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Correspondence:
Anne Skakkebæk, Department of Endocrinology and Internal Medicine (MEA), Aarhus University Hospital, 8000 Aarhus C, Denmark.
E-mail: asj@ki.au.dk

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Neuropsychology and brain morphology in Klinefelter syndrome – the impact of genetics

[1]A. Skakkebæk, [2]A. Bojesen, [3]M. K. Kristensen, [4]A. Cohen, [4]D. M. Hougaard, [5]J. M. Hertz, [6]J. Fedder, [7]P. Laurberg, [8,9]M. Wallentin, [10]J. R. Østergaard, [11,12]A. D. Pedersen and [1,13]C. H. Gravholt

[1]Department of Endocrinology and Internal Medicine (MEA), Aarhus University Hospital, Aarhus, , [2]Department of Clinical Genetics, Vejle Hospital, Sygehus Lillebaelt, Vejle, [3]Department of Mental Health, Odense University Clinic, Odense, [4]Section of Neonatal Screening and Hormones, Department of Clinical Biochemistry, Immunology and Genetics, Statens Serum Institute, Copenhagen, [5]Department of Clinical Genetics, Odense University Hospital, [6]Fertility Clinic, Department of Gynecology and Obstetrics, Odense University Hospital, Odense, , [7]Department of Endocrinology, Aalborg University Hospital, Aalborg, [8]Center of Functionally Integrative Neuroscience, Aarhus University Hospital, [9]Center for Semiotics, Aarhus University, [10]Centre for Rare Diseases, Department of Pediatrics, Aarhus University Hospital, Aarhus, [11]Vejleford Rehabilitation Center, Stouby, [12]Department of Psychology and Behavioral Sciences, Aarhus University, and [13]Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark

SUMMARY

Klinefelter syndrome (KS, 47,XXY) is associated with increased psychiatric morbidity and cognitive disabilities, although the neuropsychological phenotype shows great variability. Androgen receptor polymorphism (CAG repeat length), skewed X-chromosome inactivation and parent-of-origin of the extra X-chromosome have been suggested to influence cognitive function and psychological traits. These issues have not been clarified for KS patients. We studied X-chromosome inactivation pattern, CAG repeat length and parent-of-origin in relation to educational and cohabitation status, personality and autism traits, psychological distress, cognitive function and brain volumes in 73 KS patients and 73 controls. Grey matter (GM) volume of left insula was significantly decreased in KS patients with skewed X-inactivation ($z = 5.78$) and we observed a borderline significant difference in global brain matter volume where KS patients with skewed X-chromosome inactivation tended to have smaller brains. Skewed X-inactivation, CAG repeat length and parent-of-origin were not correlated with educational and marital status, personality traits, autism traits, and psychological distress, prevalence of depression and anxiety or cognitive function. Interestingly our results regarding brain volumes indicate that X-inactivation has an influence on GM volume in left insula and might also be related to global GM volume, indicating a possible effect of X-linked genes on the development of GM volume in KS patient. Skewed X-inactivation, CAG repeat length and parent-of-origin have no impact on the neuropsychological phenotype in KS (http://www. clinicaltrials.gov (Clinical trial NCT00999310)).

INTRODUCTION

Klinefelter syndrom (KS, 47,XXY) is the most common sex chromosome aneupleoidy in men, occurring in 1 : 660 males (Nielsen & Wohlert, 1990; Bojesen *et al.*, 2003). Characteristically, patients with KS have hypergonadotropic hypogonadism (Klinefelter *et al.*, 1942; Lanfranco *et al.*, 2004). KS is also associated with an increased prevalence of psychiatric disorders such as depression, anxiety, autism, schizophrenia and attention deficit/hyperactivity disorders (Boks *et al.*, 2007; van Rijn *et al.*, 2008; Bruining *et al.*, 2009; Tartaglia *et al.*, 2012). Furthermore, the majority of boys and men with KS display some degree of cognitive disabilities (Geschwind *et al.*, 2000; Bender *et al.*, 2001; Boone *et al.*, 2001; Ross *et al.*, 2008). The cognitive and psychological phenotype seen in patients with KS is highly variable and genetic mechanisms have been suggested to be involved.

Testosterone is known to influence brain structure and function (Rubinow & Schmidt, 1996). The biological effect of testosterone is mediated by its binding to the androgen receptor (AR) (Kovacs *et al.*, 2009). The *AR* gene contains a highly polymorphic CAG repeat in exon 1. The length of the CAG repeat is correlated negatively to the function of the AR. CAG repeat length has been associated with cognition (Yaffe *et al.*, 2003), personality (Comings *et al.*, 2000; Jonsson *et al.*, 2001; Westberg *et al.*, 2009; Aluja *et al.*, 2011)and psychological disorders (Comings *et al.*, 1999; Seidman *et al.*, 2001; Vermeersch *et al.*, 2010) and have been suggested to explain some of the phenotypic variation seen in KS patients, however, the existing evidence is sparse. The CAG

repeat length has been associated with the chance of entering partnership and with the reached educational level in one study (Zitzmann *et al.*, 2004), however, no association have been found regarding cognition (Ross *et al.*, 2008).

