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The Duchenne brain

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Chapter 2

Reduced Cerebral Gray Matter and Altered White Matter in Boys with Duchenne Muscular Dystrophy

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2.1 Abstract

Objective

Duchenne muscular dystrophy (DMD) is characterized by progressive muscle weakness caused by DMD gene mutations leading to absence of the full-length dystrophin protein in muscle. Multiple dystrophin isoforms are expressed in brain, but little is known about their function. DMD is associated with specific learning and behavioural disabilities which are more prominent in patients with mutations in the distal part of the DMD gene, predicted to affect expression of shorter protein isoforms. We used quantitative MRI to study brain microstructure in DMD.

Methods

T1-weighted and diffusion tensor images (DTI) were obtained on a 3 Tesla MR scanner from 30 patients and 22 age-matched controls (ages 8-18 years). All subjects underwent neuropsychological examination. Group comparisons on tissue volume and DTI parameters were made between DMD and controls, and between two DMD subgroups that were classified according to predicted Dp140 isoform expression (DMD_Dp140+ and DMD_Dp140-).

Results

DMD patients had smaller total brain volume, smaller grey matter volume, lower white matter fractional anisotropy, and higher white matter mean and radial diffusivity than healthy controls. DMD patients also performed worse on neuropsychological examination. Subgroup analyses showed that DMD_Dp140- contributed most to the grey matter volume differences and performed worse on information processing.

Interpretation

Both grey and white matter is affected in boys with DMD at a whole-brain level. Differences between subgroup DMD_Dp140- and controls indicate an important role for the Dp140 dystrophin isoform in cerebral development.

2.2 Introduction

The X-linked disease Duchenne muscular dystrophy (DMD) is characterized by severe and progressive muscle weakness due to mutations in the DMD gene. Mutations cause the absence of dystrophin, a 427 kDa protein (Koenig et al. 1987). Dystrophin provides structural stability by connecting the contractile filament actin and the dystrophin-associated glycoprotein complex in the sarcolemma. In addition to muscle weakness, DMD is characterized by cognitive impairment which is thought to be non-progressive. In patients, the mean full-scale intelligence quotient (FSIQ) is approximately one standard deviation below normal (Cotton, Voudouris, and Greenwood 2005). DMD patients exhibit problems with verbal short term memory, visuospatial long term memory and verbal fluency (Cyrulnik *et al.*, 2008). In addition, a higher incidence of autism spectrum disorders, attention deficit and hyperactivity disorders (ADHD) and obsessive-compulsive disorders occurs, as well as specific learning disorders such as dyslexia have been described (Hendriksen and Vles 2008; Hendriksen and Vles 2006; Wu et al. 2005).

In the central nervous system (CNS), full length dystrophin (Dp427) is expressed mainly in cortical neurons and cerebellar Purkinje cells, but little is known about its function (Lidov et al. 1990). At least four shorter isoforms of dystrophin are also present in the CNS with molecular weights of 260, 140, 116 and 71 kDa respectively. These isoforms derive from alternative tissue specific promoter sites that use the following exons as the N-terminal domain, exon 30 for Dp260, exon 45 for Dp140, exon 56 for Dp116 and exon 63 for Dp71 (Feener, Koenig, and Kunkel 1989). Cognitive impairment is more prominent in patients with mutations in the distal part of the DMD gene (downstream of exon 44) that are associated with the loss of Dp140 expression. This suggests a genotype-phenotype correlation between mutation and brain involvement (Felisari et al. 2000).

Post-mortem studies of DMD patients have not shown consistent abnormalities (Dubowitz and Crome 1969; Jagadha and Becker 2017). However, on a micro scale, architectural alterations in the cortical-spinal system and the parvalbumin and calbindin-positive cortical circuitry have been found in a mouse model for DMD (Sbriccoli et al. 1995; Carretta et al. 2004; Carretta et al. 2003). Radiological studies in DMD patients showed glucose hypometabolism using positron emission tomography (PET) and altered metabolite concentrations with magnetic resonance spectroscopy (Lee et al. 2002; Kreis et al. 2011; Rae et al. 1998). Magnetic resonance imaging (MRI) studies in DMD are limited to case reports and one quantitative study using voxel-based morphometry analysis and resting state fMRI with a region of interest in

the motor cortex (Lv et al. 2011). However, quantitative information on in vivo whole brain morphology and microstructure in DMD is not available.

