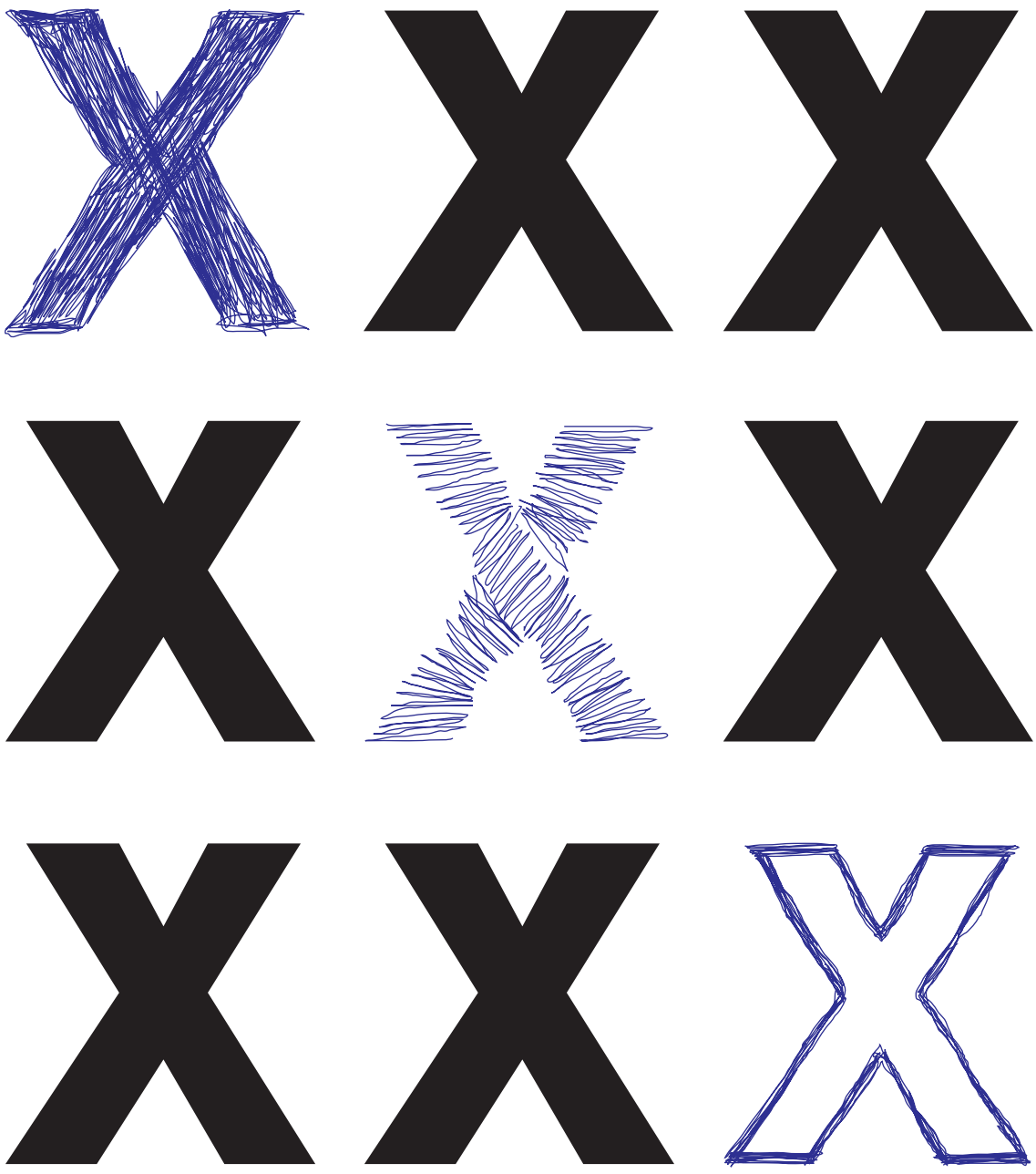
TURNER SYNDROME IN ADULTHOOD

A childhood disease grown up

Kim Freriks



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Promotor:	Prof. dr. A.R.M.M. Hermus
Copromotoren:	Dr. H.J.L.M. Timmers Dr. T.C.J. Sas (Albert Schweitzer Ziekenhuis)
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Voor mijn ouders

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Chapter 1

Introduction and Outline

Partly based on 'Turner syndrome in adulthood: the need of multidisciplinary care'

Kim Freriks[1], Catharina CM Beerendonk[2], Janneke Timmermans[3], Didi DM Braat[2], Ad RMM Hermus[1], Henri JLM Timmers[1]

Author Affiliations

Departments of [1]Internal Medicine, division of Endocrinology, [2]Obstetrics and Gynecology, [3]Cardiology of the Radboud university medical center, Nijmegen

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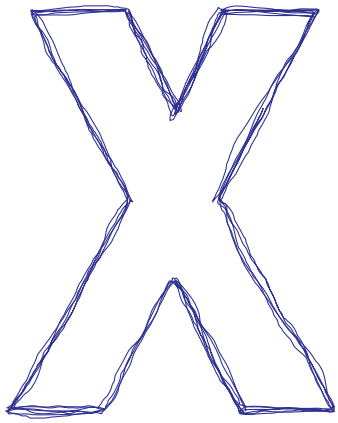
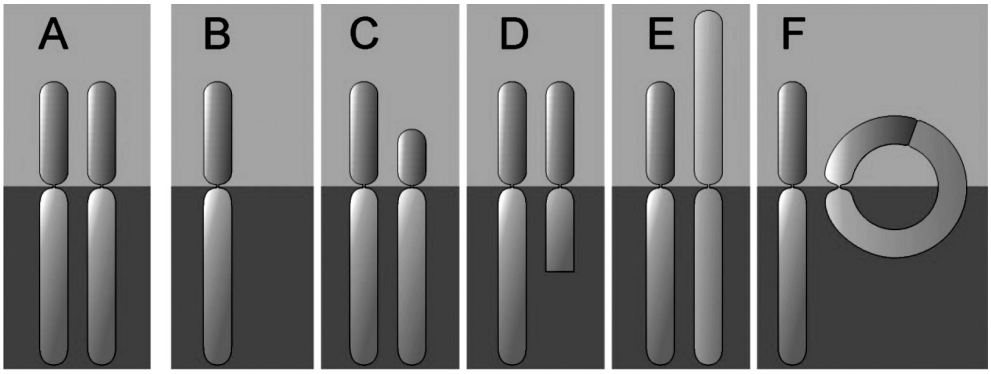


Turner syndrome

Turner syndrome (TS) is named after Henry Turner, who described the triad of short stature, 'sexual infantilism' and dysmorphic abnormalities in 1938.[1] Later on, in 1959, TS was found to be the result of the absence of one X-chromosome in women.[2] Nowadays, TS is known as a common chromosomal disorder, with an incidence in the Netherlands between 1:2000 and 1:5000 in live born girls.[3] The classic phenotype described by Henry Turner is still accurate, although it is currently recognized that the clinical picture of TS is highly variable and ranges from severe congenital lymphedema and cardiac anomalies to a milder phenotype of secondary amenorrhea and/or unexplained infertility as presenting symptoms.

The diagnosis of TS is confirmed by karyotyping of at least 30 lymphocytes. Based on this approach half of the patients are found to have a complete X chromosomal monosomy (karyotype 45,X). In 30-40% of the patients a mosaicism of different karyotypes is found, for example 45,X/46,XX, 45,X/47,XXX and 45,X/46,XY. In 5-15% of the cases one of the X chromosomes has structural anomalies including an isochromosome, ring chromosome or a deletion (Figure 1).[4-6] In general, patients with a 45,X/46,XX karyotype have a milder phenotype than those with 45,X.[4,6,7]

FIGURE 1
X-chromosomal abnormalities in Turner syndrome



A normal female karyotype (46,XX)
B complete monosomy (45,X)
C (partial) deletion short arm Xp (46,X,del(Xp))
D (partial) deletion long arm Xq (46,X,del(Xq))
E isochromosome Xq (46,X,i(Xq))
F ring chromosome (46,X,r(X))
Adapted from https://www.radboudumc.nl/Zorg/Ziektebeelden/Documents/1%20Turner.pdf

Growth and development

The disease spectrum of TS is broad and besides consultation for congenital anomalies, recurrent otitis and developmental problems, the cornerstones of childhood care are treatment with growth hormone (GH) to increase adult height and estrogen to induce puberty. Short stature is the most common characteristic of TS. Untreated TS women in the northern part of Europe reach a mean adult height of 147 cm. The main cause of reduced height is the lack of the 'short-stature homeobox-containing gene' (SHOX-gene) located on the short arm, Xp, of the X-chromosome.[8-10] Although girls with TS are not GH deficient, supra-physiological doses of GH are given resulting in an average adult height gain of 5-12 cm.[11-13] Differences in efficacy of GH treatment can be explained by age at initiation and duration of therapy, compliance, estrogen therapy regimen, and several, partly unknown, genetic factors.[11,13,14]

During fetal development the ovaries initially develop normally, but already before birth an accelerated regression with apoptosis of the oocytes and fibrosis of the stroma occurs.[4] In most patients this causes non functional ovaries resulting in absence of spontaneous puberty and infertility. Nevertheless 8-9% of the girls with monosomy 45,X and 40-47% of the girls with 45,X/46,XX develop (temporary) menstrual cycles.[15,16] Following the induction of puberty (or in case of insufficient estrogen production after a period of spontaneous puberty) hormonal replacement therapy (HRT) is indicated. Several HRT regimens are available with different formulations and doses of estrogens and different modalities of administration (continuous or cyclic / oral or transdermal). In the international guidelines, it is recommended to base the choice of HRT on age, personal preferences of the patient, complications of estrogen deficiency and additional risk factors for osteoporosis and coronary heart disease.[17] In general HRT needs to be continued until the normal age of menopause.[17]

Fertility

Spontaneous pregnancies occur in 2-8% of the women, most commonly in those with 45,X/46,XX.[18-20] When spontaneous pregnancy is not achieved, assisted pregnancy with oocyte donation, besides adoption and foster care, can be considered. Pregnancy and implantation rates after oocyte donation in TS are comparable to the normal population.[21] However, pregnancies in TS are associated with an increased risk of miscarriage and fetal abnormalities,[16,22] although not all studies confirm an increased incidence of birth defects.[21] Pregnancy in TS, spontaneous or assisted, is accompanied by an increased risk of cardiovascular morbidity, so careful counseling including cardiac evaluations before and during pregnancy is warranted.[23-25]

Structural cardiovascular disease

TS patients have an approximately 3-fold increased risk of age-related mortality, mainly due to structural cardiovascular anomalies and unfavorable cardiovascular risk factors.[26,27] A recent

review on cardiovascular disease in TS estimated that of all TS patients up to 70% have some form of congenital heart and/or aorta anomaly.[28] The most common anomalies are obstructive lesions of the left heart including a bicuspid aortic valve (15-30%) and coarctation of the aorta (up to 17%).[28] Also malformations of the left sided vessels -such as elongation of the transverse aortic arch-, abnormal aortic arch vessels and anomalies of the large thoracic veins are more common than in the general population.[29] Especially at adult age a bicuspid valve can lead to aortic valve stenosis and regurgitation.[30,31] Furthermore, patients with bicuspid aortic valve and (surgically corrected) coarctation are assumed to have an increased risk of aortic dilation and dissection.[32-34] The incidence of dilation in TS increases with age and dilation can already be present in the second decade of life.[35,36] Aortic dissection also occurs at an earlier lifespan than in the general population: it is reported already from the third decade on with a life-time chance of 1-2 percent of the TS women.[23,37,38] Regardless of the presence of a cardiac or aortic congenital anomaly, follow-up for aortic dilation is essential from childhood on.[17] In (induced) pregnancy the risk of aortic dissection is assumed to be even higher, so cardiovascular screening before and during each pregnancy is strongly advised.[24]

Non-structural cardiovascular disease

Additional to the structural abnormalities, the prevalence of atherosclerosis is doubled and the risk of ischemic heart disease is increased with a standardized mortality ratio of 3.5.[39,40] Coronary heart disease in TS is determined by the relatively high prevalence of hypertension, insulin resistance and diabetes, dyslipidaemia and estrogen deficiency.[28] Hypertension is reported in up to half of the adult women with TS and is idiopathic in the majority of the patients.[34,36] Diabetes mellitus type 1 and 2 are respectively 11 and 3-4 times more common in TS than in the normal population.[41] The prevalence of diabetes type 2 is probably related to body mass index (BMI), since Bakalov *et al.* found a prevalence of diabetes type 2 in 25% of a young American TS population (BMI 28.9 ± 7.7 kg/m^2), while Landin-Wilhelmsen *et al.* reported a prevalence of 3% in a similar age group around 30 years old (BMI 25.9 ± 5.0 kg/m^2).[42,43] Independent of BMI, total cholesterol, low density lipoprotein cholesterol and triglycerides are elevated in 37-50 percent of the TS women.[40,44,45] Furthermore, up to 21% of the adult TS women have a prolonged QTc interval.[46,47]

Osteoporosis

Decreased bone mineral density (BMD) is frequently reported in women with TS. Estrogen deficiency plays an important role in the etiology, however low BMD despite adequate HRT is reported.[48,49] Probably, achievement of optimal peak bone mass during adolescence is essential,[50,51] since it was reported that TS patients with spontaneous menstruation have normal BMD.[52] Besides estrogen deficiency, a primary defect in bone formation, most likely

due to haploinsufficiency of SHOX gene, seems to be an important contributing factor to the pathogenesis of osteoporosis as well.[53-56] A selectively decreased cortical BMD, rather than trabecular BMD, is suggested.[48,57,58] However, the exact burden of osteoporosis in the TS population is uncertain. The high frequency of osteoporosis, up to 80%, observed in TS patients is probably an overestimation, because short stature –especially a height below 150 cm– causes false low areal BMD measurements.[54,59,60] Using volumetric BMD measurements, either direct measurements with computed tomography or indirect calculations, instead of areal measurements to correct for height, can partly overcome this problem.[57-61] Nevertheless, a higher fracture risk in TS, varying from 24-32%, has been reported. The fracture risk is elevated due to the combination of diminished BMD and a higher incidence of falling.[55,62] Especially forearm fractures, probably related to frailty of cortical bone, are more common with a relative risk of 1.9.[62,63]

Auto-immunity

Diseases related to autoimmunity are frequently reported in TS and the incidence increases with age.[64,65] Mortensen *et al.* found that auto-antibodies (against gliadin, transglutaminase, adrenal cortex, intrinsic factor, thyroid peroxidase and glutamic-acid-decarboxylase 65 or a combination thereof) are present in 58% of the patients with TS.[64] Especially, autoimmunity of the thyroid (anti-TPO, 45%) and celiac disease (18%) were present. Concerning clinical disease, as many as 16-30% of the TS population develop auto-immune hypothyroidism,[64-66] and celiac disease is seen in around 5% of the adult TS population.[64,67] To a lesser extent, other autoimmune diseases like diabetes mellitus type 1 (relative risk 11) and inflammatory bowel disease (relative risk 2-3) are mentioned in relation to TS.[41] The pathophysiology of these autoimmune phenomena is unclear. It is suggested that auto-immunity is more common in patients with an isochromosome (46,X,iXq).[68] Others found that both TS women and women with premature ovarian failure have an increased incidence of Hashimoto's thyroiditis, suggesting a role for estrogen as well.[69]

Ear and hearing problems

In childhood there is a high prevalence of middle ear infections and related conductive hearing loss.[70,71] Adult patients often experience sensorineural hearing loss, which starts with a typical mid-frequency dip already during adolescence.[72,73] Also, progressive sensorineural high-frequency loss manifests earlier in TS women than in the normal population, especially in those patients with a previous mid-frequency dip.[74] The need for a hearing aid is frequent with a range from 27% in a younger population to 44% in those above 35 years old.[72] TS patients with monosomy 45,X or isochromosome 46,X,i(Xq) are supposed to be more prone to hearing problems than those with mosaicism or other structural X-chromosomal anomalies.[75,76]

This suggests that the locus for hearing impairment in TS may be located on the short arm, Xp. Furthermore, due to the presence of estrogen receptors in the human inner ear, estrogen deficiency is probably a contributing factor in hearing problems as well.[77,78]

Psychological problems

Besides the physical aspects, women with TS may report various psychological problems. Girls with TS have a distinct neuro-cognitive profile characterized by a normal to high verbal intelligence quotient (VIQ) and a decreased performance IQ (PIQ).[79,80] Cognitive problems commonly persist until adulthood and the diminished performance on non-verbal skills make adult women with TS prone to impairments in visual-motor integration, attention, (working) memory, executive function and spatial cognition.[79,80] In addition, women with TS report more social isolation, shyness, social anxiety and problems in relationships. [81-83] Furthermore, women with TS have a high rate, estimated at 36%, of lifetime depression and an increased risk for developing autism and attention-deficit hyperactivity disorder.[84-86]

For a schematic overview of TS associated morbidity see Table 1.