Imprinted genes on the X-chromosome have also been hypothesized to be involved in the phenotypic variability seen in KS (Jacobs *et al.*, 1989). Imprinted genes are predominantly expressed in brain tissue (Wilkinson *et al.*, 2007). Some evidence for an effect of X-linked imprinted genes on behaviour, cognition and brain morphology have been reported in Turner syndrome (45,X) (Skuse *et al.*, 1997; Lepage *et al.*, 2013). Imprinted genes on autosomal chromosomes have also been found to be involved in Prader–Willi and Angelman syndrome (Nicholls *et al.*, 1998), disorders with psychological and cognitive manifestations. In KS, paternal origin of the supernumerary X-chromosome has been associated with increased prevalence of motor impairment and speech/language problems (Stemkens *et al.*, 2006). Recently, a parent-of-origin effect on autistic and schizotypal traits in KS has been found, indicating that imprinted genes on the X-chromosome could play a role in the neuropsychological phenotype of KS (Bruining *et al.*, 2010).

Skewed X-chromosome inactivation defined as ≥80% inactivation of one allele has been found to have a 9–43% prevalence in KS patients (Tuttelmann & Gromoll, 2010). This epigenetic mechanism has also been proposed to account for some of the phenotypic variation in KS. Until now, skewed X-chromosome inactivation has not been found to be associated with cognitive functioning (Ross *et al.*, 2008), educational status or the chance of entering partnership (Zitzmann *et al.*, 2004) in KS. Interestingly, significantly increased prevalence of skewed X-chromosome inactivation has been reported in females with autism (Talebizadeh *et al.*, 2005) and skewed X-chromosome inactivation has also been associated with X-linked mental retardation (Plenge *et al.*, 2002), suggesting a role for skewed X-chromosome inactivation in cognitive and psychiatric disorders.

The aim of this study was to access the impact of CAG repeat length in the *AR* gene, parental origin of the supernumerary X-chromosome and the presence of skewed X-chromosome inactivation on the neuropsychological phenotype seen in KS patients.

MATERIALS AND METHODS

Materials

We studied 73 patients with KS recruited from endocrinology (n = 51), genetics (n = 20) and fertility (n = 2) clinics in Denmark and 73 age- and educational-matched control men in a prospective manner for this cross-sectional study. The participants were recruited in the period from 2009 to 2012. Inclusion criteria were (i) age between 18 and 60 years. Exclusion criteria were (i) neurological disease or head injury more severe than simple concussion, (ii) current substance abuse or (iii) colour blindness. Sixty-nine KS patients had 47,XXY karyotype and four had mosaicism. Of the 73 KS patients studied 50 (68%) received testosterone treatment [intramuscular injections (n = 43), oral (n = 2), transdermal gel (n = 5)] at the time of participation. Fifteen of the 23 KS patients (65%) who did not receive testosterone treatment had never been treated with testosterone, while seven had received testosterone therapy in the past for a period of, on average 31.5 months (range: 6 months–7.3 years) and one KS patient had received testosterone for an unspecified period when he was younger. Prior to given written consent, all participants received oral and written information. The study was approved by The Danish Data Protection Agency and local ethics committee. We have previously presented data concerning the neuroanatomy and neuropsychology from this study (Skakkebaek *et al.* 2014).

Genetic analysis

Karyotype

Of the 73 KS patients studied, 71 were registered by the Danish Cytogenetic Central Register. Two of the KS patients were not registered and were karyotyped using standard techniques. None of the 73 controls showed evidence of more than one X-chromosome in the CAG repeat or X-chromosome microsatellite analysis.

DNA extraction and purification

Genomic DNA from KS patients and controls was extracted from peripheral blood samples using QIAamp Mini Kit (Qiagen, Hilden, Germany). Saliva samples from parents of KS patients were collected with Oragene DNA Self-Collection Kit OG-250 and the DNA purification and extraction were performed using Oragene Purifier (OG-l2P) (DNA Genotek Inc., Kanata, Ontario, Canada).

Parental origin of the supernumerary X-chromosome

Saliva samples from parents were available for 41 KS patients. In 31 KS patients saliva samples from both parents were available. In 9 KS patients, only saliva sample from the mother was available and the parent-of-origin was decided to be paternal if any of the KS marker alleles were not maternal. In one KS patient, only saliva sample from the father was available, the parent-of-origin was assigned to the father as microsatellite markers from the father matched. DNA from KS patients and parents were genotyped by a panel of four highly polymorphic microsatellite markers dispersed along the length of the X-chromosome [DXS6854, HPRT, DXS8054, DXS8377 (TAG Copenhagen, Denmark)]. For six cases, additional three polymorphic microsatellite markers were analysed to allow identification of the parent-of-origin (DXS1039, DXS7132, DXS981, TAG Copenhagen, Denmark). About 3 μL DNA was added to a solution of Multiplex PCR Master Mix (Qiagen) and primers for PCR amplification which was performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA), followed by capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems). Data were analysed on Gene Mapper 3.5 software (Applied Biosystems).