The aim of the present study was to investigate the brain in DMD using quantitative MRI to assess grey and white matter volume and microstructure. Composite scores for cognitive and behavioural functioning were analysed in relation to MRI data. Finally, MRI data and composite scores were compared between two DMD subgroups with different mutations predicted to affect the expression of the Dp140 isoform and controls.

2.3 Methods

2.3.1 Participants

DMD patients were recruited from the Dutch Dystrophinopathy Database, a nationwide patient registry. Exclusion criteria were the presence of MRI contraindications and the inability to lie supine for at least 30 minutes. DMD diagnosis had previously been confirmed using DNA extracted from whole blood taken from patients by a Gentra Puregene DNA purification kit (Gentra Systems, Minneapolis, USA), following the manufacturer's instructions. Deletion/duplication analysis of the DMD gene was performed using multiplex ligation-dependant probe amplification (MPLA kit Salsa P034/P035 MCR-Holland, Amsterdam, The Netherlands). Small mutations were identified by means of high resolution melting curve analysis (HR-MCA) and subsequent sequence analysis (Almomani et al. 2009). Within the recruited patients, two patient subgroups were distinguished (Figure 2.1). Patients carrying mutations upstream of intron 44 (transcription start site) were considered Dp140+. Patients with mutations involving exon 51 (translation start site) or the genomic region downstream of exon 51 were considered Dp140-. Deletions with 3' breakpoints in intron 44 were considered Dp140+ in case the breakpoint was upstream of the Dp140 promoter and unique first exon. A polymerase chain reaction (PCR) based approach assessed whether the Dp140 promoter and first exon were deleted using the forward primer Dp140F 5'-CTTTGTGCGGAGGCATTG-3', and the reverse primer Dp140R3 5'-CCAGCTTTTAGGCACACACA-3'. A second PCR was performed for these patients to confirm the presence of exon 51 using the forward primer hEx51F 5'-GGCTTGGACAGAACTTACCG-3' and the reverse primer h51r 5'-CTTCTGCTTGATGATCATCTC-3'. Patients carrying deletions in the region between intron 44 and exon 51 were excluded due to the unpredictable effect on Dp140 expression. All protocols were approved by the local Medical Ethical Committee. All participants or their legal representatives provided written informed consent.

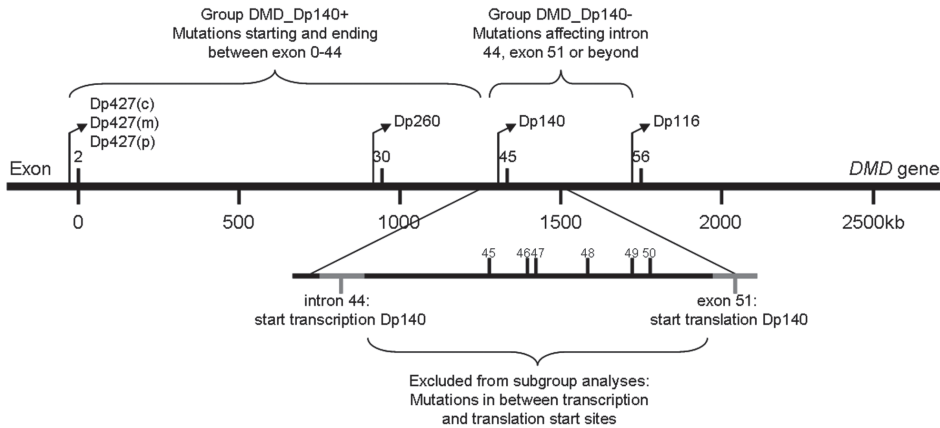


Figure 2.1 Dp140 transcription and translation sites used for subgroup classification.