TABLE 1
Abnormalities in Turner syndrome (adapted from Gravholt[5] and others[28,29,33,34,36,40,42,43,45,46,64-67,72,112-115])

Organ system	Abnormality	Frequency (%)
Reduced adult height		95-100
Gonadal dysgenesis	No pubertal development	85
	Infertility	98
	Chronic estrogen deficiency	95-98
Eyes	Epicanthus	20
	Nearsightedness	20
	Strabismus	15
	Ptosis	10
Ears	Otitis media	~60
	Conductive hearing loss	0-40
	Sensorineural hearing loss	4-66
	Deformity of the external ear	15
	Hearing loss requiring hearing aid	27-44
Mouth	Micrognatia	60
	High arched palate	35
Neck	Low posterior hairline	40
	Broad short appearing neck	40
	Pterygium colli (webbed neck)	25
	Skinfold in the back of the neck of newborns	25
Thorax	Broad chest with widely spaced nipples	30
	Inverted nipples	5
Skin, nails, hair	Increased skin ridge count	30
	Lymphedema of hands and feet	25
	Multiple pigmented naevi	25
	Nail hypoplasia	10
	Alopecia	5
	Vitiligo	5
Skeleton	Bone age retardation	85
	Osteoporosis or osteopenia	50-80
	Cubitus valgus	50
	Short 4th metacarpal bone	35
	Genu valgum	35
	Congenital hip luxation	20
	Scoliosis	10
	Madelungs deformity	5
Heart-aorta	Heart-aorta deformities (congenital)	22-70
	Bicuspid aortic valves	15-30
	Coarctatio aortae	up to 17
	Aortic dilation/aneurysm	3-42
	Elongation of the transverse aortic arch	~50
	Prolonged QTc interval	~20
Stomach, intestines, liver	Chronic inflammatory bowel disease	<1
	Celiac disease	4-6
	Elevated hepatic enzymes	36-80
Kidney	Renal anomalies	28-38
Endocrine	Autoimmune hypothyroidism	16-30
	Diabetes mellitus type 1	0.5-5
	Diabetes mellitus type 2	3-25
	Glucose intolerance	7-50
	Hypertension	up to 50
	Dyslipidemia	37-50

Multidisciplinary care in adult TS patients

Since GH treatment and induction of puberty requires strict pediatric follow-up, traditionally the care for patients with TS is focused on girls. Many patients were lost to follow-up after induction of puberty.[45,87-90] However, the need for ongoing care for adult patients is increasingly recognized. The mean life expectancy is reduced by 13 years and there is an approximately 3-fold increased age-related mortality risk.[26,41] This has lead to expert-based international guidelines for the care of TS patients, taking into account the broad spectrum of morbidity in adult women, warranting vigorous follow-up and treatment.[4,17,91] A screening protocol for medical follow-up is depicted in Table 2.

TABLE 2
Screening protocol for adults with Turner syndrome (adapted from Bondy,[17] Saenger[91] and Conway[116])

Investigation	Once	Every 1-2 yrs[c]	Every 3-5 yrs[c]
Height, weight, blood pressure, auscultation of the heart		X	
Creatinin, blood urea nitrogen, ASAT, gamma-GT, TSH,		X	
Celiac serology[a]			X
Karyotype	X		
Renal ultrasound	X		
Pelvic ultrasound	X		
Audiogram[b]	X		
Cardiac ultrasound and electrocardiogram			X
MRI aorta (thoracic and abdominal)			X
Bone mineral density measurement (DEXA)			X

ASAT=aspartate-aminotransferase; gamma-GT=gamma glutamyl transpeptidase; TSH= thyroid stimulating hormone; fT4=free thyroxine; HbA1c=glycated hemoglobin.
[a] IgA antibodies to tissue transglutaminase and endomysium; in case of IgA deficiency, IgG antibodies were measured
[b] repeat at age of 40
[c] frequencies are increased when abnormalities are found

The morbidities associated with TS in adults lie within the fields of different medical and paramedical professions, making multidisciplinary collaboration essential. Proper transition from pediatric to adult care is important, considering the fact that TS-related conditions diagnosed during childhood require ongoing follow-up. For example congenital anomalies of the heart and/or aorta require regular investigations at adult age because of the risk of late complications.[32,91,92] Collaboration is particularly important during pregnancy. Considering the increased risk of aortic dilation and dissection during pregnancy, cardiac evaluations before and during pregnancy are called for, especially when congenital heart disease and/or hypertension

is present.[23-25] Since medical care for TS throughout adult life is advocated, several specialized TS clinics have been established in the United States and Europe.[4,17,91] Nevertheless, the medical care for TS patients needs improvement. A French group reported that only 3.5% of the adult population received appropriate medical care and in a Belgian cohort 12.7% of the women lacked medical attention despite reported health problems.[87,88] It was reported that in the United States only one third of the adult TS women had undergone the three examinations considered standard care (renal and cardiac ultrasound and audiology).[89] Also, an inventory in the Netherlands showed that only a minority of the patients continued to receive adequate medical care after discharge from pediatricians' follow-up.[90] In order to provide appropriate medical care for TS women, the Radboudumc opened a multidisciplinary Turner clinic in 2005. The aim was to ensure an adequate transition from pediatric to adult care, optimize screening and treatment of TS related conditions in adults, and to provide optimal HRT and fertility counseling.

In **Chapter 2** we describe our study on the effectiveness of an approach to medical care of TS women as advocated in published expert opinion based guidelines. The aim of this study was to investigate the yield of an initial comprehensive screening for TS-associated morbidity in a large group of adult patients in a single Dutch center in order to answer the question if the burden of screening is justified by the yield of new diagnoses.

Karyotyping in Turner syndrome

With standard karyotyping of 30 blood lymphocytes a 10% chromosomal mosaicism can be identified with a 95% confidence interval.[17,93,94] However, standard karyotyping might not be sufficient for comprehensive genotyping. Firstly, it does not rule out low-grade mosaicism in blood lymphocytes and secondly, it does not rule out tissue mosaicism, *i.e.* the coexistence of different karyotypes across different body tissues. The idea of tissue mosaicism is established by the assumption that fetuses with apparent 45,X must have a parallel normal cell line to survive to term. This theory is strengthened by the fact that 45,X is much more common in still-born fetuses than in live born girls with TS.[95,96] Several reports on TS patients confirm the presence of tissue specific mosaicism.[97-99] Comprehensive karyotyping is important for gaining insight in genotype/phenotype correlations, but also appears to be relevant for individual patients when it comes to mosaicisms that include a Y chromosome. Y chromosomal material in TS is associated with the development of gonadoblastoma in the streak ovaries and when found on standard karyotyping, prophylactic gonadectomy is performed, to prevent the development into a malignant dysgerminoma.[100] The need for gonadectomy when a Y chromosome is found in another tissue besides blood lymphocytes has not been established.

In **chapter 3** we describe our study that was aimed at evaluating whether conventional karyotyping of blood lymphocytes is accurate for full understanding of the karyotype. We investigated the role of additional cytogenetic investigations using different techniques including fluorescent in situ hybridization and real-time polymerase chain reaction in additional tissues such as buccal mucosa cells.

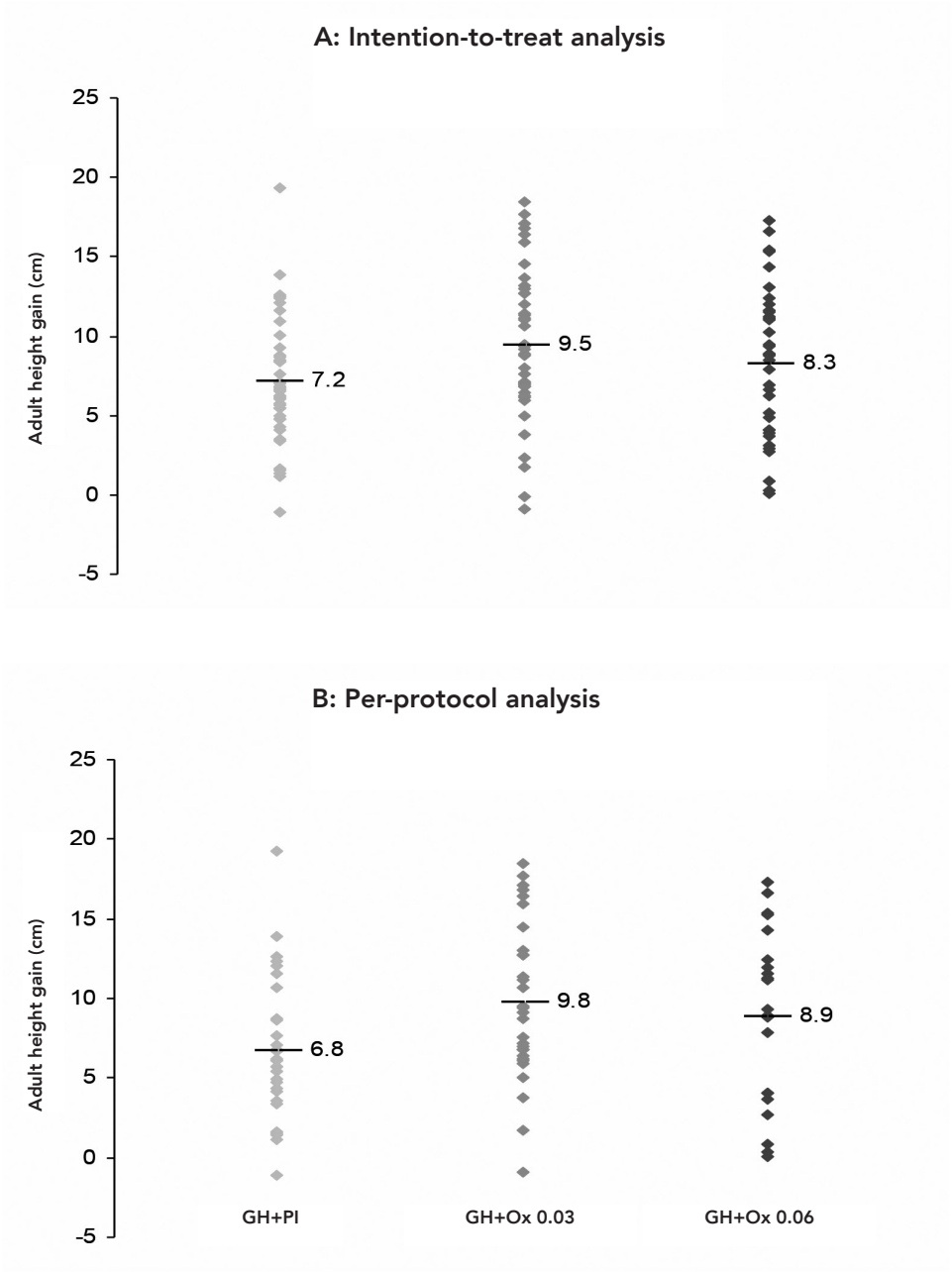
Long term effects of oxandrolone treatment

The results of routine GH treatment in TS girls are not always optimal. Therefore, in previous studies higher doses of GH have been investigated, as well as the addition of androgens. Given the fact that androgens are important mediators of growth, it seems worthwhile to investigate the effects of androgen treatments on adult height gain. Oxandrolone (Ox), a synthetic, non-aromatizable anabolic androgenic steroid derived from testosterone, has been previously tested to enhance growth in TS. Ox has a high anabolic to androgenic ratio (10:1), which means that Ox gives less virilizing effects compared to testosterone.[101] Nevertheless, in the initial studies on Ox in TS, where dosages of 0.1 mg/kg per day or higher were used, the dose had to be frequently lowered due to virilization and early bone maturation.[102-104]

More recently three randomized controlled studies on the growth enhancing effect of lower than conventional doses of Ox in addition to GH have been published.[105-107] This includes the Dutch Turner Oxandrolone Study, a collaborative initiative of ten pediatric endocrine centers in the Netherlands. Participants of this randomized double-blind placebo controlled trial were recruited between 1991 and 2003.[105] In total 133 girls were included and blindly randomized to receive Ox in a dose of 0.03 mg/kg/day (Ox 0.03) or 0.06 mg/kg/day (Ox 0.06) or placebo (Pl) orally after reaching the age of 8 years. Furthermore the girls were treated with GH (1.33 mg/m^2/day) from baseline and, when spontaneous puberty was absent, with estrogen from the age of 12 years.

The main results of the pediatric study were that compared to GH+Pl, GH+Ox 0.03 increased adult height gain –final minus predicted adult height– (9.5 vs. 7.2cm in GH+Pl) at the cost of mild deceleration of breast development (Figure 2). At the higher dose, Ox 0.06, no significant increase in height gain was found (8.3 vs. 7.2cm in GH+Pl), probably due to faster bone maturation and premature discontinuation of Ox because of virilizing side effects. In the GH+Ox 0.06 group significantly more girls reported subjective virilization. Findings in both Ox groups included a decrease in fat mass, an increase in muscle mass and lowering of the voice pitch, which were initially in the higher range, to normal voice frequencies in most cases.[108,109] There were no effects of Ox on behaviour, aggression, romantic and sexual interest, mood, and gender role.[110] Furthermore no effects on glucose metabolism were established.[111]

FIGURE 2
Adult height gain in the intention-to-treat analysis (A) and per-protocol analysis (B). Diamonds represent adult height gain of the individual patients; lines represent mean adult height gain per dosage group.