X-inactivation

Analyses of methylation at the *AR* and *FMR1* genes were used to examine X-inactivation patterns. X-inactivation analysis was performed according to the principle described by Bojesen *et al.* (2011). Fifty ng DNA was digested with the methylation-sensitive restriction enzyme *Hpa*II (Thermo Scientific, Waltham, MA, USA) or mock digested. *Hpa*II cleaves only un-methylated DNA, thus only the active X-chromosome is digested. *Hpa*II cleaves two restriction sites near the CAG repeat of the *AR* gene and two restriction sites near CGG repeat of the *FMR1* gene.

Subsequently, samples were followed by PCR amplification on a 96-Well Thermal Cycler (Applied Biosystems) and capillary electrophoresis performed on a 3130 Genetic Analyzer (Applied Biosystems). GeneMapper 3.5 Software (Applied Biosystems) was used to calculate the fluorescent peak areas for alleles in digested and undigested samples. X-chromosome inactivation pattern was then calculated as described by Thouin *et al.* (2003). Skewed X-chromosome inactivation was defined as X-inactivation patterns of 80 : 20 or more.

AR CAG repeat length

Fifty ng of DNA was added to primer mix containing AR, forward and AR, reverse primers, followed by PCR amplification on an 96-Well Thermal Cycler (Applied Biosystems) and capillary electrophoresis on an 3130 Genetic analyzer (Applied Biosystems). The CAG repeat length was determined by comparing the mobility of PCR products on electrophoresis to reference sample from individuals with known CAG repeat lengths (13, 22, 23, 30). Mean CAG repeat length was calculated as the sum of allele 1 and allele 2 divided by 2. Physiological CAG repeat length was calculated by the following formula described in Hickey *et al.* (2002): Physiological CAG repeat length = (activity of allele 1 × CAG repeat length allele 1) × (activity of allele 2 × CAG repeat length allele 2).

Hormone analysis

Testosterone was measured by liquid chromatography tandem mass spectrometry using Perkin Elmer's CHS Steroid MS kit. Oestradiol was measured using in house liquid chromatography tandem mass spectrometry method. Free testosterone was calculated based on testosterone and SHBG values (Bartsch, 1980). SHBG were analysed on the Architect i200 platform (Abbott, North Chicago, IL, USA) by chemiluminescence micro particle immunoassay method. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were analyed on a Cobas e601 platform (Roche, Basel, Switzerland) by electrochemiluminescence immunoassay method.

Cognitive and psychological assessment

Participants were administered a 3-h battery of standardized cognitive tests. The tests were administered by trained research assistants under supervision of a psychologist and a specialist in clinical neuropsychology. The test battery assessed the following cognitive abilities: Processing speed, working memory, visual/spatial construction and performance, Visual memory and learning, verbal memory and learning, verbal fluency, response inhibition, executive function, dyslexia and reading speed. The tests administered were described in detail previously (Skakkebaek *et al.*, 2014).

Psychological questionnaires

A week before attending the trial, participants received self-administered psychological questionnaires used to assess psychological distress, autistic traits and personality traits. The psychological questionnaires used were described in detail previously (Skakkebaek *et al.*, 2014).

General questionnaires

Participants were also asked to complete a general questionnaire to assess specific characteristics such as education, employment status, problems with writing, reading and math in elementary school, need for special help in math and Danish in elementary school, cohabitation status, psychological diagnosis and number of friends.

Magnetic resonance image (MRI) scanning

MR scans were available for 65 of the 73 KS patients. A 3-T General Electric Medical systems (Milwaukee, WI, USA) MR system with a standard head coil was used to acquire high-resolution 3D GR contiguous T1-weighted anatomical scans, consisting of 256 × 256 × 134 voxels with a 0.94 × 0.94 × 1.2 mm^3 voxel size, obtained with a TR of 6.552 ms, a 2.824 ms TE and a 14° flip angle.

MRI data analysis

Voxel-based morphometry analysis was performed using Statistical Parametric Mapping (SPM8). T1-weighted images were converted from DICOM to NIfTI format. Images were inspected to rule out abnormalities and acquisition artefacts and co-registered to the anterior commissure – posterior commissure axis. Images were classified into grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) using the unified segmentation tool in SPM8 (Ashburner & Friston, 2005) with an affine transformation to the ICBM space template (European brains). We considered the pre-processed tissue classification probability in each voxel to be an estimate of regional GM/WM/CSF volume. Global differences in GM, WM, CFS and total brain volumes between groups were analysed using two-sample *t*-tests on the sum of tissue classification probability across all voxels for a given structure type. Spearman's rank correlation coefficient was used to test the relationship between global brain volumes and the CAG repeat length. Age effect on global brain volumes were analysed by Spearman correlation within and between the KS and control groups. We did not find any significant effect of age on global brain volumes (all *p*-values >0.05). To enable voxel-wise comparisons, GM images were further normalized to the templates generated from Diffeomorphic Anatomical Registration using Exponentiated Lie algebra (DARTEL) (Ashburner, 2007). A study-specific template was generated using DARTEL to minimize between scan variations (Klein *et al.*, 2009). The template was made using the complete set (n = 130) of segmented GM and WM scans. Finally, the segmented and normalized images were smoothed with a default 8 mm FWHM Gaussian filter. Voxel-based statistical analyses were conducted using a general linear model approach. Correction for global brain volume (GM + WM) was carried out using proportional scaling. Initial analyses included age as a covariate, however, no significant voxel-wise effect was seen (all *p*-values > 0.7 FWE corrected) and the age covariate was removed from the final analysis. Analysis of GM was performed using explicit mask, including all voxels with a probability above 5% for the given tissue class. Four between-group contrasts were defined for GM: Skew > no skew, no skew > skew, paternal > maternal and maternal > paternal and tested with two-sample *t*-test. Anatomical regions were located using WFU (Wake Forest University School of Medicine) Pickatlas referencing the aal atlas (Tzourio-Mazoyer *et al.*, 2002). To test the relationship between regional brain volumes and CAG repeat length, CAG repeat length was inserted as a covariate in the general linear model in SPM8. $p < 0.05$ was used as significance

threshold, whole brain corrected for family-wise error and cluster size >20 voxels.