The prediction of Dp140 expression is based on mutation location in the DMD gene. There is a large gap between the start of transcription and start of translation for Dp140. Patients with a mutation starting and ending exactly in between transcription and translation were excluded from the analyses due to unpredictability of the mutation effect on Dp140 expression.

2.3.2 Procedures

The protocol was designed such that all tests could be performed on the same day. The Brooke scale for upper extremity function and Vignos scale for lower extremity function were used as a measure of muscle function (Brooke et al. 1987). Participants were taken to a dummy scanner to familiarize themselves with the sounds and layout of an MRI machine. Subsequent neuropsychological evaluation (NPE) lasted a maximum of one hour. MRI scanning was then performed on a 3T scanner (Philips Achieva, Philips Healthcare, Best, The Netherlands) using an 8 channel receive-only headcoil. Participants were placed supine with legs slightly elevated for comfort. 3D T1-weighted (T1w; echo time (TE) and repetition time (TR) 4.6/9.8 ms; spatial resolution 1.17x0.92x1.2 mm; 4:55 min), T2-weighted (T2w; TE/TR 80/4000 ms; spatial resolution 0.5x0.46x3.6 mm; 2:46 min), fluid attenuated inversion recovery (FLAIR; TE/TR 120/10000 ms; spatial resolution 1x0.8x3.6 mm; 4:00 min), and diffusion tensor (DTI; TE/TR 56/9440 ms; spatial resolution 1.96x2x2 mm; 32 directions, b=0 and 1000 s/mm²; 6:40 min) images were obtained parallel to the corpus callosum. An independent neuroradiologist assessed the scans for gross structural abnormalities and incidental findings.

2.3.2 Neuropsychology

Three composite scores were constructed using predefined parts of standardized and validated instruments reflecting the most important aspects of neuropsychological and behavioural/emotional functioning in DMD (Snow, Anderson, and Jakobson 2013). The first composite score represented reading (scores standardized for age with a range of 1-19, mean 10 and standard deviation 3 in healthy controls) and was based on the mono-syllabic word reading test and the one minute reading test derived from CB&WL: “continu benoemen en woorden lezen” (Bos & Lutje Spelberg, Boom test uitgevers, Amsterdam, The Netherlands). The second composite score represented information processing (scores standardized for age with a range of 1-19, mean 10 and standard deviation 3 in healthy controls), and used two subtests from the Kaufman Assessment battery (number recall for auditory working memory and block counting for conceptual thinking) and one subtest from the Wechsler Intelligence Scale for Children (symbol search). The third composite score for emotional and behavioural problems (range 0-40) was constructed on the basis of the four problem based subscales from the Dutch version of the Strengths and Difficulties Questionnaire for parents (Goodman and Goodman 2012). The Personal Adjustment and Role Skills scale (PARS) –III, a parent report measure studied in 287 DMD patients, was used to assess psychosocial adjustment (J. G. M. Hendriksen et al. 2007). General intellectual level was assessed by the Peabody Picture Vocabulary test (PPVT-III-NL). This test measures receptive vocabulary, is normalized for age, and requires no motor response. It was previously used in 130 Duchenne boys (Cyrulnik *et al.*, 2008).

2.3.3 MRI

T1w and DTI scans were analyzed with FSL software v5.0 (Smith et al. 2004). Intracranial volumes were obtained using the Brain Extraction Tool with BET2 + betsurf (BET v2.1) (Smith 2002) with a fractional intensity threshold (f) of 0.35. Grey matter, white matter and cerebrospinal fluid (CSF) were segmented using the automated segmentation tool FAST (Zhang, Brady, and Smith 2001). Quantification of the volumes was performed with `fsstats -V` using the partial volume corrected output from FAST. Diffusion tensor imaging scans were corrected for motion and distortion using ExploreDTI (Leemans, Jeurissen, and J 2009). ExploreDTI descriptive statistics was used to calculate mean whole brain FA and MD. Maps of axial diffusivity (AD), radial diffusivity (RD), fractional anisotropy (FA) and mean diffusivity (MD) were also created. These maps were then exported for tract-based spatial statistics (TBSS) in FSL (Smith et al. 2006).

