lesions (hyperintense on 3D-FLAIR) were segmented. Subsequently such WM lesion areas were masked out to derive summary statistics for fractional anisotropy (FA) and mean diffusion (MD) of the normal appearing white matter (NAWM).

Group comparisons on NAWM diffusion properties

HIV-infected (HIV+) patients compared to HIV-uninfected controls showed significantly lower NAWM FA ($p=0.02$) and higher NAWM MD ($p=0.02$), for further details see supplementary Table 1. Interaction effects were assessed and no interaction effect of age and HIV serostatus was found for these NAWM DTI-metrics (FA: $p=0.44$, MD: $p=0.55$).

Determinants and confounders of altered NAWM diffusion properties

Joint analysis of HIV+ patients and controls showed that besides the effects of HIV serostatus, the number of antihypertensive medications used was also significantly associated with lower NAWM FA and higher NAWM MD (supplementary Table 2: Model 1). Remaining possible confounding variables did not contribute sufficiently to the WM DTI-metrics and were therefore not selected for the final model.

When restricting the analysis to the HIV+ group, the number of antihypertensive medications used remained significantly associated. Additionally, higher LDL-cholesterol and duration spent with a CD4 count below 500 cells/mm$^3$ were significantly associated with higher NAWM MD (supplementary Table 2: Model 2).

Altered NAWM diffusion properties and its associations with cognitive deficits

Comparing cognitively impaired and cognitively unimpaired HIV+ patients, no significant differences in NAWM FA ($p=0.97$) or NAWM MD ($p=0.89$) were found. Overall poorer cognitive performance was neither associated with NAWM FA ($\beta=0.016, p=0.87, \eta^2<0.001$) nor with NAWM MD ($\beta=-0.040, p=0.69, \eta^2=0.002$).
Supplementary Table 1: Group comparisons on normal appearing white matter (NAWM) diffusion properties

<table>
<thead>
<tr>
<th></th>
<th>HIV-infected patients (n=100)</th>
<th>HIV-uninfected controls (n=70)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAWM FA(^1)</td>
<td>0.424 (0.415-0.437)</td>
<td>0.433 (0.421-0.441)</td>
<td>0.02(^a)</td>
</tr>
<tr>
<td>NAWM MD(^1) (10(^{-3}) mm(^2)/s)</td>
<td>0.767 (0.747-0.783)</td>
<td>0.754 (0.742-0.765)</td>
<td>0.02(^a)</td>
</tr>
</tbody>
</table>

Data presented as median (IQR), p = p-value, bold indicates statistical significance.

Test type used: *Mann-Whitney U test

Abbreviations: NAWM=Normal Appearing White Matter, FA=Fractional Anisotropy, MD=Mean Diffusion

\(^1\)White matter (WM) lesions (hyperintense on 3D-FLAIR) were segmented. Subsequently such WM lesion areas were masked out. For each individual subject, its fractional anisotropy (FA) and mean diffusion (MD) maps were projected on a skeletonised population-based FA-map. This FA and MD maps were averaged into normal appearing white matter (NAWM) summary statistics. Results displayed are adjusted for age, brain volume and scanner system.

Supplementary Table 2: Models of determinants of normal appearing white matter (NAWM) DTI-metrics

<table>
<thead>
<tr>
<th></th>
<th>NAWM FA</th>
<th>NAWM MD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\beta)</td>
<td>p</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Age (years)</td>
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<td>&lt;0.001</td>
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<td>Brain Volume(^1)</td>
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<td>&lt;0.001</td>
</tr>
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<td>Scanner (1/2)(^2)</td>
<td>-0.087</td>
<td>0.14</td>
</tr>
<tr>
<td>HIV serostatus (0/1)(^3)</td>
<td>-0.148</td>
<td>0.01</td>
</tr>
<tr>
<td>Antihypertensives (n)</td>
<td>-0.193</td>
<td>0.02</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
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<td>Model 2: Multiple linear regression model of effects of HIV/ART-related factors (HIV+ patients only)</td>
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<tr>
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<td>-0.193</td>
<td>0.02</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>-0.063</td>
<td>0.42</td>
</tr>
<tr>
<td>CD4-count &lt;500 cells/mm(^2) (years)</td>
<td>-0.112</td>
<td>0.18</td>
</tr>
</tbody>
</table>

\(\beta\)=standardized beta coefficients; \(\eta^2\)=partial eta squared; p = p-value, bold indicates statistical significance

Abbreviations: NAWM=normal appearing white matter, FA=fractional anisotropy, MD=mean diffusion

\(^1\)Relative total brain volume consist of the ratio of total brain volume (sum of total grey and white matter volume) over intracranial volume (the sum of grey matter, white matter and cerebrospinal fluid) by SPM8\(^{[49]}\), to account for inter-subject variability in brain volume by (age-related) neurodegenerative processes.

\(^2\)Scans were performed on either a 3T Intera (1) or 3T Ingenia (2) system (Philips Healthcare, Best, the Netherlands), using the exact same DTI-protocol.

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