So far, previous studies on Ox in TS were focusing on the short-term effects in children and adolescents and long-term follow-up data were lacking. In this part of the thesis we report the results of our follow-up investigations in the Dutch Turner Oxandrolone Study cohort. The aim of these follow-up studies was to assess the long-term effects of the different treatment regimens of the trial (GH and estrogen with or without Ox 0.03 or Ox 0.06). The above mentioned beneficial and adverse effects on growth, body proportions, body composition, metabolic parameters and psychological items may or may not carry on beyond adolescence. The original participants of the Dutch Turner Oxandrolone Study, who have now reached an adult age, were investigated. We explored whether beneficial effects of auxiliary treatment with Ox are permanent and outweigh adverse effects that may be temporary. A broad spectrum of outcome parameters was investigated. In **chapter 4** we discuss the long-term effects of Ox on height, body proportions and composition, virilization and cardiovascular risk profile. In **chapter 5** the effects on neurocognition, quality of life and social-emotional functioning are discussed. In **chapter 6** we focus on the influence of Ox on hearing with additional analyses to establish the genetic basis for hearing problems in TS.

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Chapter 2

Standardized multidisciplinary evaluation yields significant previously undiagnosed morbidity in adult women with Turner syndrome

Kim Freriks[1], Janneke Timmermans[2], Catharina CM Beerendonk[3], Chris M Verhaak[4], Romana T Netea-Maier[1], Barto J Otten[5], Didi DM Braat[3], Dominique FCM Smeets[6], Dirk HPM Kunst[7], Ad RMM Hermus[1], Henri JLM Timmers[1]

Author Affiliations

Departments of [1]Internal medicine, division of Endocrinology, [2]Cardiology, [3]Obstetrics and Gynecology, [4]Psychology, [5]Pediatric endocrinology, [6]Human Genetics, [7]Otolaryngology, of the Radboud university medical center, Nijmegen

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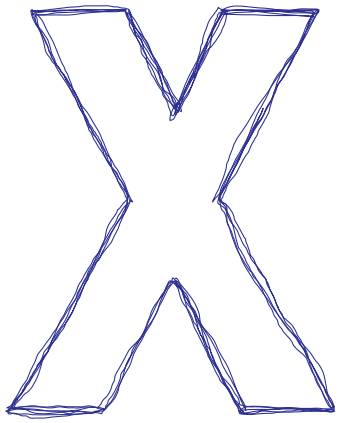


ABSTRACT

Context

Besides short stature and gonadal dysgenesis Turner syndrome (TS) is associated with various abnormalities. Adults with TS have a reduced life expectancy mainly related to structural abnormalities of the heart and aorta, and an increased risk of atherosclerosis.

Objective

Our objective was to investigate the yield of an initial standardized multidisciplinary screening in adult TS patients.

Design

This was an observational study at a multidisciplinary care unit for adult women with TS.

Participants

Participants were adult women with TS (n=150). Mean age 31.0±10.4 years, 47% karyotype 45,X.

Interventions

All women were consulted by an endocrinologist, a gynecologist, a cardiologist, an otorhinolaryngologist, and when indicated, a psychologist. The screening included magnetic resonance imaging of the heart and aorta, echocardiography, electrocardiogram, dual-energy x-ray absorptiometry, renal ultrasound, audiogram and laboratory investigations according to international expert recommendations.

Main outcome measures

New diagnoses and prevalences of TS associated morbidity were evaluated.

Results

Thirty percent of patients currently lacked medical follow-up and 15% lacked estrogen replacement therapy in the recent last years. The following disorders were newly diagnosed: bicuspid aortic valve (n=13), coarctation of the aorta (n=9), elongation of the transverse aortic arch (n=27), dilation of the aorta (n=34), osteoporosis (n=8), osteopenia (n=56), renal abnormalities (n=7), subclinical hypothyroidism (n=33), celiac disease (n=3), glucose intolerance (n=12), dyslipidaemia (n=52), hypertension (n=39), and hearing loss warranting a hearing aid (n=8). Psychological consultation was needed in 23 cases.

Conclusions

Standardized multidisciplinary evaluation of adult women with TS as advocated by expert opinion is effective and identifies significant morbidity. Girls with TS benefit from a careful transition to ongoing adult medical care.

INTRODUCTION

Turner syndrome (TS) is the result of complete or partial absence of one X chromosome and one of the most common sex chromosomal abnormalities with an incidence of approximately 1:2000 in live born girls.[1] Besides short stature, gonadal dysgenesis, and dysmorphic features TS is associated with a wide range of abnormalities affecting nearly every organ system. Girls with TS are usually treated with growth hormone to increase adult height and with oestrogens to induce puberty. Hormone replacement therapy (HRT) has to be started either following induction of puberty or when oestrogen production becomes insufficient after initial spontaneous puberty.

Also in adulthood, TS is associated with significant morbidity. There is a ~3 fold increased age related risk of mortality mainly caused by structural cardiovascular anomalies and atherosclerosis related to hypertension, diabetes and dyslipidaemia.[2] In addition, women with TS are prone to develop hypothyroidism, osteoporosis, hearing loss, neurocognitive deficits, and emotional problems.[3,4] In recent years the enhanced morbidity and mortality in adults with TS as well as the ensuing need for regular medical attention are increasingly recognized. Especially the transition from pediatric to adult care is a point of concern. Many young women are lost to regular medical follow-up after discharge from the pediatric clinic. Several specialized TS clinics have been established in the US and Europe to provide a coordinated multidisciplinary care service. Based on the experience of these centers, recommendations for standard of care and periodic screening have been put forward. [5-7] Despite these efforts, however, adult women with TS often lack appropriate medical attention.[8-12] In a young French TS population, only 3.5% received appropriate medical care.[8] In an adult US TS population, only one third had undergone the three examinations considered standard care for TS women, *i.e.* cardiac and renal ultrasound, and audiology.[10] In a Belgian cohort, 12.7% lacked medical attention despite reported health problems and 14.5% lacked HRT.[9] An inventory among Dutch TS women previously followed at a single pediatric clinic showed that appropriate specialist care was continued in only a minority.[12]

Our multidisciplinary care unit for TS women was established in 2005 and is the largest facility in the Netherlands. Our approach was adapted from international guidelines for care in TS patients.[5-7] The yield of screening as advocated in these guidelines was not previously examined. In light of the question whether the burden of screening is justified by the yield of new diagnoses, and whether the approach is cost-effective, the aim of this study was to investigate the yield of an initial comprehensive screening for TS associated morbidity in a large group of adult patients in a single Dutch center.

PATIENTS AND METHODS

Patients

The study included 150 consecutive adult TS patients who underwent an initial evaluation between May 2005 and June 2009. Mean age was 31.0 ± 10.4 years. The diagnosis TS was established by standard karyotyping of 30 peripheral lymphocytes (Table 1).[5]

TABLE 1
Patients characteristics (n=150)

Physical examination	**Mean (SD)**
Age (yr)	31.0 (10.4)
Height (cm)	153.2 (7.5)
Weight (kg)	61.6 (13.6)
BMI (kg/m^2)[a]	26.2 (5.5)
Karyotype	**Percentage**
45,X	47%
45,X/46,XX[b]	12%
45,X/47,XXX	3%
45,X/46,X,i(Xq)	14%
45,X/46,X,del(X)	5%
45,X/46,XY or marker Y	3%
45,X/46,X,r(X)	3%
46,X,i(Xq)	3%
46,X,del(X)	2%
Others[c]	5%
Pending[d]	3%
Medical care prior to investigation	**Percentage**
Direct transition from pediatric care	17%
Specialist care[e]	53%
Gynecologist	36%
Internist	7%
Cardiologist	2%
Combination of more than one specialist	8%
No medical care	30%

BMI= body mass index
[a] No difference in patients with and without diabetes
[b] Including one patient with mosaicism trisomie 21 as well
[c] Four patients with a mosaicism containing more than two cell lines, two patients with a marker chromosome, and two patients with a translocation with chromosome 13
[d] Patients were diagnosed with TS elsewhere; confirmation in our laboratory is pending
[e] Of these patients 6 had lacked medical attention during a mean period of 18.5 years earlier in life

Seventy-six percent of the women had received regular pediatric care during childhood and 56% of the women had been treated with growth hormone. Of 150 patients, 26 were directly referred for transition from pediatric to adult care within our centre. Seventy-nine patients already received regular medical care by either a gynecologist, cardiologist, and/or endocrinologist. Forty-five patients had lacked medical follow-up by any specialist, with a mean period of approximately 12 years before attending the clinic. The patients lacking medical follow-up were older than those with medical follow-up (37.5 vs. 28.2 years, P value < 0.01) and mean time since diagnosis was longer (26.7 vs. 17.0, P value < 0.01).

Screening

All patients underwent a standardized medical evaluation adapted from expert opinion recommendations,[5-7] which consisted of consultation (history and physical) by an endocrinologist, gynecologist, cardiologist, otorhinolaryngologist and, in a selected group of patients, a psychologist. The work-up included laboratory investigations and imaging, as indicated in Table 2.

TABLE 2
Screening protocol for adults with Turner syndrome (adapted from Bondy[5], Saenger[6], Conway[7])

Investigation	Once	Every 1-2 yrs[c]	Every 3-5 yrs[c]
Height, weight, blood pressure, auscultation of the heart		X	
Creatinin, blood urea nitrogen, ASAT, gamma-GT, TSH, fT4, total cholesterol, low-density cholesterol, high-density cholesterol, triglycerides, glucose, HbA1c, urine dipstick analysis		X	
Celiac serology[a]			X
Karyotype	X		
Renal ultrasound	X		
Pelvic ultrasound	X		
Audiogram[b]	X		
Cardiac ultrasound, including electrocardiogram			X
MRI aorta (thoracic and abdominal)			X
Bone mineral density measurement (DEXA)			X

ASAT=aspartate-aminotransferase; gamma-GT=gamma glutamyl transpeptidase; TSH= thyroid stimulating hormone; fT4=free thyroxine four; HbA1c=glycated hemoglobin.
[a] IgA antibodies to tissue transglutaminase and endomysium; in case of IgA deficiency, IgG antibodies were measured
[b] repeat at age of 40
[c] frequencies are increased when abnormalities are found

MRI scans were performed using Avanto 1.5T (Siemens, Erlangen, Germany). The protocol consists of dark blood (TSE db T1) and TRUFI images. Diameters of the aorta were measured at the level of the sinuses of Valsalva, the right pulmonary artery, the origin of the left subclavian artery, the left atrium, the diaphragm, the kidney arteries, and just above the bifurcation of the abdominal aorta. Also, the largest cross sectional diameters of the thoracic and abdominal aorta were measured.

Echocardiographic evaluation was performed according to the American Society of Echocardiography recommendations.[13] All examinations were performed by the same clinician (J.T.), using the echocardiography machine Vivid 7 or System Five (GE-Vingmed, Horten, Norway) connected to a phased-array probe (2.5 and 3.5 MHz). Digitized measurements of the aortic root were made in 2-dimensional parasternal long-axis views at end-diastole using the leading-edge technique at four aortic levels: the annulus, the sinuses of Valsalva, the supra-aortic ridge, and the proximal ascending aorta. The largest aortic diameter obtained in these views was described.

A twelve lead electrocardiogram (ECG) was performed to diagnose conduction or repolarization abnormalities.

Bone mineral density was measured at the lumbar spine (L1-L4) and the right femoral neck using a QDR 4500 densitometer (Hologic, Zaventem, Belgium). Bone mineral density was expressed as T- and Z-scores based on a normal reference population.

Standard renal ultrasound was performed for assessment of morphological abnormalities.

Hearing measurements were conducted according to standard audiometric methods (ISO 389) in a sound proof room. Air conduction thresholds were measured in dB hearing level at 0.25, 0.5, 1, 2, 4, and 8 kHz and bone conduction thresholds were measured in dB hearing level at 0.5, 1, 2, 4, and 8 kHz.

Data Analysis and Statistics

The analysis was restricted to the findings of the initial evaluation. No data of subsequent follow-up visits are presented. We recorded all newly identified diagnoses. In case of previous diagnoses, relevant changes in treatment were recorded. Case finding definitions are given in Table 3. For individual patients TS-related disease burden was calculated as the total number of diagnoses among the following principal stigmata (maximum score of fourteen): bicuspid aortic valve, coarctation of the aorta, elongation of the transverse aortic arch, dilation of the aorta, diabetes, glucose intolerance, hypertension, dyslipidaemia, osteoporosis or osteopenia, (subclinical) hypothyroidism, celiac disease, renal anomalies, liver enzyme disturbances and hearing loss requiring a hearing aid.[14] Results are expressed as mean (standard deviation), unless mentioned otherwise. In order to analyze which patients benefit most from the standardized

screening, we performed a subgroup analysis and compared the yield of diagnoses between patients with different times since initial diagnosis, patients with or without previous care and patients with monosomy 45,X versus patients with other karyotypes. For subgroup analyses and comparisons of means, Student's *t*-test or Chi-square tests were used where appropriate. We used Statistical Package for the Social Sciences version 16.0 (SPSS, Inc., Chicago, Illinois).

Data were collected under conditions of regular clinical care, with institutional review board approval obtained for the use of these data for scientific reasons.