2.3.4 Statistical analysis

PARS and PPVT-III-NL scores of the DMD group were compared to known DMD populations using one sample t-tests. One sample t-tests were also used to test volumetric differences in whole brain intracranial, white matter, grey matter, and CSF volumes, as well as NPE scores between patients and controls. To correct for multiple comparisons, we used the method of Benjamini and Hochberg which limits the false discovery rate (FDR) to 5% (Benjamini and Y 1995). One-way ANOVA was used to assess differences in these same parameters between the subgroups DMD_Dp140+, DMD_Dp140- and controls.

A univariate general linear model with Duchenne status yes/no was used to assess the relation between three whole brain MR measures decided upon before the analysis to reduce multiple comparisons (grey matter volume, mean FA and mean MD) and the neuropsychological composite scores for each group, and to assess the relation between the whole brain MR measures and age. All analyses were performed using SPSS v20.0 (IBM Corp., NY, USA) and considered significant at $p < 0.05$.

To localize MRI findings in specific areas of the brain, voxelwise statistical analyses were performed. For grey matter, voxel-based morphometry analysis (VBM) was performed with FSL-VBM (Good et al. 2001). For white matter, FA, MD, RD and AD, data were analyzed with age as covariate using Tract-Based Spatial Statistics (TBSS) (Smith et al. 2006). An additional TBSS analysis was performed with ICV and TBV as covariates to assess the relationship between volume and white matter microstructure. The aligned maps from each subject were projected onto the FA skeleton, and the resulting data used for voxel-wise cross-subject statistics using RANDOMISE ttest_2 with multiple comparison correction tcf_corr (Bullmore et al. 1999).

2.4 Results

2.4.1 Participants

Thirty-three DMD boys (ages 8-18 years) and twenty-two healthy age-matched control boys (ages 8-16 years) participated in the study. Three DMD boys were excluded because they withdrew informed consent prior to the MRI scan ($n=2$), or the scans could not be made due to technical problems ($n=1$). In five DMD boys, one or more of the MRI scans could not be evaluated due to motion artifacts (one T1w and five DTI), leaving 29 DMD boys for T1w analysis and 25 for DTI analysis. In the controls, one DTI scan was also excluded due to motion artifacts. Some NPE tests could not be completed within one hour prior to the MRI, resulting in the missing

Table 2.1 Patient characteristics and available assessments

	Control	DMD	DMD_Dp140+	DMD_Dp140-
Participants (n)	22	29	12	12
Age (years)*	13.1 (2.0)	12.3 (2.8)	13.1 (3.3)	12.0 (2.7)
Range (years)	8-16	8-18	9-18	8-16
Steroid treatment (n)	-	24	10	10
On/off 10 cycle (n)	-	22	10	9
Brooke scale*	1	2.1 (1.6)	2.0 (1.6)	2.5 (1.7)
Vignos scale*	1	5.9 (3.1)	6.2 (3.1)	6.1 (3.2)
Wheelchair bound (n)	-	15	7	6
Age of loss of ambulation (years)*	-	10.2 (2.1)	11.0 (2.6)	9.9 (1.3)
Regular education (n)	22	17	8	6
Reading composite score (n)	21	25	11	12
Information composite score (n)	21	27	11	12
SDQ composite score (n)	22	28	11	12
3D T1 scans (n)	22	29	12	12
DTI scans (n)	21	25	10	10

* Data are presented as mean with SD.

composite scores. Clinical characteristics and an overview of the available MRI and NPE assessments are summarized (Table 2.1). Representative T1w images are shown (Figure 2.2). DMD general intellectual level was not different from data reported in the literature using the PPVT-III (97.56 vs 98.15 respectively) (Cyrulnik *et al.*, 2008). Psychosocial adjustment, reflected by the PARS-III score, was significantly better than in historical controls (91.62 vs 84.43, $p < 0.001$) (J. G. M. Hendriksen *et al.* 2007). From the 29 DMD patients, 12 were classified as DMD_Dp140+ and 12 as DMD_Dp140-. Five DMD patients were excluded from the subgroup analysis because of the unpredictability of the mutation effect on Dp140 expression. No mutations downstream of exon 56 containing promoter sites of Dp116 and Dp71 were found.