Statistical analysis

SPSS version 19.0 (SPSS Inc., Chicago, Ill., USA) was used for statistical data analysis. Differences between groups were analysed with Student's *t*-test and Mann–Whitney test. χ^2 test was used for nominal variables. Wilcoxon signed rank test was used for paired test. Spearman correlation was used to investigate correlations between CAG repeat length and measured parameters. Bonferroni–Holm correction for multiple comparisons was performed within cognitive variables (30 variables), psychological variables (14 or 16 variables) and global brain morphometry (3 variables). $p < 0.05$ was considered statistically significant.

RESULTS

Parent-of-origin

The supernumerary X-chromosome was maternal in 21 KS patients and paternal in 20 KS patients. Mean age was lower in KS patients with a maternal supernumerary X-chromosome (Table 1). No difference was seen regarding education, testosterone treatment, sex hormones, learning disabilities, employment and cohabit status, number of friends, personality and autism traits, psychological distress, cognitive performance, and brain volume between KS patients with maternal vs. paternal origin of the supernumerary X-chromosome (Tables 1 and 2, Table S1, Fig. 1).

X-chromosome inactivation

Of the 73 KS patients, 42 were heterozygous for the *AR* polymorphism and 31 were homozygous. Of those 31 homozygous patients, 22 were also homozygous for the *FMR1*, whereas nine of the 31 KS patients homozygous for the *AR* polymorphism were heterozygous for the *FMR1* polymorphism. Of the 51 KS patients heterozygous for the *AR* and/or *FMR1*, 11 had skewed X-chromosome inactivation. No difference was seen regarding education, testosterone treatment, sex hormones, learning disabilities, employment and cohabitation status, number of friends, personality and autism traits, psychological distress and cognitive performance between KS patients with skewed X-chromosome inactivation and KS patients with random X-chromosome inactivation (Table 1 and Table S1, Fig. 2). We observed a borderline significant difference in global brain matter volume (Table 2) where KS patients with skewed X-chromosome inactivation tended to have smaller brains ($p < 0.07$). Furthermore, KS patients with skewed X-chromosome inactivation had significantly smaller left insula (Montreal Neurological Institute peak coordinates (X,Y,Z) −39,18,−5; *Z*-value (peak voxel) 5.78; Cluster size = 28) than KS patients with random X-inactivation (Fig. 3).

AR CAG repeat length

In the KS patients heterozygous for the *AR* polymorphism, the median CAG repeat length of allele 1 were 20 (18–24) and 24 (19–31) for allele 2. The median CAG repeat length was 21 (17–26) in patients homozygous for the *AR* polymorphism. The mean activity for allele 1 was 0.49 ± 0.24 and for allele 2 it was 0.51 ± 0.24. In the controls, the median CAG repeat length was 22 (14–30). No significant differences were found between mean CAG repeat length [22 (18.5–27.5)] and the physiological CAG repeat length [22.5 (18.1–29.4)] from the same KS patient ($p = 0.13$), or between physiological CAG repeat length and CAG repeat length of the controls ($p = 0.76$) or KS patients homozygous for the *AR* polymorphism ($p = 0.23$). No correlations were seen between CAG repeat length and educational length, employment and cohabitation status, personality traits autism traits, psychological distress, cognitive performance and brain volumes were seen in the KS patients or in the controls after correction for multiple testing (Table S2). Neither, did we find any correlation between CAG repeat length and sex hormones (Table 3).

DISCUSSION

This study is the first to access the influence of CAG length, X-chromosome inactivation pattern and parental origin of the supernumerary X-chromosome on psychological traits, personality traits and regional and global brain volumes in KS patients. Interestingly our results regarding brain volumes indicate that X-inactivation has an influence on GM volume in left insula and might also be related to global GM volume. We did not find evidence that skewed X-chromosome inactivation, CAG repeat length nor parent-of-origin have any impact on the cognitive and psychological phenotypic variation seen in KS patients.