TABLE 3
Case finding definitions

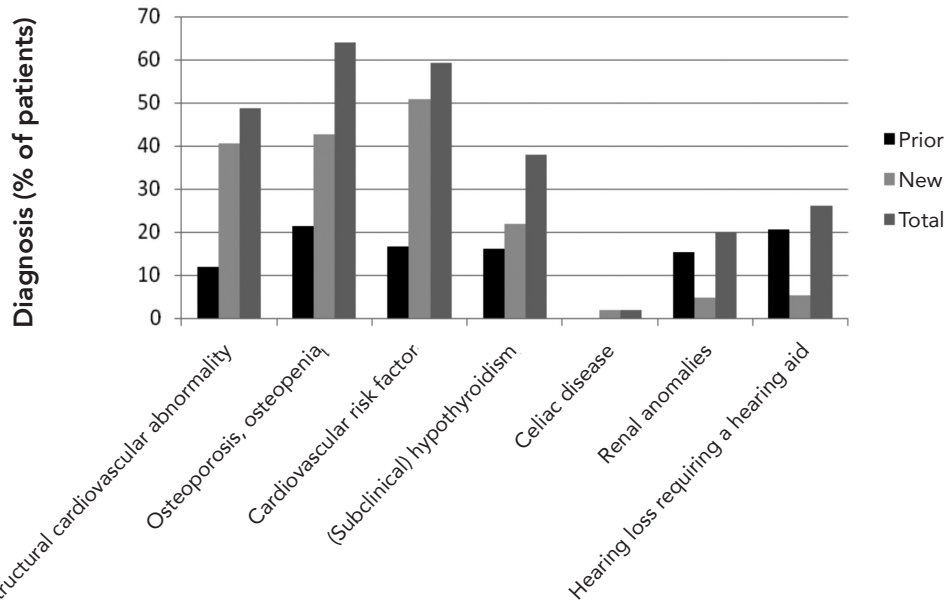
	Case finding definition
Congenital anomalies of the heart/aorta	Bicuspid aortic valve, coarctation of the aorta, elongation of the transverse aortic arch
Dilation of the aorta	Dilation of the ascending aorta based on BSA corrected values[17,40] and/or local or fusiform dilation of the descending aorta
Diabetes	Diabetes: random glucose > 11,1 mmol/l or Hba1c ≥ 6.5 mmol/l Impaired glucose tolerance: random glucose between 7.8 mmol/l and 11.1 mmol/l or HbA1c between 5.7% and 6.4%[41]
Hypertension	Use of antihypertensive medication or systolic blood pressure > 140 mmHg and/or a diastolic blood pressure > 90 mmHg
Dyslipidemia	Total cholesterol (TC) ≥ 5.50 mmol/l and/or high density lipoprotein cholesterol (HDL-C) ≤ 1.10 mmol/l and/or low density lipoprotein (LDL-C) ≥ 3.50 mmol/l and/or triglycerides (TG) ≥ 2.20 mmol/l (random lipid profiles)
Diminished bone mineral density	Osteoporosis: T-score below or equal to -2.5 SD. Osteopenia: T-score between the -1.0 and -2.5 SD
Hypothyroidism	Hypothyroidism: thyroid stimulating hormone above upper reference limit of 4.0 mE/l and free thyroxin below lower reference limit of 8.0 pmol/l Subclinical hypothyroidism: thyroid stimulating hormone > 4.0 mE/l and free thyroxin within normal range (8.0-22.0 pmol/l)
Structural anomalies of the kidney	Horse shoe kidney, duplication of the collecting system, agenesis and rotation as TS specific renal malformations
Liver enzyme elevation	Gamma-GT > 35 U/l and/or ASAT > 40 U/l

BSA=body surface area; gamma-GT=gamma-glutamyl transpeptidase; ASAT= aspartate-aminotransferase; TS=Turner syndrome

RESULTS

New diagnoses and total prevalence of morbidity are summarized in Table 4 and Figure 1. The disease burden in individual patients was 1.2±1.2 diagnoses before screening and 3.5±1.9 diagnoses after screening ($p<0.01$). The mean increase in disease burden was 2.3±1.5. When comparing patients with 45,X with those with other karyotypes, the former had a disease burden of 3.7±1.8 diagnoses after screening and the latter 3.3±2.0. Patients with 45,X had significantly more new diagnoses during screening when compared to patients with other karyotypes (2.6±1.5 vs 2.0±1.5). Age correlated positively with the disease burden before screening (r 0.485), after screening (r 0.689) and the number of new diagnosis during screening (r 0.403).

FIGURE 1
Frequencies of TS associated morbidities in 150 TS patients



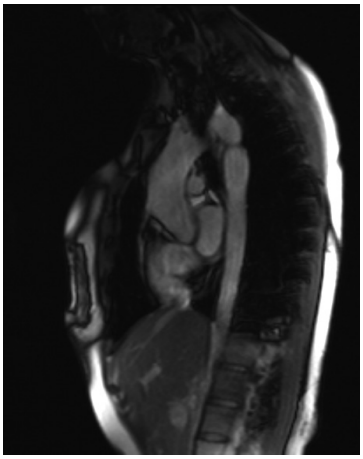
Anomalies of the Heart and Aorta

Prior to the current investigations, 81 patients (54%) had undergone cardiac ultrasound and 16 (10.7%) MRI. Eighteen had a previous diagnosis of structural cardiac and/or aortic anomalies. Five had undergone surgery. Re-evaluation yielded additional aortic anomalies in six. Screening of 132 patients without previously known cardiac or aortic anomalies consisted of MRI and cardiac ultrasound (n=100), MRI only (n=12), and ultrasound only (n=9). MRI results are pending in nine. In addition, no MRI was performed in six because of claustrophobia and in five because of recent cardiovascular screening including MRI. This yielded one or more congenital

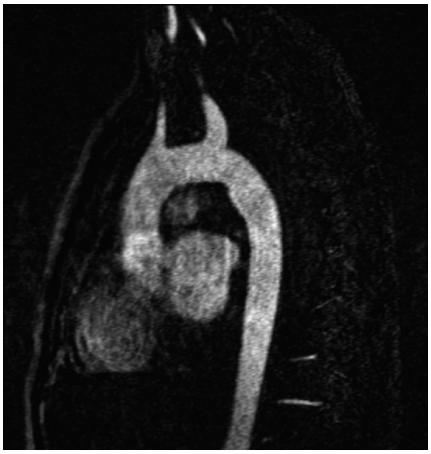
anomalies in 40 patients (30.3%). Fifteen of these patients had an additional dilation of the aorta. In 15 other patients aortic dilation was an isolated finding. In the group with new discovered cardiac and aortic abnormalities (n=55), 25 had previously undergone cardiac screening by cardiac ultrasound and MRI in one. In five patients a bicuspid aortic valve was missed by previous screening. All other new diagnoses were attributable to screening with an additional MRI: elongation, mild coarctation and dilation of the aorta. See Figure 2 for images.

FIGURE 2
MRI and MRA images of anomalies of the heart and aorta:
a. coarctation of the aorta
b. dilation of the aorta
c. bicuspid aortic valve
d. elongation of the transverse aortic arch
Published with permission of the 'Nederlands Tijdschrift voor Geneeskunde[44]

2a >



2b >



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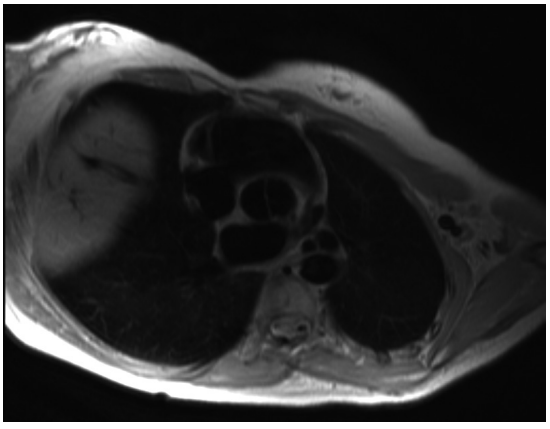

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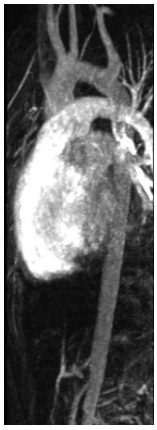

In women with karyotype 45,X significantly more structural cardiovascular abnormalities were diagnosed compared to women with other karyotypes (Supplemental data table A). Furthermore the time since the initial diagnosis of TS correlated positively with a higher number of newly diagnosed structural cardiovascular abnormalities. (Supplemental data table B)

An ECG was performed in 134 patients. All patients showed a sinus rhythm, including eight patients with sinustachycardia (>100 beats per minute) and one with sinusbradycardia (< 60 beats per minute). In 53.4% of the patients the ECG was normal, 37.3% showed repolarisation abnormalities (STT abnormalities), 17.2% QTc prolongation, 4.5% other conduction abnormalities, 3.0% left axis deviation and 0.7% right axis deviation.

Concerning the diastolic and systolic function we found that 2.0% of the women (n=3) had an impaired cardiac function: one patient had an ejection fraction of 43.8% and two patients had a dilated right ventricle: one due to an atrium septum defect and one due to a partial anomalous pulmonary venous return. We found one patient with left ventricular hypertrophy secondary to a bicuspid aortic valve with stenosis. [15]

Atherosclerosis and Risk Factors

Two patients had a history of myocardial infarction at 31 and 44 years of age. Seventeen patients (11.3%) were current smokers. Mean BMI was 26.2 ± 5.5 (52.7% had a BMI > 25 kg/m^2, 20.7% had a BMI > 30 kg/m^2).

Seven patients were previously diagnosed with diabetes (four insulin dependent) and two with impaired glucose intolerance. No new cases of diabetes were encountered.

Seventeen patients were known with hypertension, 10 of whom with insufficient blood pressure control. An additional 39 patients had an elevated blood pressure based on supine office sphygmanometric measurements at time of consultation. In the context of individual cardiovascular risk, medication was started in seven.

Eight patients were previously diagnosed with dyslipidaemia, three of them were currently taking a statin. In the others, lipid profile was abnormal in 36.6%.

Fertility and Hormone Replacement Therapy

In 36 patients spontaneous menarche had occurred. In the remaining 114 patients, puberty was induced by estrogen-therapy in 107. In three patients with 45,X fertility treatment with egg donation resulted in one successful pregnancy. Spontaneous pregnancies occurred in two patients with mosaicism. Two other patients with mosaicism attempted to become pregnant with fertility treatment, one including egg donation, but did not succeed. Five patients were known with a (partial) Y chromosome. Prophylactic gonadectomy took place except in two: one

refused, in the other the SRY gene was absent.

Gynecological ultrasound in 117 patients revealed a uterus subseptus in two, a uterine myoma in two, and a polyp in one.

At referral, 146 women were below 52 years of age, the median age for menopause. In this group, 23 women lacked any form of HRT, including 7 with a spontaneous menstrual cycle and 1 because of current fertility treatment.

The different regimes of the 124 (one above 52 years of age) patients already on HRT were: continuous combined (15.3%), sequential combined (54.0%), oral contraceptives (28.2%), and estrogen only (2.4%). Eight of them previously lacked HRT for a mean duration of six years. In 31 patients the regimen was intensified or otherwise adjusted because of decreased bone mineral density (n=5), metrorrhagia, dysmenorrhea, and/or the patient's preference to switch to a non-cyclic regimen (n=26).

Bone Mineral Density

Forty-six patients (30.7%) had a history of trauma associated fractures. Forty-three patients (28.7%) had previously undergone a bone mineral densitometry, yielding osteoporosis in 10 and osteopenia in 22. Ten of these patients were taking bisphosphonates. Based on 26 repeated bone mineral density measurements four patients needed adjustment of therapy. In 118 patients without a prior diagnosis, 111 bone mineral density measurements were performed. In patients with osteoporosis (n=8) or osteopenia (n=56) life style interventions enhancing bone health were advocated, *i.e.* adequate calcium intake, exposure to sunlight, and weight bearing exercise. Additionally, in eight HRT was started or intensified, in eight calcium/vitamin D supplementation was started, in two bisphosphonates were prescribed, and in two a combination of calcium/vitamin D supplementation and change in HRT.

Thyroid Disease

Seventeen patients had a history of hypothyroidism and were taking thyroid hormone. The dose needed adjustment in 11 patients. We found 33 cases of new subclinical hypothyroidism, prompting supplementation of thyroid hormone in five symptomatic cases.

TABLE 4
TS-associated morbidity (n=150)

	Prior diagnoses (n)	Changes in treatment (n)	New diagnoses (n)	Observed Frequency (%)	Frequency literature (%) [3-5,11,17,23,25-27,34,38,42,43]
Bicuspid aortic valve	12	0	10	19[i]	14-34
Aortic coarctation	2	0	6	9[i]	7-14
Bicuspid valve and coarctation	3	0	4[c]		
Elongation of the transverse aortic arch	0	0	27	18[i]	~50
Dilation of the aorta	4[a]	0	34	25[i]	3-42
Diabetes type 1	2	0	0	1	0.5-5
Diabetes type 2	5	0	0	3	3-25
Glucose intolerance	2	0	12	9	7-50
Hypertension	17	5	39[d]	37	24-50
Dyslipidemia	8	0	52	40	37-50
Osteoporosis	10		8	12[j]	
Osteopenia	22		56	52[j]	
Osteoporosis or osteopenia	32	4	64	64[j]	50-80
Hypothyroidism	17	11	0	11	16-30
Subclinical hypothyroidism	7	2	33[e]	27	
Celiac disease	0	0	3	2	4-6
Renal anomalies	23[b]	0	7[f]	20[k]	28-38
Liver enzyme disturbances	5	0	45[g]	33	36-80
Hearing loss requiring aid	31	1	8[h]	26[l]	27-44

[a] 3 in patients with a bicuspid aortic valve and/or coarctation
[b] horseshoe kidney (n=15), duplication of the collecting system (n=5), unilateral renal agenesis (n=2) and malrotation (n=1)
[c] including one recoarctation of the aorta
[d] 7 warranting medication
[e] 5 warranting medication
[f] horseshoe kidney (n=3), duplication of the collecting system (n=4)
[g] 46 patients had a mild elevation of gamma-GT and/or ASAT. ASAT was below twice the upper limit in all cases
[h] additional 35 with hearing impairment
[i] possible underestimation because of cardiac ultrasound in all, in 20 no MRI
[j] possible underestimation because of bone mineral density measurement in 146 patients
[k] possible underestimation because of renal ultrasound in 142
[l] possible underestimation because of ENT consult in 110 patients

Kidney Malformations

Seventy-two patients (48%) had previously undergone renal ultrasound showing anomalies in 23 patients. Three patients had undergone surgical interventions.
Of the remaining 78 patients, 70 underwent a renal ultrasound yielding anomalies in seven. Renal function was normal in all patients. Isolated erythrocyturia was found in two patients without structural anomalies or proteinuria. In eight patients renal ultrasound results are pending.

Hearing Loss

Thirty-one patients already had a hearing aid, including four patients with a bone anchored hearing aid. Of 119 without a prior diagnosis, 78 underwent audiology indicating significant hearing impairment in 43 patients, with the requirement of a hearing aid in eight.

Psychological problems

Twenty-three patients (mean age 38 yr) consulted the psychologist with the following reasons: need for support in accepting TS related limitations in daily life (70%), low self esteem (55%), non assertiveness (45%), lack of social support (25%), and infertility related emotional problems (10%).

Abnormalities regarding celiac disease and liver enzyme disturbances are specified in Table 4.

When comparing the women without previous medical care and the women with adult specialist care to the women with direct transition from pediatric care we found that a larger number of new diagnoses were found in the first group (Table 5).

TABLE 5
Impact of previous care on percentage new diagnosis

	Direct transition from pediatric care (mean age 19.0±1.5)	**Preceding adult specialist care** (mean age 31.3±9.1)	**No specialist care** (mean age 37.5±9.2)
Structural cardiovascular abnormalities	34.6%	31.7%	60.0%*
Osteoporosis or osteopenia	53.9%	32.9%*	53.3%
Cardiovascular risk factors	34.6%	51.9%	57.8%*
(Subclinical) Hypothyroidism	15.4%	15.2%	37.8%*

* Significant compared to women directly referred from pediatric care

DISCUSSION

We investigated the yield of comprehensive screening for TS associated morbidity in a large group of adult TS patients in a single Dutch centre. Multidisciplinary evaluation identified many patients with previously unknown diagnoses, including cardio-aortic anomalies (40.7%), hypertension (26.0%), dyslipidemia (34.7%), impaired glucose tolerance (8.0%), current or previous lack of HRT despite oestrogen deficiency (15.3%), osteoporosis (5.3%), osteopenia (37.3%), subclinical hypothyroidism (22.0%), and hearing loss requiring a hearing aid (5.3%). In individual patients on average two new major TS related diagnoses were found. Patients without previous specialist care benefited most from the screening.