2.4.2 MRI

Visual assessment of the T1w, T2w and FLAIR images did not reveal any gross structural brain abnormalities. Quantitative analysis of the T1w images showed a significantly smaller total brain and grey matter volume in DMD patients compared to controls (Table 2.2). Intracranial volume was also smaller in DMD, although this difference failed to reach statistical significance ($p = 0.056$). Adding intracranial

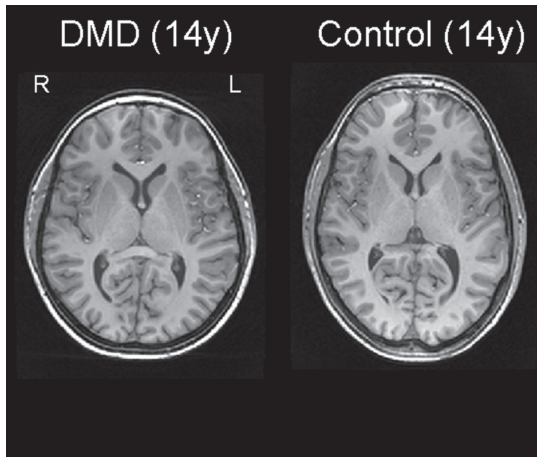


Figure 2.2 Representative T1w-images
A 14 year old boy with DMD is depicted on the left and an age-matched healthy control on the right. Images are displayed in the radiological convention with the left hemisphere on the right of the image. Note the absence of gross structural differences between the patient and the age-matched healthy control, which explains why routine visual assessment of the MR images did not show abnormalities.

Table 2.2 Brain structure volumes in DMD patients and age-matched controls

Volumes	Control	DMD	DMD_Dp140+	DMD_Dp140-
Intracranial (cc)	1651 ± 95	1584 ± 137	1633 ± 149	1550 ± 123
Total brain (cc)	1430 ± 78	1355 ± 118 *	1401 ± 121	1324 ± 114 *
Grey matter (cc)	811 ± 42	757 ± 56 ***	770 ± 53	749 ± 63 ***
White matter (cc)	619 ± 45	598 ± 70	631 ± 75	576 ± 61
CSF (cc)	222 ± 30	229 ± 32	232 ± 39	226 ± 29

Significant differences between patient groups and controls are indicated by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$ after multiple comparison correction.

volume as a covariate in the analysis of grey matter and total brain volume showed that these structures were not smaller compared to total intracranial volume. VBM analysis showed small regions within the occipital cortex and the left insula that were significantly smaller in patients compared to controls (Figure 2.3a).

White matter FA was significantly lower in DMD in the occipital lobe (Figure 2.4). Significantly higher MD in DMD was found mainly in the corpus callosum. RD was significantly higher in DMD throughout the white matter. Taking ICV and TBV as covariates in the TBSS analysis revealed a relationship between these parameters and all three DTI measures, while part of the occipital lobe showed changes in FA and RD independent of volumetric parameters (data not shown).

DMD_Dp140- patients had the smallest grey matter, total brain and intracranial volumes (Figure 2.5a-c). VBM analysis showed differences between DMD_Dp140- and controls in a different but similarly small area as found in the whole DMD group versus control analysis (Figure 2.3b). TBSS showed similar results for DMD_Dp140+ and DMD_Dp140- versus control with significantly lower FA and higher RD, but no differences in MD. There was no effect of age on grey matter, white matter or total brain volumes. However, age did correlate with white matter microstructure, with higher FA and lower MD and RD with increasing age.

2.4.3 Neuropsychological parameters

All three NPE composite scores were significantly different between DMD patients and controls, with DMD patients performing worse. Subgroup analysis showed that DMD_Dp140- patients performed worst on all composite scores, although this difference was statistically significant only for information processing. Scores of DMD_Dp140+ patients were between DMD_Dp140- and controls (Figure 2.5d-f). Within each subgroup, there were no significant correlations between the three composite scores and whole brain grey matter volume, whole brain FA or whole brain MD using the univariate general linear model.