There was no parent-of-origin effect or an effect of CAG repeat length on brain volume; however we did find that KS patients with skewed X-chromosome inactivation had smaller GM volume in the left insula and we observed a borderline significant difference in global brain matter volume where KS patients with skewed X-chromosome inactivation tended to have smaller brains. Our previous analyses (Skakkebaek *et al.*, 2014) have demonstrated that KS patients as a group have smaller global GM volume compared to controls and decreased GM density in a number of brain regions, including insula. The KS patients with skewed X-chromosome inactivation display an extreme version of this pattern, pointing towards a potential link between KS and skewed X-chromosome inactivation in development of macro-scale neuro-architecture. Skewed X-chromosome inactivation has been shown to be associated with different diseases in women, although there have not been any conclusions about the association between clinical severity and the X-inactivation pattern (Orstavik, 2006). However, it is plausible that skewed X-chromosome inactivation could be related to brain volumes in KS also, as evidence for genes involved in brain size is evolving, not just for autosomal genes such as *ASPM, STIL, MCPH1, CENPJ, CDK5RAP2* which have been associated with autosomal recessive primary microcephaly (Thornton & Woods, 2009), but also for X-linked gene such as *ATRX* which have been associated with the size of the forebrain in mice (Berube *et al.*, 2005). As to the functional significance of reduced insular volume seen in our KS patients, the insular cortex have been found to play an important role in social, emotional and mental processing (Nagai *et al.*, 2007), areas where patients with KS have been show to exhibit deficits. Decreased insular volume has also been documented in patients with Williams syndrome which is associated with reduced social and emotional processing (Cohen *et al.*, 2010) and in patients with schizophrenia (Nagai *et al.*, 2007). Thus, it is plausible that the decreased insular volume seen in patients with KS (Skakkebaek *et al.*, 2014) and the extreme version of this in KS patients with skewed X-inactivation could have a functional significance in relation to psychiatric symptoms and social and emotional

Table 1 Comparison between KS patients with maternal and paternal origin of the supernumerary X-chromosome and without and with skewed X-chromosome inactivation on age, education, sex hormones, employment and cohabitation status and friends

	Maternal origin of extra X-chromosome (n = 21)	Paternal origin of extra X-chromosome (n = 20)	No skewed X-chromosome inactivation (n = 40)	Skewed X-chromosome inactivation (n = 11)	p-value mat vs. pat	p-value No skew vs. skew
N						
Age (years)	30.0 ± 8.1	35.3 ± 6.8	37.2 ± 8.9	35.4 ± 10.4	0.03[a]	0.58[a]
Education (years)	13 (8–15)	13.5 (9–16)	13 (6–18)	13 (9–17)	0.18[b]	0.54[b]
Testosterone treatment (%)	67 (14/21)	75 (15/20)	58 (23/40)	73 (8/11)	0.56	0.36
Sex hormones						
Testosterone (nmol/L)	16.0 (3.4–27.7)	17.0 (2.7–61.3)	14.7 (1.8–35.9)	18.3 (4.7–61.3)	0.35[b]	0.34[b]
Free testosterone (nmol/L)	0.40 (0.06–0.76)	0.48 (0.04–2.06)	0.32 (0.04–1.03)	0.40 (0.09–2.06)	0.20[b]	0.33[b]
17β-Oestradiol (pmol/L)	81.8 (0.0–461.2)	67.6 (9.5–300.5)	62.9 (0.0–461.2)	87.6 (0.0–300.5)	0.74[b]	0.58[b]
SHBG (nmol/L)	31.8 (12.6–62.5)	27.5 (10.7–108.8)	32.1 (14.5–59.5)	31.6 (4.0–108.8)	0.64[b]	0.76[b]
LH (IU/L)	12.4 (0.1–33.1)	5.0 (0.1–24.4)	15.9 (0.1–33.1)	13.4 (0.1–32.1)	0.09[b]	0.95[b]
FSH (IU/L)	31.6 (0.2–61.5)	10.2 (0.2–53.0)	19.4 (0.1–53.0)	8.6 (0.3–61.5)	0.05[b]	0.95[b]
Learning disability						
Self-reported learning disabilities in reading/writing (%)	62 (13/21)	80 (16/20)	75 (30/40)	55 (6/11)	0.20	0.19
Special education						
Special education in school in reading/writing (%)	57 (12/21)	75 (15/20)	60 (24/40)	45 (5/11)	0.23	0.39
Special education in school in mathematics (%)	38 (8/21)	25 (5/20)	25 (10/40)	27 (3/11)	0.37	0.88
Highest attained education					0.35	0.68
Basic school (%)	33 (7/21)	25 (5/20)	8/40	27 (3/11)		
General upper-secondary education (%)	5 (1/21)	0 (0/20)	3 (1/40)	9 (1/11)		
Vocational education and training (%)	62 (13/21)	65 (13/20)	68 (27/40)	64 (7/11)		
Short-cycle higher education (%)	0 (0/21)	0 (0/20)	0 (0/40)	0 (0/11)		
Medium-cycle higher education (%)	0 (0/21)	10 (2/20)	8 (3/40)	0 (0/11)		
Bachelor (%)	0 (0/21)	0 (0/20)	0 (0/40)	0 (0/11)		
Long-cycle higher education (%)	0 (0/21)	0 (0/20)	3 (1/40)	0 (0/11)		
PhD-degree (%)	0 (0/21)	0 (0/20)	0 (0/40)	0 (0/11)		
Employment status					0.14	0.33
Employed (%)	48 (10/21)	70 (14/20)	27/40	45 (5/11)		
Subsidized employment (%)	5 (1/21)	10 (2/20)	2/40	9 (1/11)		
Unemployed (%)	19 (4/21)	20 (4/20)	7/40	27 (3/11)		
Disability pension (%)	5 (1/21)	0/20	2/40	0 (0/11)		
Retired (%)	0 (0/21)	0/20	1/40	0 (0/11)		
Under education (%)	24 (5/21)	0/20	1/40	18 (2/11)		
Cohabitation status					0.70	0.09
Living with spouse (%)	43 (9/21)	11/20	58 (23/40)	45 (5/11)		
Living with parents (%)	19 (4/21)	10 (2/20)	5 (2/40)	27 (3/11)		
Living with other than spouse/parents (%)	0 (0/21)	0 (0/20)	0 (0/40)	9 (1/11)		
Living alone (widower/divorced) (%)	19 (4/21)	20 (4/20)	23 (9/40)	9 (1/11)		
Living alone (%)	14 (3/21)	5 (1/20)	10 (4/40)	9 (1/11)		
Other	5 (1/21)	10 (2/20)	5 (2/40)	0 (0/11)		
Friends					0.77	0.95
11 or more friends (%)	45 (9/20)	40 (8/20)	48 (19/40)	45 (5/11)		
6–10 friends (%)	35 (7/20)	30 (6/20)	30 (12/40)	27 (3/11)		
5 or fewer friends (%)	20 (4/20)	30 (6/20)	23 (9/40)	27 (3/11)		