In the majority of patients, 76% in our cohort, TS was diagnosed in childhood triggered by dysmorphic features, impaired growth, and/or delayed puberty. Ensuing growth hormone treatment and induction of puberty requires strict pediatric follow-up. Many patients are lost to follow-up after discharge from pediatric care.[8-11] In the present cohort, one third lacked any form of ongoing specialist care. Lack of care was most prevalent among older patients. It is unclear which patients are prone to lack ongoing medical care. It might be that socio-economic factors play a role. We have insufficient data to investigate this. However in the Netherlands health insurance is obligatory by law and entitles all patients to identical care.

The importance of a well organized transition from pediatric to adult care was stressed by the Turner Syndrome Consensus Study Group.[5] Besides a well organized transition, a multidisciplinary periodic screening of adult women with TS is advocated in international expert based guidelines, mainly because many disorders do not present until adult age.[5-7] Our current finding of a significant previously unrecognized morbidity demonstrates the effectiveness of such an approach.

Medical care in adult women with TS is mainly aimed at reduction of the ~3 fold increase in age related risk of cardiovascular mortality.[2] The necessary screening for structural cardiac and aortic anomalies had been omitted in half of our patients. Current screening revealed unknown cardiac and/or aortic anomalies in approximately 40% of the patients. Cardio-aortic anomalies and hypertension are assumed to increase the risk of aorta dilation and dissection.[4,16,17] The prevalence of aortic dilation increases with age, but dilation in TS can already be present in the second decade of life.[18,19] Aortic dissection occurs at relatively young age (third decade) with an age dependent incidence of 15-50 cases in 100.000 TS years compared to 6 cases in the normal population.[20] Considering the fact that aortic abnormalities are usually asymptomatic until complications occur, periodic screening of the aortic diameter appears to be justified. Cardiac follow-up can be performed using ultrasound and MRI, the latter having the advantage of superior imaging of the coarctation site and the distal aorta.[16,19] In a recent report Matura *et al.* advise to use aorta size index (aortic diameter/body surface area) instead of absolute aortic diameter because of limited availability of reference values for aortic diameters.[17] The

authors suggest strict follow-up in case of an aorta size index ≥ 2.0 cm/m² and evaluation for prophylactic intervention in case of an aorta size index of ≥ 2.5 cm/m² in combination with an absolute aorta diameter above 3.5 cm. In (induced) pregnancy, the risk of dissection is even higher and in one retrospective study the pregnancy related mortality is estimated at two percent.[21] Cardiovascular screening is strongly advised before and during each pregnancy. In fact, pregnancy is discouraged in patients with congenital cardiac and aortic anomalies, hypertension and/or an aortic size index (aortic diameter/body surface area) of ≥ 2.0 cm/m².[22]

Besides structural anomalies, TS in adults is associated with an unfavorable risk profile for cardiovascular disease. The prevalence of atherosclerosis is doubled.[23] In the present young population 37% had hypertension, 40% had an abnormal non-fasting lipid profile, 5% had diabetes, and half of the patients were overweight. Prevalence of diabetes type 2 in the TS population is increased compared with the general population. Focusing on the age group around 30 incidence numbers vary. Bakalov *et al.* found a prevalence of diabetes type 2 in 25% of a young TS population (age 35.4 ± 11.3 years, BMI 28.9 ± 7.7 kg/m²), while Landin-Wilhelmsen *et al.* reported a prevalence of 3% in a similar age group (age 33.7 ± 11 years, mean BMI 25.9 ± 5.0 kg/m²).[24,25] Probably this difference is related to differences in BMI. Nevertheless, the clearly unfavorable cardiovascular risk profile at young age suggests that patients benefit from screening and careful management. Intervention trials, however, are lacking.

The pathophysiology of the increased prevalence of autoimmune disease, especially in association with Xi(Xq) genotypes, remains unknown. We confirm a high prevalence (38%) of hypothyroidism, including subclinical cases. Previous studies have indicated that 16-30% of the TS patients have elevated TSH levels and that 27-45% of the TS patients have positive thyroid antibody titers.[26,27] Furthermore we found 3 patients with proven celiac disease. It has been shown that celiac disease is more common in the TS population with a prevalence of 4-6%.[28] Ongoing periodic screening for both hypothyroidism and celiac disease seems reasonable because incidences increase with age. Vigilance for additional less common TS-related autoimmune diseases is essential. [28-30]

Another important issue that needs ongoing medical attention, at least until normal age of menopause, is HRT. HRT in estrogen deficient women has a positive effect on bone mineral density, prevents vaginal atrophy and was shown to have a favorable impact on cardiovascular risk in TS.[31] In our cohort 15% lacked HRT, which is similar to observations in other countries.[9,32] In about half of the cases HRT was discontinued by the patient because of self reported side effects or unawareness of the reasons for HRT. In others HRT was never started. One reason could be that physicians might be unjustly reserved regarding HRT because of the relationship with breast cancer in *elderly* postmenopausal women.[33]

Two thirds of our TS patients were found to have a low bone mineral density correlated with older age and lack of HRT. TS is associated with an intrinsic structural bone defect, which is

worsened by estrogen deficiency.[34] Because short stature can cause false low areal bone mineral density, it is possible that the high frequency of osteoporosis observed in TS patients based on bone mineral density measurements is in fact an overestimation.[35] Nevertheless, a higher fracture risk in TS, varying from 24-32%, has been established warranting careful observation and treatment of bone health, including optimal HRT.[32,36]

During childhood, the vast majority of TS patients suffer from recurrent glue ear, otitis media and resulting conductive hearing loss, probably related to an abnormal Eustachian tube.[37] During adulthood, conductive and perceptive hearing loss frequently occurs, the latter often with either a mid- or high frequency dip. In our group more than 25% of patients suffered from significant hearing impairment warranting the use of a hearing aid, which is similar to previous reports. Hultcrantz et al found that 27% of TS patients needed a hearing aid (n=324; age range 4-68), and as many as 44% when focusing on those aged above 35 years.[38]

Besides physical health issues women with TS often deal with (neuro)psychological problems.[39] Our study confirms that many TS women experience difficulties in regards to acceptance of limitations related to TS and low self esteem. Remarkably, emotional problems related to infertility were mentioned by only a small minority.

In conclusion, standardized multidisciplinary evaluation of adult women with TS yields significant previously undiagnosed morbidity. TS patients are therefore likely to benefit from a careful transition from pediatric into adult medical care, consisting of a multidisciplinary service with standardized screening for TS associated morbidity. To what level our approach improves long-term morbidity, mortality, and health related quality of life remains to be investigated. The actual benefit should also be weighed against the costs of screening and the risk of medicalisation.

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DISCLOSURE STATEMENT

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Supplemental data

TABLE A
Impact of karyotype on percentage new diagnosis

	45,X (n=71)	Other karyotypes (n=79)
Structural cardiovascular abnormalities	56.3%	26.6%*
Osteoporosis or osteopenia	40.9%	44.3%
Cardiovascular risk factors	54.9%	46.8%
(Subclinical) Hypothyroidism	26.8%	17.7%

*Significant difference between both groups (significance stated as $p<0.05$)

TABLE B
Impact of time since initial diagnosis on percentage new diagnosis

	0-10 years (n=25) (age 23.6±7.2)	11-20 years (n=55) (age 25.9±6.6)	21-30 years (n=47) (age 34.5±8.5)	31-40 years (n=16) (age 44.0±6.4)	>41 years (n=6) (age 49.4±7.0)
Structural cardiovascular abn*	32.0%	34.6%	40.4%	62.5%	83.3%
Osteoporosis or osteopenia	52.0%	38.2%	46.8%	31.3%	33.3%
Cardiovascular risk factors	36.0%	45.5%	57.5%	68.8%	66.7%
(Subclinical) Hypothyroidism	8.0%	20.0%	29.8%	25.0%	33.3%

* Significant difference for structural cardiovascular abnormalities. Total number of patients does not equal 150, due to one missing value.

TABLE C
Impact of age at initial diagnosis on percentage new diagnosis

	0-10 years (n=68) (age 26.4±8.5)	11-20 years (n=68) (age 33.5±9.8)	21-30 years (n=10) (age 43.5±8.8)	31-40 years (n=3) (age 42.0±9.2)
Structural cardiovascular abnormalities	44.1%	39.7%	40%	0%
Osteoporosis or osteopenia	42.7%	41.2%	50%	33%
Cardiovascular risk factors*	38.2%	55.9%	90%	100%
(Subclinical) Hypothyroidism	27.9%	20.6%	0%	0%

* Significant difference for cardiovascular risk factors. Total number of patients does not equal 150, due to one missing value.

Chapter 3

Buccal cell FISH and blood PCR-Y detect high rates of X chromosomal mosaicism and Y chromosomal derivatives in patients with Turner syndrome

Kim Freriks[1], Henri JLM Timmers[1], Romana T Netea-Maier[1], Catharina CM Beerendonk[2], Barto J Otten[3], Janiëlle AEM van Alfen-van der Velden[3], Maaike AF Traas[2*], Hanneke Mieloo[4], Guillaume WHJFL van de Zande[4], Lies H Hoefsloot[4], Ad RMM Hermus[1], Dominique FCM Smeets[4]

Author Affiliations

Departments of [1]Internal medicine, division of Endocrinology, [2]Obstetrics and Gynecology, [3]Pediatric Endocrinology and [4]Human Genetics of the Radboud university medical center, Nijmegen

*Present address: Gelre Hospital Apeldoorn, Department of Obstetrics and Gynecology, Apeldoorn

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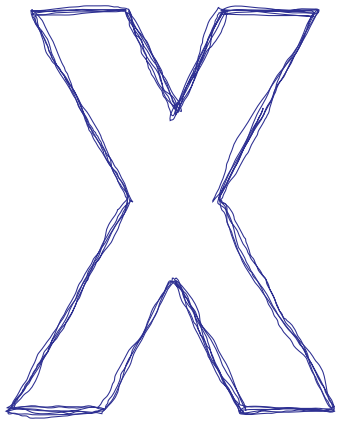


ABSTRACT

Turner syndrome (TS) is the result of (partial) X chromosome monosomy. In general, the diagnosis is based on karyotyping of 30 blood lymphocytes. This technique, however, does not rule out tissue mosaicism or low grade mosaicism in the blood. Because of the associated risk of gonadoblastoma, mosaicism is especially important in case this involves a Y chromosome. We investigated different approaches to improve the detection of mosaicisms in 162 adult women with TS (mean age 29.9±10.3). Standard karyotyping identified 75 patients (46.3%) with a non-mosaic monosomy 45,X. Of these 75 patients, 63 underwent additional investigations including FISH on buccal cells with X- and Y-specific probes and PCR-Y on blood. FISH analysis of buccal cells revealed a mosaicism in 19 of the 63 patients (30.2%). In five patients the additional cell lines contained a (derivative) Y chromosome. With sensitive real-time PCR we confirmed the presence of this Y chromosome in blood in three of the five cases. Although Y chromosome material was established in ovarian tissue in two patients, no gonadoblastoma was found. Our results confirm the notion that TS patients with 45,X on conventional karyotyping often have tissue specific mosaicisms, some of which include a Y chromosome. Although further investigations are needed to estimate the risk of gonadoblastoma in patients with Y chromosome material in buccal cells, we conclude that FISH or real-time PCR on buccal cells should be considered in TS patients with 45,X on standard karyotyping.

INTRODUCTION

Turner syndrome (TS) is the result of (partial) X chromosome monosomy and one of the most common chromosomal disorders with an incidence of approximately 1 in 2000 to 2500 live born girls.[1] Besides short stature and gonadal dysgenesis, TS is associated with a wide range of abnormalities affecting nearly every organ system. The phenotype is highly variable which is partly explained by differences in karyotype. Furthermore the presence of tissue mosaicism *i.e.* genetic variability among different body tissues can explain phenotypic variation. Tissue mosaicisms may also involve tissue-specific presence of a (derivative) Y chromosome.

When Y chromosomal material is encountered upon karyotyping in TS, usually a prophylactic gonadectomy is recommended because of the increased risk of gonadoblastoma, a benign neoplasm of the dysgenetic gonad.[2,3] Gonadoblastoma in TS is assumed to develop early in life and may develop into malignant tumors such as dysgerminoma.[2,4] The incidence of gonadoblastoma in TS patients carrying a Y chromosome is estimated at 12%, although higher incidences up to 30% have been documented.[2-4] However, gonadoblastoma is also reported in patients with TS lacking a Y chromosome on conventional cytological analysis.[4-6] In these studies Y chromosomal material was revealed only after applying additional cytogenetic and/or molecular techniques.

Diagnosis of TS is generally based on cytogenetic analysis of 30 cultured blood lymphocytes, by which a 10% chromosomal mosaicism can be identified with a 95% confidence interval.[3,7,8] According to this approach, about half of the patients appear to have a pure monosomy 45,X, 30 to 40 percent have a mosaicism and yet another 5-15% have a structurally abnormal X chromosome. When using conventional karyotyping, approximately 5-6% of the TS patients are found to carry a complete or derivative Y chromosome.[10] Standard karyotyping of merely blood lymphocytes in patients with TS is probably not sufficient in a large number of cases.[4-7] Firstly, it does not rule out tissue mosaicism. The idea of tissue mosaicism is established by the assumption that for a fetus to survive to term it is necessary to have a second sex chromosome. This implies that most, if not all, live born girls with apparent 45,X have additional cell lines in other tissues. This assumption is strengthened by the fact that complete 45,X is more common in fetuses who do not survive to term than in live born girls with TS.[11,12] Several studies confirm the presence of tissue mosaicism in live born patients with TS.[13-15] Secondly, a low grade mosaicism (<10%) in blood lymphocytes can easily be missed during karyotyping. For that reason it is advocated to use additional techniques.[4-7,16-19] A meta-analysis of 541 patients showed that, when using polymerase chain reaction (PCR) techniques on blood samples of patients with apparent 45,X, Y chromosomal sequences can be found in approximately 5%.[2] Alternatively, fluorescent in situ hybridization (FISH) with X- and Y- specific probes is used to find mosaicisms of the sex chromosomes or derivatives thereof.[5,16,19]

The clinical guideline for TS advises to perform conventional karyotyping of blood lymphocytes in patients with clinical suspicion of TS.[3] At the moment, additional FISH is recommended only when conventional karyotyping reveals a marker chromosome or when clinical virilization is present. Another, more recent, guideline advises additional FISH with X- and Y-specific probes in all patients with 45,X.[7] Although both guidelines suggest cytogenetic studies of a second cell line in case of clinical suspicion of TS and a normal female karyotype, they do not comment upon the investigation of other tissues besides lymphocytes in patients with proven TS, without virilization or marker chromosomes.