2.5 Discussion

In DMD patients, grey matter and total brain volume are smaller, and white matter microstructural integrity is altered compared to healthy age-matched controls. Strikingly, comparison of DMD patients with and without Dp140 expression with controls shows that DMD_Dp140- patients, in whom the expression of the Dp140 isoform is predicted to be impaired, were more severely affected in terms of total brain and grey matter volumes as well as neuropsychological scores.

The smaller grey matter volume in DMD is global, as the regions in the occipital lobe and left insula identified by VBM are too small to account for the difference. This is in contrast to a previous quantitative MRI study in DMD brain, in which VBM identified a smaller volume in the left primary sensorimotor cortex, although no analysis of absolute grey matter volumes was performed (Lv et al. 2011). One CT study reported smaller grey matter volume in 20 out of 30 DMD patients and contributed this difference to cortical atrophy. In the present study, skull size was proportional to total brain volume. There is a dynamic interaction between the developing brain and skull growth (Courchesne et al. 2000; Reiss et al. 1996), with 25-27% increase in whole brain and intracranial volume between early childhood

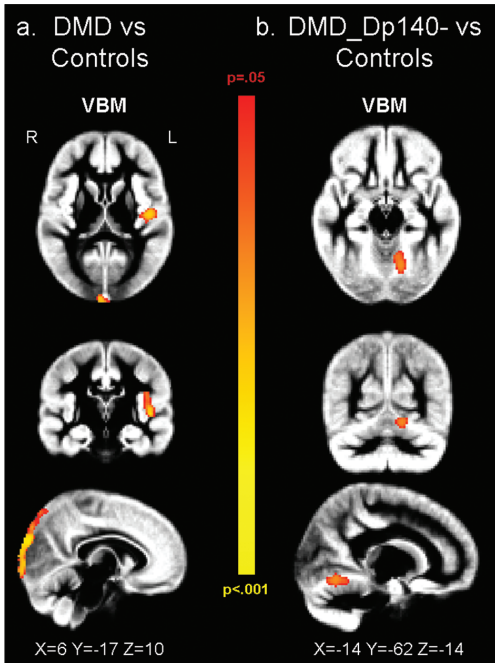


Figure 2.3 Grey matter voxel-based morphometry results

Local grey matter reductions in part of the left insula and occipital lobe are demonstrated by VBM when comparing the whole patient group to controls (a). Local grey matter reductions in part of the lingual gyrus and left cerebellar cortex are demonstrated by VBM when comparing the DMD_Dp140- subgroup to controls (b). No differences were found between DMD_Dp140+ and controls (*data not shown*). Images are displayed in the radiological convention with the left hemisphere on the right side.

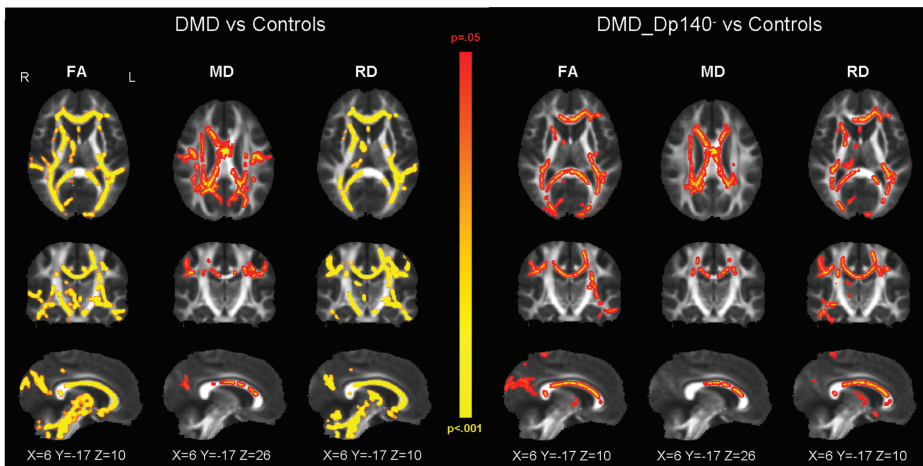


Figure 2.4 DTI analyses of the white matter

Widespread alterations in white matter with reduced fractional anisotropy (FA) in the occipital lobe, and increased mean diffusivity (MD) and radial diffusivity (RD) throughout as demonstrated by tract-based spatial statistics (TBSS).