Mat, maternal; Pat, paternal. Data are medians (total range) or means ± SD. χ^2 test. [a]Student's t-test. [b]Mann–Whitney test rank sum test.

function. However, caution should be taken in interpreting our results as X-inactivation can vary between tissues. Our analysis of X-inactivation was carried out in peripheral blood cells, which may not be the relevant tissue, to assess the relationship with brain volumes. Furthermore, samples of patients KS including larger group of KS patients with skewed X-chromosome may be needed to detect subtle differences in global and other regional brain volumes.

We did not find any influence of parent-of-origin of the X-chromosome on the cognitive phenotype in our cohort of KS patients, in line with the study by Ross *et al.* (2008), and in line Stemkens *et al.* (2006) and Ratcliffe *et al.* (1990), who reported no parent-of-origin effect on intelligence. However, Stemkens *et al.* reported an increased prevalence of motor impairment and speech/language problems in KS patients with a paternal origin of the extra X-chromosome (Stemkens *et al.*, 2006), a finding we could not replicate in line with others (Lorda-Sanchez *et al.*, 1992; Ross *et al.*, 2008; Zeger *et al.*, 2008). No parent-of-origin effects have been found in relation to psychiatric morbidity in KS patients. However, a recent study suggested that schizotypal traits were associated with maternal origin of the extra X-chromosome, and that a parent-of-origin effect was seen in the pattern of autistic traits (Bruining *et al.*, 2010). Our results regarding prevalence psychological distress, autism traits and personality profile showed no parent-of-origin effect. Conclusively, the evidence for a parent-of-origin effect on the cognitive

Table 2 Comparison between KS patients with maternal and paternal origin of the supernumerary X-chromosome and without and with skewed X-chromosome inactivation on global brain volumes

	Maternal origin of extra X-chromosome	Paternal origin of extra X-chromosome	No skewed X-chromosome inactivation	Skewed X-chromosome inactivation	*p*-value mat vs. pat	*p*-value (mat vs. pat) corrected for multiple testing	*p*-value No skew vs. skew	*p*-value (no skew vs. skew) corrected for multiple testing
n	19	18	35	10				
Age (years)	29.1 ± 7.98	36.2 ± 6.6	37.1 ± 8.8	35.2 ± 10.9	0.006		0.57	
Total grey matter volume (mL)	739.2 (656.0–815.8)	736.4 (624.0–778.8)	744.7 (630.1–798.5)	680.7 (624.0–788.4)	1.00	1	0.02	0.07
Total white matter volume (mL)	534.1 (452.2–600.0)	530.2 (455.7–565.6)	536.3 (430.7–605.9)	507.2 (425.2–592.4)	0.81	1	0.06	0.11
Cerebro-spinal fluid (mL)	334.2 (279.0–420.8)	331.4 (291.7–382.8)	325.7 (279.0–406.5)	326.8 (278.5–438.8)	0.72	1	0.62	0.62

Mat, maternal; Pat, paternal.

Figure 1 Autism Spectrum Quotient scores (A), Revised NEO Personality Inventory scores (B) and Symptom Check List (C) scores for parent-of-origin KS patients and controls.