Considering the high percentage of (tissue) mosaicism in TS and the possible clinical impact of a Y chromosome or derivatives thereof, we set out this study to determine the value of additional genetic studies (FISH, conventional and real-time PCR) and the inclusion of a second, easy accessible tissue (buccal cells) in search for mosaicisms in TS patients with apparent complete 45,X karyotype on standard lymphocyte karyotyping.

PATIENTS AND METHODS

Patients

Between 2005 and 2009, 162 adult women (mean age 29.9 ± 10.3) with a previously established diagnosis of TS were evaluated at our multidisciplinary facility. Standard postnatal karyotyping of 30 blood lymphocytes had identified 75 patients (46.3%) with 45,X in all examined cells (Table 1).

TABLE 1
Conventional lymphocyte karyotype (n=162)

Karyotype	Number (%)
45,X	75 (46.3)
45,X/46,XY or derivative Y	6 (3.7)
45,X/46,XX	17 (10.5)
45,X/47,XXX	5 (3.1)
45,X/46,Xi(Xq)	23 (14.2)
45,X/46,X,del(X)	9 (5.6)
45,X/46,X,r(X)	7 (4.3)
46,X,i(Xq)	7 (4.3)
46,X,del(X)	5 (3.1)
Others[a]	8 (4.9)

[a] Four patients with a mosaicism containing more than two cell lines, two patients with a marker chromosome and two patients with a translocation with chromosome 13

In the other 87 patients (53.7%) we found other karyotypes including mosaicisms and structural X chromosomal anomalies. In six patients (3.7%) the presence of a (derivative) Y chromosome was encountered. Four of the six had previously undergone bilateral gonadectomy. Histological evaluations yielded no malignancies in three patients and bilateral gonadoblastoma and unilateral dysgerminoma in one patient. In two patients, gonadectomy was omitted; one patient refused surgery and in the other patient surgery was not performed because of absence of the part of the Y chromosome that is typically associated with gonadoblastoma. This patient had karyotype 45,X/46,X,i(Yq), missing the entire short arm of the Y chromosome, where most gonadoblastoma associated genes are located.[18]

Of the 75 patients with 45,X, 63 (mean age 29.0 ± 10.4) agreed to participate in additional cytogenetic investigations. None of these patients showed signs of virilization.

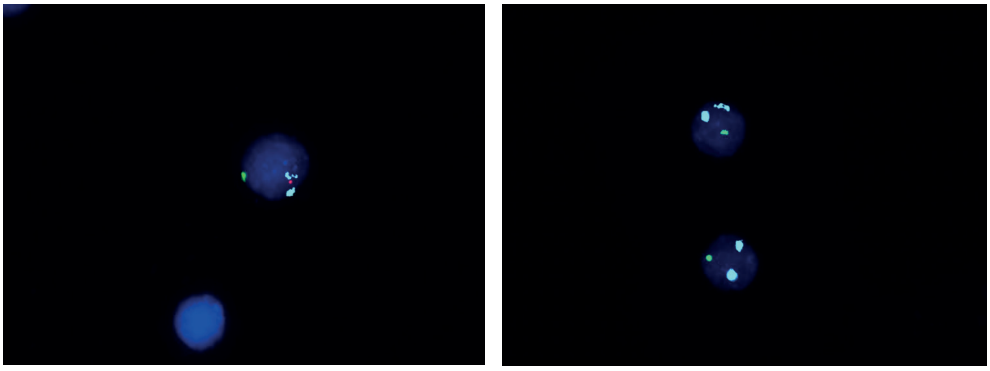
Methods

FISH analysis of buccal cells

In all patients, buccal smears were collected from the inner lining of the cheeks. Buccal cells were stored in standard culture tissue medium and subsequently centrifuged. The samples were incubated at 37 degrees Celsius with 0.075 M kaliumchloride for 10 minutes followed by prefixation with 1 ml fixative (3:1 methanol/acetic acid). Fixation was repeated three times. The cell suspension was applied on a slide in 2 drops, 15 microliter each. FISH was performed with X- and Y-specific centromeric probes (CEP X and CEP Y; Vysis, Abbott, USA) and an additional probe of the SRY-region (LSI SRY, Vysis, Abbott, USA). One female and one male sample were used to define the intensity of the signals. As an example of FISH on buccal cells with X- and Y-probes see figure 1.

FIGURE 1

A. FISH on buccal cell with one Y chromosome specific signal (red), one X chromosome specific signal (green) and two chromosome 18 signals (light blue).
B. FISH on buccal cells with one X chromosome specific signal (green) and two chromosome 18 signals (light blue).



In patients with Y chromosomal signals in buccal cells additional FISH on interphase blood lymphocytes was performed and/or the number of cells for conventional karyotyping was extended. When possible we performed standard PCR-Y and/or FISH on ovarian tissue.

PCR-Y of blood lymphocytes

We were able to collect 20cc EDTA –ethylene diamine tetraacetic acid- blood for DNA extraction in 56 of the 63 patients with apparent 45,X. To detect Y chromosome sequences DNA was amplified after extraction in two separate reactions according to the protocol described by Simoni *et al.*[20] After amplification the resulting fragments were separated on a 1.4% agarose gel,

and photographed. This method not only detects the presence of the SRY gene on the Yp-arm, but also sequences on Yq euchromatin (sY84, sY86, sY127, sY134, sY254 and sY255).

Besides this conventional PCR to detect Y chromosomal sequences, a more sensitive real-time PCR protocol was used. This protocol is in essence the same as it is used for the detection of fetal Y chromosomal sequences in the plasma of pregnant women, as described by Scheffer *et al.*[21] In short, with real time PCR with probes for the SRY gene and a separate assay for the multicopy DYS14 marker the concentration of Y chromosome specific sequences was measured compared to a control.

Data were collected under conditions of regular clinical care, with ethical committee approval obtained for the use of those data for scientific purposes.

RESULTS

FISH analysis of buccal cells

In patients with complete monosomy X (45,X) on conventional lymphocyte karyotyping (n=63), FISH analysis of buccal smears revealed second or third cell lines in 19 patients (30.2%) (Figure 2). In five patients (7.9%), the additional cell lines included the presence of a (derivative) Y chromosome. In 4 of the 5 Y positive cases, prophylactic gonadectomy was performed. FISH analysis of ovarian tissue was performed in two patients, revealing Y positive signals in both cases (Table 2). Histological evaluation of the ovarian tissue was normal in all cases. In one patient (Table 2, patient 5) we performed no gonadectomy because of absence of the gonadoblastoma associated region of the Y chromosome.

In the 5 cases with Y positive buccal cells, FISH was also performed on interphase blood lymphocytes, revealing Y chromosomal material in one patient only (Table 2, patient 5). Repeating the chromosome studies on cultured lymphocytes in this patient revealed a marker chromosome (derivative Y) in 6% of the cells.

FIGURE 2
Karyotype of buccal cells (as interpreted from the FISH signals) in patients with 45,X on conventional karyotyping

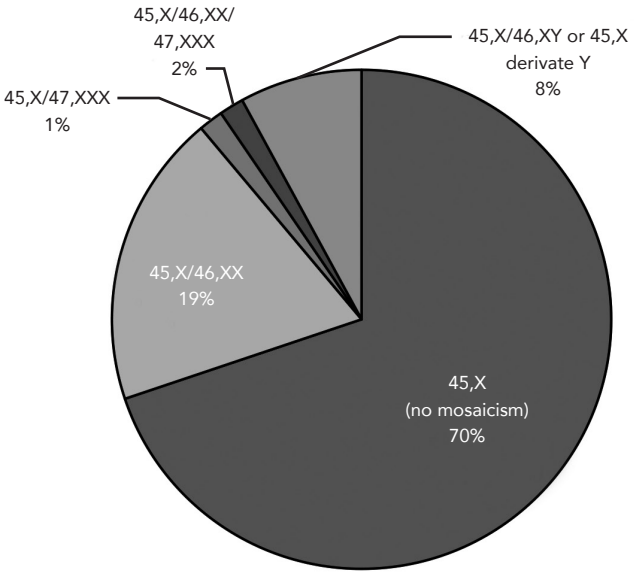


TABLE 2
Cytogenetic and histological results in five patients with (derivative) Y chromosome in buccal smear

Age (years)	Karyotype blood lymphocytes	FISH blood lymphocytes	PCR-Y blood lymphocytes	Real-time PCR SRY/DYS14 blood lymphocytes	FISH buccal cells	FISH ovarian tissue	PCR-Y ovarian tissue	Histology ovarian tissue
38	45,X[30]	45,X[100]	neg	neg/neg	45,X[95]/46,XY[5]	45,X[94]/46,XY[6]	Neg	No gonadoblastoma
21	45,X[30]	nt	neg	pos/pos	45,X[77]/46,XY[23]	45,X[172]/46,XY[28]	Pos	No gonadoblastoma
33	45,X[30]	45,X[100]	neg	neg/neg	45,X [73]/46,XY[27]	Nt	Nt	No gonadoblastoma[a]
36	45,X[30]	45,X[100]	neg	pos/pos	45,X[82]/46,XY[18]	Nt	Nt	No gonadoblastoma[b]
29	45,X[c]	45,X[168]/46, XY[32]	neg	neg/pos	45,X[58]/X, der Y[42]	Nt	Nt	No gonadectomy

Nt=not tested [a]Performed in other centre [b]No material available for cytogenetic analysis [c]Performed elsewhere. Repeated karyotyping in our hospital because of findings in buccal cells, revealed 45,X[61]/46,X,+mar[4]

PCR-Y analysis of blood lymphocytes

Conventional PCR-Y analysis of blood lymphocytes was negative in all 56 cases, including those in whom FISH in buccal cells had revealed the presence of a Y chromosome (Table 2). Of the four patients who underwent prophylactic gonadectomy prompted by buccal FISH results, PCR-Y was also performed on ovarian cells in two, yielding one positive result.

Real-time PCR revealed the presence of SRY and DYS14 sequences in blood in 2 of the 5 patients with a Y chromosome positive FISH result. In one patient, only a low concentration of the DYS14 sequences could be detected (Table 2). This was expected since the Y chromosome was incomplete (marker Y). All other patients had a negative real-time PCR result.

DISCUSSION

In this study we show that in patients with a full-blown monosomy 45,X on conventional karyotyping of blood lymphocytes, FISH analysis of buccal cells with X and Y chromosome specific probes reveals tissue-specific sex-chromosomal mosaicism in 30 percent of the cases. This includes the detection of a (derivative) Y chromosome in 8% of the patients. Regular PCR-Y using 7 different Y-specific markers failed to identify Y chromosomal derivatives in blood samples, including those from patients whose buccal cells were Y positive. However, a much more sensitive real-time PCR could confirm the presence of Y chromosomal sequences in the blood in three of the five Y positive cases. Our results imply that if a full blown monosomy 45,X is detected after conventional karyotyping of blood lymphocytes, a second tissue like easy accessible buccal cells should be examined for full appreciation of the genotype.

Karyotyping blood cells versus buccal cell FISH

The main limitation of karyotyping blood lymphocytes only, is the fact that low-grade mosaicisms and tissue specific mosaicisms, including those with a Y chromosome, are likely to be missed.[13-15] Conventional karyotyping identifies Y chromosomal mosaicism in approximately 5-6% of TS patients.[9,10] In our group, 3.6% of the patients had a Y chromosome or derivate thereof on conventional karyotyping and FISH on buccal cells doubled the number of patients carrying Y chromosomal material. A previous study drew the attention to superior results of FISH on buccal cells in a group of 12 patients with Y chromosome material found by conventional karyotyping. Concerning the level of Y positive cells, FISH on buccal cells showed significant differences compared with FISH on lymphocytes in half of the cases, while gonadal karyotypes were better reflected by buccal FISH results than by regular lymphocyte karyotyping.[15] Another study of 21 patients with apparent 45,X and negative PCR-Y (DYZ and DYZ3) reported that FISH on buccal cells revealed an extra cell line in 6 patients. Only 3 of these additional cell lines

could be confirmed by FISH on blood lymphocytes.[13] The additional value of performing FISH in patients with an already established chromosomal mosaicism or structural abnormality on regular karyotyping is limited. We performed FISH on buccal cells of 13 patients with TS and various X chromosomal mosaicisms and found that all mosaicisms were confirmed in buccal cells, although proportions of the different cell lines varied between lymphocytes and buccal cells and additional cell lines were encountered (data not shown). No additional Y chromosomal signals were detected which in fact was what we anticipated.

Molecular analysis (PCR-Y)

Tissue-specific and low-grade mosaicisms, in particular of Y chromosomal sequences, can also be detected by molecular techniques like a PCR-Y. One study showed that in 35% of 20 patients with apparent 45,X, PCR-Y (SRY and DYZ3) could reveal Y chromosome sequences in at least one tissue. PCR-Y results varied between different tissues, *i.e.* blood, buccal cells and hair roots.[14] A meta-analysis of 541 patients with TS who did not show Y chromosomal material on regular cytogenetic analysis, revealed that 5% had a Y chromosome containing cell line when using PCR-Y on lymphocyte extracted DNA.[2] It is therefore tempting to apply such a PCR-Y on DNA extracted from blood cells in order to detect low frequencies of a Y chromosome. However, in our cohort regular PCR-Y on blood DNA did not identify any additional patients carrying Y chromosomal material, which has also been reported by others.[13,22-24] The most probable explanation is that the standard PCR-Y that we used has a too low sensitivity, since we know it is difficult to detect mosaicisms below 10%. Even in one patient with a marker chromosome, the standard PCR-Y was not able to detect the proven low grade Y chromosomal mosaicism. Therefore, we decided to set up another, much more sensitive, real time PCR technique to analyze the blood DNA and in this way we were able to detect Y chromosome specific sequences in three of the five patients with Y chromosome material earlier found with FISH on buccal cells, while no additional patients were encountered using this technique. These results implicate that this appears to be a very promising technique to use on other tissues as well and it is perfectly conceivable that applying real-time PCR on DNA extracted from buccal cells is even more sensitive to detect a (derivative) Y chromosome than FISH, since in blood lymphocytes the real-time PCR revealed Y chromosomal sequences while FISH did not (Table 2). Our negative real time PCR-Y results in the remaining two patients with a Y chromosome detected by FISH in buccal cells, is most likely caused by a complete absence of Y chromosome material in the blood or an extremely low frequency. This assumption is strengthened by the fact that extended FISH studies on blood lymphocytes, metaphases as well as interphases, also failed to detect Y chromosomal sequences in all but one patient (Table 2). Further improving the search for Y chromosome derivatives in blood by performing a nested PCR does not seem to be a very promising approach regarding the high rate of false positive results.[25,26]