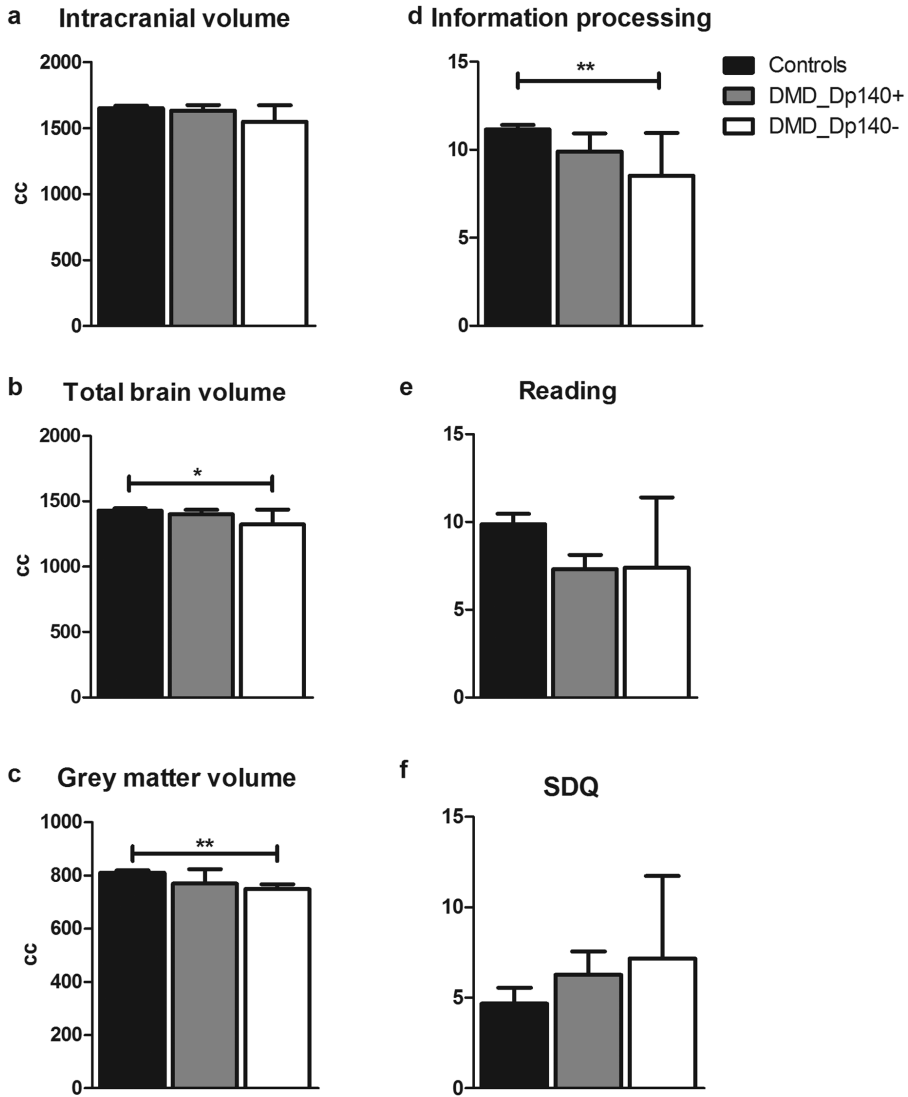


Figure 2.5 T1w volumes and composite scores

Subgroup analyses for controls (black) DMD_Dp140+ (grey) and DMD_Dp140- (white). Volumes (cc) of the intracranial (a), total brain (b) and grey matter (c) are depicted on the left. Composite scores for information processing (d), reading (e) and emotional/behavioral problems (SDQ) (f) are shown on the right. Significant differences between groups are indicated by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$ after multiple comparison correction.

and adolescence. Therefore, correcting total brain volume with respect to intracranial volume enables the detection of atrophy. This implies that, smaller brains in DMD are due to differences in maturation rather than atrophy.