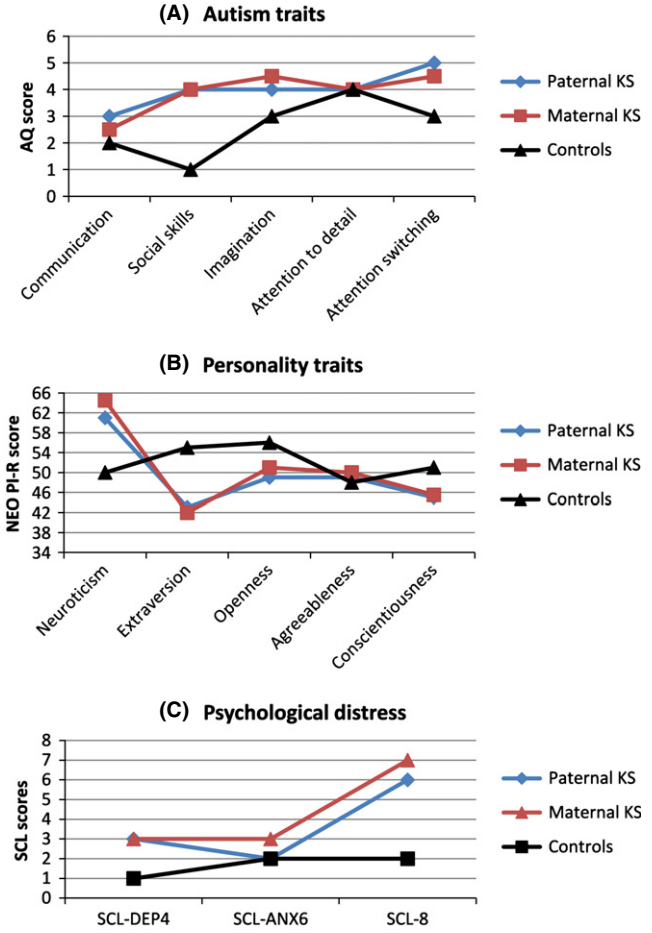
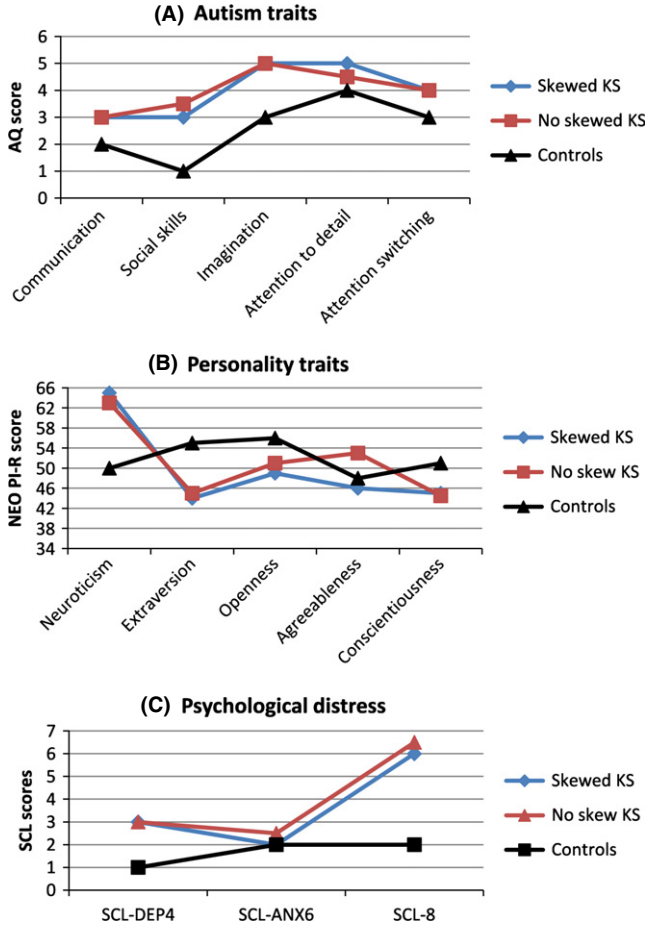


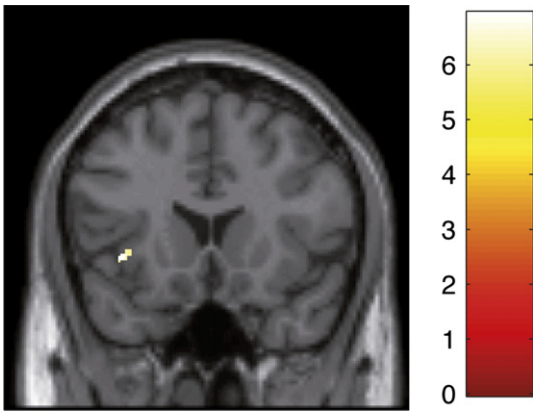
Figure 2 Autism Spectrum Quotient scores (A), Revised NEO Personality Inventory scores (B) and Symptom Check List (C) scores for KS patients with skewed and random X-chromosome inactivation and controls.



and psychological phenotype seen in KS does not seem likely, indicating that parent-of-origin might not account for the majority of phenotypic variation seen in KS.

Studies regarding the impact of skewed X-chromosome inactivation on the cognitive phenotype in KS are limited. The only study investigating this had only two participants with skewed X-chromosome inactivation making the conclusion of no association weak (Ross *et al.*, 2008). Another study investigated the impact on educational and marital status in KS patients with skewed X-chromosome inactivation and found no relation either (n = 5) (Zitzmann *et al.*, 2004). Our study is the largest

Figure 3 T-map displaying significant grey matter cluster differences between the KS patients with skewed X-chromosome inactivation and KS patients with random X-chromosome inactivation ($p = 0.05$, FEW corrected) is overlaid brain template. KS<C is displayed in yellow-red colours (see online pdf version for colours) [t (df), df = 43].



study of the impact of skewed X-chromosome inactivation on the neuropsychological phenotype in KS. In line with the above mentioned studies, we did not find an impact of skewed X-chromosome inactivation on cognition, autism traits, personality traits and psychological distress, indicating that skewed X-chromosome inactivation might not contribute substantially to the psychological phenotypic variation in KS. However, further studies including higher number of participants with skewed X-chromosome inactivation are needed.