Y chromosomal mosaicism and gonadoblastoma

In several studies positive PCR-Y in patients with apparent 45,X was associated with gonadoblastoma.[4-6,14,18] Out of 161 patients with Y negative results on conventional karyotyping, four had a (derivative) Y chromosome when using a PCR with SRY and DYZ3 markers, while two of them had a gonadoblastoma.[4] In another study, 107 TS patients with apparent 45,X were investigated and 2 gonadoblastomas were detected in 10 PCR-Y (SRY, ZFY, Yc, Yq, PABY markers) positive patients.[6] Furthermore, in a group of 78 non-selected TS patients it was shown that 18.5% had a positive PCR-Y (SRY, TSPY and DYZ3), while 2 of them had a bilateral gonadoblastoma.[18] Others combined FISH and PCR-Y and in two out of 50 patients lacking a Y chromosome on conventional karyotyping positive results and gonadoblastoma were found.[5]

Regardless the technique used, Y chromosomal material in patients with dysgenetic gonads is associated with a risk of gonadoblastoma. It is generally accepted that the finding of a Y chromosome on conventional karyotyping urges for gonadectomy to prevent the development of a gonadoblastoma and the associated malignant dysgerminoma.[2] The need for gonadectomy when a Y chromosome is found in another tissue or with another technique than conventional karyotyping on blood lymphocytes is not yet fully established. The incidence of gonadoblastoma in patients with TS and a (derivative) Y chromosome in blood is estimated at 12%.[2] However, the risk of developing gonadoblastoma and the associated dysgerminoma is uncertain since not all patients with a Y chromosome revealed by other techniques than conventional karyotyping consistently underwent gonadectomy. Furthermore, gonadectomy is performed mostly prophylactically *i.e.* before a gonadoblastoma and especially dysgerminoma actually develops in the dysgenetic gonad. We performed gonadectomy in 4 patients with apparent monosomy 45,X and Y chromosomal material present in buccal cells, and found no gonadoblastoma. Cytogenetic analysis of the ovarian tissue was performed in two and revealed Y chromosome material in both samples. This suggests that the streak ovaries were indeed prone to developing gonadoblastoma. Nevertheless, the beneficial effects of gonadectomy should be weighed against potential negative consequences. Although a high percentage of women with TS are infertile and already need lifelong hormone replacement therapy, gonadectomy induces early oestrogen deficiency. In this context it is not established whether gonadectomy is necessary when Y chromosomal material is found with a more sensitive technique than conventional karyotyping or identified in another tissue besides blood lymphocytes, including buccal mucosa. However, increased vigilance towards the development of gonadoblastoma would at least warrant careful gynecological follow-up, for example with (transvaginal) ultrasound of the gonads.

In conclusion our results confirm that TS patients with apparent 45,X on conventional karyotyping of blood lymphocytes often have various tissue specific mosaicisms, which may include the presence of a (derivative) Y chromosome. These mosaicisms can rather quickly be detected with high sensitivity by applying FISH with X- and Y-specific probes on non-invasively

obtainable buccal cells. The results of this approach seem to correlate well with the karyotype of ovarian tissue and could as such serve as a predictor of the risk of developing gonadoblastoma. Buccal FISH studies, or alternatively real-time PCR on DNA extracted form buccal cells, should therefore be considered as an additional routine investigation in TS patients with a 45,X karyotype in blood lymphocytes. Further investigations are needed to estimate the exact risk of developing gonadoblastoma in patients with Y chromosomal material in buccal cells.

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Chapter 4

Long-term effects of previous oxandrolone treatment in adult women with Turner syndrome

Kim Freriks[1], Theo CJ Sas[2,3], Maaike AF Traas[4], Romana T Netea-Maier[1], Martin den Heijer[1], Ad RMM Hermus[1], Jan M Wit[5], Janiëlle AEM van Alfen-van der Velden[6], Barto J Otten[6], Sabine MPF de Muinck Keizer-Schrama[3], Martin Gotthardt[7], Philippe H Dejonckere[8], Gladys RJ Zandwijken[9], Leonie A Menke[5,10], Henri JLM Timmers[1]

Author affiliations

[1]Radboud university medical center, Nijmegen, Department of Internal Medicine, division of Endocrinology, [2]Albert Schweitzer Hospital, Dordrecht, Department of Pediatrics, [3]Erasmus Medical Centre, Rotterdam, Department of Pediatrics, [4]Gelre Hospital Apeldoorn, Department of Obstetrics and Gynecology, [5]Leiden University Medical Center, Leiden, Department of Pediatrics, [6]Radboud university medical center, Nijmegen, Department of Pediatrics, [7]Radboud university medical center, Nijmegen, Department of Nuclear Medicine, Nijmegen, [8]University Medical Centre Utrecht, Department of Phoniatrics, [9]Dutch Growth Research Foundation, Rotterdam, [10]Haga Hospital, Den Haag, Department of Pediatrics

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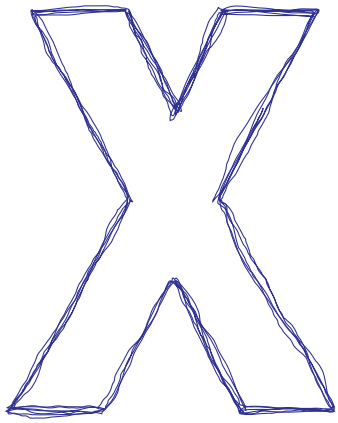


ABSTRACT

Objective

Short stature is a prominent feature of Turner syndrome (TS), which is partially overcome by growth hormone (GH) treatment. We have previously reported the results of a trial on the effect of oxandrolone (Ox) in girls with TS. Ox in a dose of 0.03 mg/kg/day (Ox 0.03) significantly increased adult height gain, whereas Ox 0.06 did not, at the cost of deceleration of breast development and mild virilization. The aim of this follow-up study in adult participants of the pediatric trial was to investigate the long-term effects of previous Ox treatment.

Design and methods

During the previous randomized controlled trial, 133 girls were treated with GH combined with either placebo (Pl), Ox 0.03, or Ox 0.06 from 8 years of age and estrogen from 12 years. Sixty-eight women (Pl n=23, Ox 0.03 n=27, Ox 0.06 n=18) participated in the double-blind follow-up study (mean age 24.0 years, mean time since stopping GH 8.7 years, mean time of Ox/Pl use 4.9 years). We assessed height, body proportions, breast size, virilization and body composition.

Results

Height gain (final minus predicted adult height) was maintained at follow-up (Ox 0.03 10.2±4.9cm, Ox 0.06 9.7±4.4cm vs. Pl 8.0±4.6cm). Breast size, Tanner breast stage and body composition were not different between groups. Ox-treated women reported more subjective virilization and had a lower voice frequency.

Conclusion

Ox 0.03 mg/kg/day has a beneficial effect on adult height gain in TS patients. Despite previously reported deceleration of breast development during Ox 0.03 treatment, adult breast size is not affected. Mild virilization persists in only a small minority of the patients. The long-term evaluation indicates that Ox 0.03 treatment is effective and safe.

INTRODUCTION

Turner syndrome (TS) has an incidence of approximately 1 in 2000 live born girls and is the result of complete or partial absence of one X chromosome.[1] Short stature, together with ovarian failure and dysmorphic features, is one of the most prominent aspects. Without growth hormone (GH) treatment, adult women with TS are on average 20 cm shorter than women without TS.[2] Although TS is not associated with GH deficiency, supraphysiological GH doses increase adult height by 5-12 cm.[3-5] Differences in efficacy of GH treatment can be explained by age at initiation and duration of therapy, compliance, estrogen therapy regimen and several, partly unknown, genetic factors.[4,6,7]

Oxandrolone (Ox) is a synthetic non-aromatizable anabolic steroid with weak virilizing effects compared with testosterone. Ox has been shown to increase adult height and growth

velocity in TS.[8-14] Some of the initial studies used Ox dosages of 0.1 mg/kg/day or higher which were subsequently lowered due to virilization and early bone maturation.[9-11]

We have previously reported the results of a randomized placebo-controlled double blind trial on the effect of the addition of Ox in lower dosages to standard GH and estrogen treatment in girls with TS.[12] Intention to treat analysis showed that compared to GH+placebo (Pl), GH+Ox in a dose of 0.03 mg/kg/day (Ox 0.03) increased adult height gain –final minus predicted adult height- (9.5 vs. 7.2cm in Pl) at the cost of mild deceleration of breast development. At a higher dose of 0.06 mg/kg/day (Ox 0.06), no significant increase in height gain was found (8.3 vs. 7.2cm in Pl), probably due to faster bone maturation and premature discontinuation of Ox because of virilizing side effects. In the Ox 0.06 group significantly more girls reported subjective virilization. Findings in both Ox groups included a decrease in fat mass, an increase in muscle mass and lowering of the voice pitch, which were initially in the higher range, to normal voice frequencies in most cases.[15,16] To assess whether these effects were transient or definitive, we conducted this follow-up study. We investigated the long term side effects of Ox in the two different dosages (0.03 and 0.06 mg/kg/day) compared to Pl in GH and estrogen treated TS women several years after discontinuation of GH and Ox therapy. We examined height, body proportion and composition, virilization (including voice frequency) and breast development.

SUBJECTS AND METHODS

Participants and previous treatment

Participants of the initial pediatric randomized double-blind placebo controlled trial were recruited between 1991 and 2003 in ten pediatric endocrine centers in the Netherlands.[12] Inclusion criteria were a TS compatible karyotype (except for cytogenetical evidence of Y-chromosomal material), a calendar age between 2.00 and 15.99 years, and a bone age younger than 12.00 years. Exclusion criteria were growth failure due to other causes, use of medication that could interfere with study medication and previous GH or sex steroid therapy.

In total 133 girls were included and assigned to age group 1 (2.00-7.99), 2 (8.00-11.99), or 3 (12.00-15.99 years). After stratification for calendar age and height standard deviation score (SDS), they were randomized and blindly assigned to receive Ox 0.03, Ox 0.06 or Pl orally at bedtime after reaching the age of 8 years. Furthermore the girls were treated with GH (1.33 mg/m^2/day) from baseline and, when spontaneous puberty was absent, with estrogen from the age of 12 years. 17-ß-Estradiol was prescribed in age groups 1 and 2 and ethinyl-estradiol in age group 3 (5 and 0.05 μg/kg/day orally, increasing to 10 and 0.1 μg/kg/day after two years, respectively). When ethinyl-estradiol became unavailable in 2002, 17-ß-estradiol was also prescribed in age group 3. For more detailed participant information, randomization and treatment modalities see Menke *et al.*[12]

All participants in the original trial were eligible for inclusion in the current follow-up study, provided that they had been off GH treatment at least six months prior to the investigation. Additional exclusion criteria for the follow-up study were participation in another drug study within two months of entry, malignancy, severely disabling disease, psychiatric illness and current pregnancy or fertility treatment.

Investigations

Participants and investigators were blinded to the treatment arms, so data were collected in a double-blind fashion. Participants were invited for a one-day visit in the out-patient department of the Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands. We used a questionnaire, including an extensive medical history and questions about possible side effects, which was completed at home. All examinations were performed by a single researcher (KF), except for the gynecological examinations, which were done by a gynecologist (MAFT).

Height

Height was measured using an electronic stadiometer. Adult height gain was calculated as the mean of three height measurements minus the predicted adult height. Predicted adult height was calculated using the modified projected adult height (mPAH): 146.95 + 6.37 * (-0.2 + 0.836 * height SDS at baseline).[12,17] Height SDS for untreated Northern European girls with TS at adult age was calculated by: (adult height – 146.95)/ 6.37.[2]

Body composition and proportions

Sitting height was measured using an electronic stadiometer and a sitting height table. Subischial leg length was calculated by height minus sitting height. Biacromial and biiliacal distance and hand and foot length were measured using a Harpenden anthropometer. Skinfolds were measured with a Harpenden skinfold caliper at the biceps, triceps, subscapular and suprailiacal level. We measured the arm span and circumferences of the head, left arm, waist, hip and thigh with a conventional measuring tape. All measurements were performed according to Gerver *et al.*[18] Body weight was measured on a scale, with the patient in underwear and barefoot. Body mass index (BMI) was calculated by dividing weight (kg) by squared height (m). Upper arm muscle area (UAMA) was calculated according to Frisancho: UAMA = (mid upper arm circumference - (π x triceps skinfold thickness))^2/4π.[19] Total body fat percentage was calculated using the sum of four skinfolds and *c* and *m* values according to Durnin and Womersley.[20] Total body fat percentage was calculated by (4.95/(c – (m* log sum of skinfolds)))-4.5)*100. A total body dual-energy X-ray absorptiometry (DXA) (Hologic QDR 4500 densitometer, Zaventem, Belgie) was performed to estimate body composition including percentages of total body fat

and lean body mass. To asses 'android' fat percentage, a region of interest spanning from the pelvis cut (lower boundary) to a line defined as 20% of the distance between the pelvis and neck cuts (upper boundary) was used, as defined by the manufacturer.

Breast size and sexual maturation

Breast size was estimated by subtraction of the widest chest circumference (at the level of the nipples) minus the smallest chest circumference (under the breasts) with the patient in supine position. Pubertal stage was estimated according to Tanner for breast development and pubic hair.[21]

Subjective virilization

Subjective virilization was scored by the following questions: Do you experience excessive hair growth (yes/no)? Do you suffer from acne (yes/no)? Do you experience a greasy skin (yes/no)? How do you consider your clitoral size (too small/small/normal/large/too large)? How do you consider your voice (low/normal/high)?

Objective virilization

The Ferriman and Gallwey score was used to assess hirsutism. A score of six or higher was considered as hirsutism.[22] The existence of acne, greasy hair and/or skin was scored. The gynecologist measured clitoral size (large/normal/small/absent, a clitoris size above 1 cm was considered large). To assess voice frequencies recordings were performed in a quiet room. The voice recording consisted of a backward count from 10 to 0 and five standardized sentences. Both measurements were at a comfortable pitch and loudness. Fundamental voice frequency was assessed using a multidimensional voice program (Kay Elemetrics Corp., Lincoln Park, New Yersey, USA). Based on Traunmüller *et al.* we considered a fundamental voice frequency below 201 Hz as abnormally low.[23]

Laboratory investigations

To assess long-term safety and to identify possible confounders of outcome measures, venous blood samples were assayed for lipid spectrum, kidney- and liver function, glucose metabolism (fasting glucose and insulin levels, glycosylated haemoglobin), insulin like growth factor-I (IGF-I), androgen levels (dehydroepiandrosterone, androstenedione and testosterone) and thyroid function. The homeostatic model assessment (HOMA) index for insulin resistance was calculated according to the formula: (insulin mU/l * glucose mmol/l)/ 22.5.[24]

Statistical analyses

Differences in patient characteristics between the participants and non-participants of the follow-up study were assessed using student's T test. Differences in outcome between dosage groups were tested by linear regression using two dummies (for GH+Ox 0.03 and GH+Ox 0.06) for continuous variables, and by Pearson Chi Square test for nominal data. We performed an intention to treat analysis for height related items (primary endpoint) and, in order to prevent underestimation of the side-effects of Ox, a modified intention to treat analysis, i.e. including only those patients who took at least one dose of the study medication, for the secondary endpoints (Figure 1). A P value <0.05 was considered significant. We used Statistical Package for the Social Sciences version 16.0 (SPSS, Inc., Chicago, Illinois).