In contrast to grey matter, white matter showed no difference in volume, but was significantly affected on a microstructural level as shown by DTI. The smaller FA and higher MD found in DMD indicate compromised structural complexity, and suggest reduced fiber density, increased membrane permeability, and/or decreased structural organization (Assaf and Pasternak 2008). The higher RD in DMD indicates more fiber branching, decreased structural organization, or demyelination which can lead to increased membrane permeability (Vandermosten et al. 2012; Song et al. 2005). The white matter microstructural differences between DMD and controls were associated with intracranial and total brain volume, although these cross-sectional data do not allow any conclusions regarding a causal relationship between the two. The global nature of the reduction in grey matter volume and microscopic nature of the white matter alterations could explain why these changes have not been described in previous post mortem studies and conventional MRI studies, as they can only be observed with whole brain quantitative MRI.

The global reduction in grey matter volume of patients could be related to the fact that full length Dp427, affected in all patients, is normally located in cortical and hippocampal pyramidal cells and the fetal cerebral cortex and cerebellar purkinje cells (Lidov et al. 1990). In our DMD_Dp140- subgroup, no mutations downstream of exon 56 were found. This implies that differences in MRI and NPE assessments in this study are mainly due to a lack of expression of the Dp140 isoform and do not include Dp116 and Dp71. The involvement of the Dp140 isoform in cognitive impairment was previously identified (Felisari et al. 2000). Dp140 is predominantly active during fetal development in the astroglial processes (Lidov et al. 1990). DMD patients with mutations disturbing Dp71 expression were even more severely affected mentally (Moizard et al. 2000). Therefore, the loss of more dystrophin isoforms may well have a cumulative effect on altered brain maturation.

The correlation analyses did not reveal a significant association between mean whole brain grey matter volume, FA or MD and the neuropsychological composite scores within the groups. A modest, yet significant association between grey matter volume and total IQ was previously reported by Reiss et al (Reiss et al. 1996) in 69 healthy children. Our study was not powered to find such function to morphology associations within the group of DMD patients or controls, but aimed at finding differences between these groups.

There are two potentially confounding issues in this study, which need to be specifically addressed. First, the brain still undergoes crucial developments in the age range of our subjects. Maximum global grey matter volume is reached between 6-9 years of age, after which it linearly declines approximately 5% per decade (Courchesne et al. 2000). White matter volume increases substantially from infancy through early adulthood with changes in myelination and axonal packing (Lebel et al. 2008). Age had a significant effect on FA, MD and RD which is in line with recent results (Lebel et al. 2008). This made a correction for age essential in the TBSS analysis despite matching for this parameter upon recruitment.

Secondly, the steroid treatment could have affected brain morphology. Volume reductions of hippocampus and amygdala have been observed in patients with asthma or rheumatic diseases receiving steroid treatment (Brown, Woolston, and Frol 2008). Children with excess endogenous glucocorticoids due to Cushing syndrome showed a significantly smaller total brain volume, larger ventricles, and smaller amygdala volumes than controls (Coburn-Litvak et al. 2004). Five DMD patients in our study were steroid naïve or had only been on steroid treatment for a short period of time at least ten years before participating. Two of these patients were in DMD_Dp140+ and two in DMD_Dp140-. Given that DMD_Dp140- showed the most extensive differences with controls in all tests, this strongly suggests that steroid use cannot be the main contributor to the morphological findings. However, a secondary contributing effect of the steroids on the morphological MRI findings cannot be excluded.

In conclusion, we observed significant global morphological and microstructural differences in the brain between DMD boys and controls. Results suggest that these differences arise from altered brain maturation rather than atrophy. Differences between DMD_Dp140+ and DMD_Dp140- patients indicate an important role for the Dp140 dystrophin isoform in cerebral development. Future studies could include longitudinal data to confirm the lack of atrophy or focus on assessment of the structural and functional connectivity between different brain networks (the connectome) and hopefully further dissect the role of each of the dystrophin brain isoforms.