The mean CAG repeat length found in this study is in line with previous studies (Zitzmann & Nieschlag, 2003; Huhtaniemi *et al.*, 2009; Bojesen *et al.*, 2011). Furthermore, no difference between median CAG repeat length and physiological CAG repeat length was found. We did not find an influence of CAG repeat length on cognitive phenotype in KS, in agreement with another study (Ross *et al.*, 2008). However, our finding of no correlation with education and cohabitation status is at odds with Zitzmann *et al.* (2004) ,who found short CAG repeat length to be associated with stable partnership and professions requiring higher level of education. Different study design may account for the difference. The association between cognitive function and CAG repeat length has been investigated in middle-aged and older men, with equivocal results. Lee *et al.* (2010) failed to detect any association between CAG repeat length and spatial awareness, memory and processing speed, investigating middle-aged or old men, however, Yaffe *et al.* (2003) reported that a higher CAG repeat length was associated with lower cognitive function and processing speed in older men. To conclude, no association has been found between CAG repeat length and cognitive function in boys or adults (age < 60 years) neither in KS patients nor in men from the general population, indicating that it is unlikely that CAG repeat length contributes to the cognitive variation in KS patients.

Our results do not support a relation between CAG repeat length and personality traits in KS patients or in controls. In agreement with our findings in controls, Jonsson *et al.* (2001) did not find any association with personality traits. However, other studies have found correlation between CAG repeat length and extraversion (Westberg *et al.*, 2009), impulsive-disinhibited (Aluja *et al.*, 2011) and antisocial traits (Comings *et al.*, 2000; Prichard *et al.*, 2007), indicating that CAG repeat length could influence personality traits. In this study, we used the short form of the NEO PI-R test, precluding conclusions on a putative association with sub-traits. Use of the long version including sub-scales may give a more nuanced picture of personality in relation to CAG repeat length. We found no correlation between the prevalence of depression and anxiety, psychological distress, autism traits and the CAG repeat length in our KS patients or in our controls. Previously, CAG repeat length have been associated with specific symptoms of depression (Sankar & Hampson, 2012) and Seidman *et al.* (2001) reported that depression was negatively associated with testosterone in men with shorter CAG repeat length (<21). On the other hand long CAG repeat length has been associated with lower scores of tests assessing ADHD and conduct disorders (Comings *et al.*, 1999). The above mentioned studies indicate that CAG repeat length might affect personality traits and psychological disorders in the general population. Our study does not support an impact of CAG repeat length on the neuropsychological phenotype in KS.

The majority of our KS patients were treated with testosterone which could influence our results. However, we did not find any correlation between testosterone and CAG repeat length in KS patients treated with testosterone and untreated KS patients. Neither did we find any difference between testosterone-treated KS patients nor untreated KS patients regarding global and regional brain volumes (Skakkebaek *et al.*, 2014) or education, learning disabilities, employment, cohabit status, personality and autism traits, psychological distress, cognitive performance (unpublished data). Thus, it is not plausible that the fact that the majority of our patients were treated with testosterone influence our results in any significant way.

In conclusion, we do not find any evidence for an influence of parent-of-origin, CAG repeat length and skewed X-chromosome

Table 3 Spearman correlations between sex hormones and CAG repeat length in 23 untreated Klinefelter syndrome patients (U-KS), 50 treated Klinefelter syndrome patients (T-KS) and 73 controls

	U-KS		T-KS		Controls	
	Spearman's rho	*p*-value	Spearman' s rho	*p*-value	Spearman's rho	*p*-value
Testosterone (nmol/L)	0.22	0.31	−0.01	0.99	0.12	0.32
Free testosterone (nmol/L)	0.08	0.71	−0.04	0.81	0.21	0.07
17β-Oestradiol (pmol/L)	−0.102	0.64	−0.04	0.81	0.07	0.57
SHBG (nmol/L)	0.33	0.12	0.12	0.40	−0.08	0.52
LH (IU/L)	−0.34	0.11	0.03	0.86	−0.08	0.50
FSH (IU/L)	0.14	0.52	−0.01	0.94	−0.05	0.67

inactivation on the cognitive and psychological phenotype seen in adult KS patients. However, skewed X-chromosome inactivation could have an impact on GM volume in KS, findings which are to be replicated in another and larger cohort of KS patients.

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AUTHOR CONTRIBUTIONS

All authors contributed substantial to research design and to the acquisition, analysis and interpretation of the data. Furthermore, all authors contributed to drafting the paper, revising it and approved the submitted and final version.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Comparison between KS patients with maternal and paternal origin of the supernumerary X-chromosome and without and with skewed X-chromosome inactivation on psychological and cognitive variables. Mat, maternal; Pat, paternal. Data are medians (total range) or means ± SD. Mann–Whitney test rank sum test. †Student's *t*-test.

Table S2. Spearman correlations between CAG repeat length and psychological and cognitive variables and brain volumes.