This follow-up study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the ethics committee of the Radboud University Nijmegen Medical Centre. Written informed consent was obtained for each participant.

RESULTS

Recruitment

Of the 133 original participants in the trial, 68 were eligible for inclusion in the follow-up study (Pl n=23, Ox 0.03 n=27, Ox 0.06 n=18). Their data are shown in Figure 1 and Table 1. Besides lost to follow-up (n=9), excluded (n=7) and deceased (n=2), 47 women refused to participate. Causes of death in the two deceased patients were severe pneumonia at the age of 15 years and colorectal carcinoma at the age of 28 years. The main reasons for declining to participate were the burden of a full day of examination besides regular medical follow-up (at the cost of work or study) and travel distance. Table 2 compares the 68 participants in the follow-up study with the 65 non-participants. The participants used Ox for a longer period and had a higher height gain at the end of the pediatric study compared to the non-participants. The main results for the participants are summarized in Table 3. The mean age was 24.0±3.8 years and the mean interval since the end of GH treatment was 8.7±3.3 years.

TABLE 1
Patient characteristics of 68 patients previously treated with placebo or oxandrolone (0.03 or 0.06 mg/kg/day)

	GH+Pl (n=23)	GH+Ox 0.03 (n=27)	GH+Ox 0.03 vs GH+Pl P value	GH+Ox 0.06 (n=18)	GH+Ox 0.06 vs GH+Pl P value
Age	24.6±4.2	23.7±3.7	0.425	23.6±3.4	0.425
% spontaneous puberty[a]	4/23=17.4%	8/27=29.6%	0.313	4/18=22.2%	0.698
% 45,X	14/23=60.9%	10/27=37.0%	0.093	8/18=44.4%	0.295
% HRT users[b]	20/23=87.0%	24/27=88.9%	0.834	16/18=88.9%	0.851
Time since stopping GH	8.7±3.7	8.6±3.2	0.916	8.8±3.0	0.899
Age at starting GH	9.9±3.7	9.0±3.8	0.402	8.6±3.0	0.259
Age at starting Ox/Pl[b]	10.7±2.5	10.4±2.2	0.593	9.7±1.7	0.140
Duration of GH therapy	5.8±2.7	6.2±3.4	0.601	6.2±2.3	0.639
Duration of Ox/Pl therapy[c]	5.1±1.6	4.7±1.5	0.315	5.0±1.3	0.709

HRT=hormone replacement therapy; GH=growth hormone; Ox=oxandrolone

[a] defined as no need for estrogen treatment before or during GH treatment

[b] two patients, one in the Ox 0.03 group and one in the Ox 0.06, *in*correctly used no HRT. There are no significant differences in HRT regimens. For the total group different regimens were continuous combined n=9, sequential combined n=33, oral anticonceptive containing estrogen and progestogen n=13 and other n=5. One patient in the Ox 0.03 group used no HRT for four years between the original and follow-up study.

[c] two patients, both in the Ox 0.03 group, never started Ox

TABLE 2
Characteristics of participants versus non-participants in the follow-up study

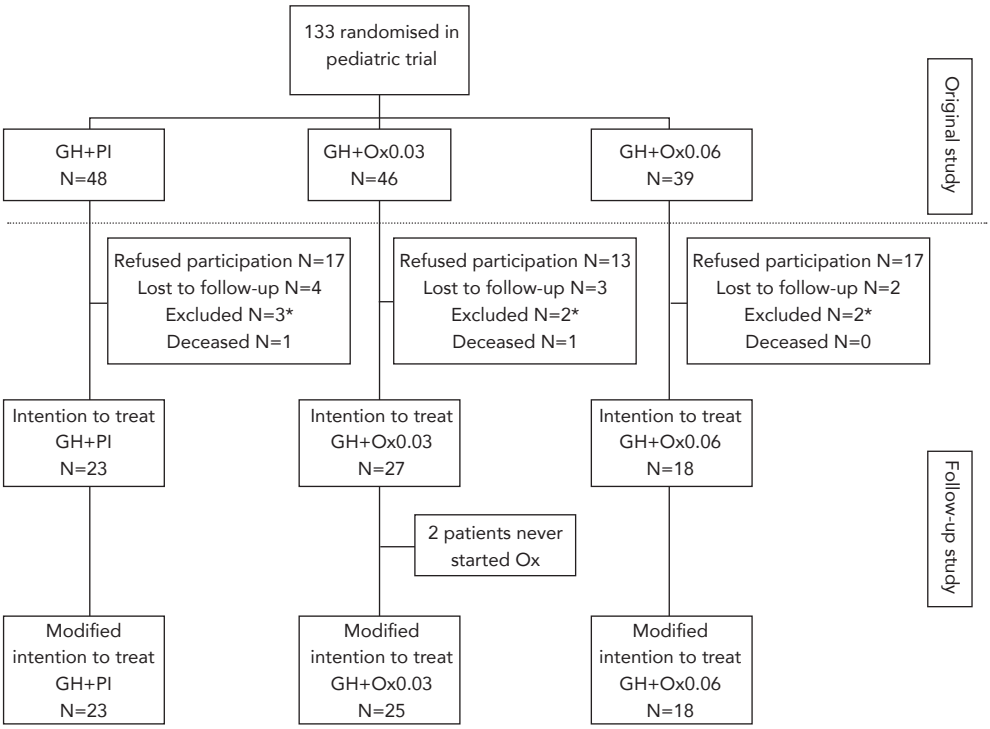
	Participants (n=68)	Non-participants (=65)	P value
Age at start GH	9.2±3.6	7.9±4.2	0.061
Age at start Ox	10.3±2.2	10.3±2.4	0.947
Height SDS (ref TS) start	0.6±0.9	0.3±1.0	0.064
Duration of GH use[a]	6.2±2.9	6.9±3.5	0.258
Duration of Ox use[ab]	5.0±1.4	4.3±1.8	0.040
Height SDS (ref TS) last pediatric visit[a]	1.7±0.9	1.2±1.0	0.002
Height gain last pediatric visit[a]	9.4±4.6	7.5±4.3	0.017
% 45,X	47.1%	53.9%	0.434

GH=growth hormone; Ox=oxandrolone; SDS=standard deviation score; TS=Turner syndrome

[a] Five patients in the non-participant group were still on GH treatment and four were lost to follow-up. These nine were excluded for these comparisons.

[b] 6 patients in the non-participant group and 2 patients in the participant group never started Ox.

FIGURE 1
Follow-up of original study participants and inclusion to the current study



*Exclusion in the Pl group due to current GH treatment (n=2) and multiple handicaps (n=1), in the Ox 0.03 group due to current GH treatment (n=2) and in the Ox 0.06 group due to current GH treatment (n=1) and psychiatric illness (n=1).
Pl=placebo; GH=growth hormone; Ox=oxandrolone

Height gain

For the total group mean adult height gain was 9.2±4.7 cm as compared to 9.1±4.8 cm at the last visit of the pediatric trial. Differences in adult height gain between both Ox treated groups and the Pl group were comparable with the data of the pediatric trial. Duration of Ox use was positively correlated to adult height gain (P value 0.011).

Body composition and proportions

Weight, BMI and percentage lean body mass/fat mass were equal across the three treatment modalities. Regarding body proportions, sitting height-to-height ratio was higher in the GH+Ox 0.06 group. Biiliacal distance was not different between the Pl and Ox groups nor was biacromial distance. Hand and foot lengths were similar in all groups, but the foot length/height ratio was slightly lower, indicating relatively smaller foot length, in the GH+Ox 0.03 group. Head circumference was higher in both Ox treated groups. No differences in left arm, waist, hip and left thigh circumferences were found.

Breast size and sexual maturation

Four patients underwent breast surgery: one patient underwent a breast reduction (GH+Ox 0.06), one patient a breast enlargement (GH+Ox 0.06) and two patients a breast reconstruction because of shape (one in the GH+Ox 0.03 group, one in the GH+Ox 0.06 group). Two additional patients did not want to have their breast size measured. These six patients were excluded from the breast size analysis. Breast size and Tanner stages did not differ between the different treatment modalities. Fifty percent of the women reported a subjective delay of breast development in puberty as compared to peers, with equal distributions across groups. At the time of reassessment, five patients regarded breast size as being too small (one in the GH+Pl group, three in the GH+Ox 0.03 group and one in the GH+Ox 0.06 group).

Subjective virilization

Hirsutism was reported by 19 women (GH+Pl n=0, GH+Ox 0.03 n=9, GH+Ox 0.06 n=10). The frequency was significantly higher in both Ox groups as compared to Pl (Table 3). Acne was reported by five women (GH+Pl n=0, GH+Ox 0.03 n=4, GH+Ox 0.06 n=1) and greasy skin by five (GH+Pl n=0, GH+Ox 0.03 n=3, GH+Ox 0.06 n=2). A low voice was reported by 15 women (GH+Pl n=6, GH+Ox 0.03 n=4, GH+Ox 0.06 n=5) and clitoromegaly by two (GH+Pl n=2, GH+Ox 0.03 n=0, GH+Ox 0.06 n=0). Women treated with Ox 0.03 or 0.06 had a relative risk of 1.4 (P = 0.342) and 2.4 (P = 0.004), respectively, to report one or more virilizing effects.

Objective virilization

Hirsutism, defined as a Ferriman and Gallweyscore above six, was confirmed objectively in three women (GH+Pl n=0, GH+Ox 0.03 n=2, GH+Ox 0.06 n=1). None of them had elevated androgen levels. Women in both Ox groups had a lower mean voice frequency compared to the Pl group. Nine women had a voice frequency below the reference values for both counting backwards and reading standardized sentences (GH+Pl n=2, GH+Ox 0.03 n=5, GH+Ox 0.06 n=2, mean voice frequency 189.1±6.9). Three (GH+Ox 0.03 n=1, GH+Ox 0.06 n=2) had objective evidence of mild clitoromegaly (clitoral size just over 1 cm). Four patients had more than one sign of virilization: two in the GH+Ox 0.03 group (one patient with a greasy skin, acne, hirsutism and a low voice, and one patient with acne, hirsutism and a low voice) and two in the GH+Ox 0.06 group (one patient with clitoromegaly and low voice, and one with greasy skin, acne and hirsutism).

Correlation between subjective and objective virilization

Virilization was objectively confirmed in a minority of the patients with subjective symptoms. The proportions of objective virilization in patients with subjective symptoms were as follows: hirsutism 3/19, greasy skin 3/5, acne 5/5, clitoromegaly 0/2 and low voice 4/15. Patients who reported a low voice were confirmed to have a lower mean voice frequency than those who reported a normal or high voice (205.5 Hz vs. 219.5 Hz, P = 0.016). Vice versa, actual virilization was recognized in most cases, except for clitoromegaly. The proportions of patients with subjective symptoms in cases of objective virilization were as follows: hirsutism 3/3, greasy skin 3/3, acne 5/8, clitoromegaly 0/3 and low voice 4/9.

Laboratory investigations

The lipid spectrum was equal in all groups, except for high density lipoprotein cholesterol (HDL) which was lower in both Ox groups compared to placebo (Table 3). Ten patients, equally distributed in the treatment modalities, had a HDL below the 1.10 mmol/l. Thyroid function, after exclusion of 11 levothyroxine users, was similar between groups. Glucose metabolism did not differ between the treatment groups. There was one patient with type 2 diabetes in the GH+Ox 0.03 group. All patients had a creatinine level within the reference values. Ten patients had one or more liver enzymes above twice the upper reference limit: three in the Pl group (13.0%), four in the GH+Ox 0.03 group (16.0%) and three in the GH+Ox 0.06 group (16.7%).

TABLE 3
Main results of 68 patients previously treated with growth hormone in combination with placebo or oxandrolone (0.03 or 0.06 mg/kg/day). Mean±SD or %.

	GH+Pl (n=23)	GH+Ox 0.03 (n=25)	GH+Ox 0.03 vs GH+Pl P value	CI difference GH+Ox 0.03 vs GH+Pl	GH+Ox 0.06 (n=18)	GH+Ox 0.06 vs GH+Pl P value	CI difference GH+ Ox 0.06 vs GH+Pl
Height[c]							
Adult height (cm)[c]	156.7±4.7	159.0±6.4	0.150	-0.86 to 5.45	157.8±5.2	0.527	-2.38 to 4.61
Height SDS (untreated TS women)[c]	1.5±0.7	1.9±1.0	0.150	-0.13 to 0.86	1.7±0.8	0.527	-0.37 to 0.72
Predicted height (cm)[c]	148.7±5.4	148.8±4.7	0.965	-2.65 to 2.77	148.2±4.1	0.713	-3.57 to 2.45
Adult height gain (cm)[c]	8.0±4.6	10.2±4.9	0.094	-0.39 to 4.86	9.7±4.4	0.256	-1.24 to 4.58
Height gain last pediatric visit (cm)[c]	7.8±4.4	9.8±5.3	0.137	-0.66 to 4.66	10.0±4.1	0.135	-0.71 to 5.19
Body composition and proportions							
Weight (kg)	66.1±13.9	70.9±18.9	0.282	-4.05 to 13.70	66.3±10.9	0.979	-9.54 to 9.79
BMI (kg/m^2)	26.9±5.2	28.0±6.9	0.487	-2.14 to 4.44	26.6±4.2	0.879	-3.86 to 3.31
% fat (skinfold)	35.5±6.2	35.7±5.6	0.904	-3.08 to 3.48	34.8±5.0	0.693	-4.35 to 2.91
% fat (DXA)	39.0±6.6	38.6±6.5	0.807	-4.16 to 3.25	38.8±5.5	0.895	-4.29 to 3.75
Fat mass/m^2 (DXA)	12.2±9.0	9.5±5.5	0.191	-6.64 to 1.35	8.1±5.1	0.066	-8.39 to 0.28
Android fatpercentage (DXA)	36.4±14.5	40.5±9.2	0.189	-2.11 to 10.43	39.5±6.8	0.366	-3.71 to 9.93
Waist-hipratio	0.87±0.08	0.89±0.07	0.579	-0.03 to 0.05	0.87±0.07	0.734	-0.05 to 0.04
UAMA	34.0±5.2	37.2±10.7	0.168	-1.41 to 7.91	33.7±6.6	0.907	-5.46 to 4.85
Head circumference (cm)	54.5±1.6	55.7±1.9	0.024	0.17 to 2.28	55.5±2.0	0.076	-0.11 to 2.19
Hand length (cm)	18.3±1.0	18.8±1.2	0.111	-0.12 to 1.13	18.6±1.1	0.382	- 0.38 to 0.98
Foot length (cm)	24.5±0.9	24.4±1.5	0.769	-0.77 to 0.57	24.5±0.9	0.912	-0.69 to 0.77
Sitting height (cm)	86.0±2.8	87.8±3.0	0.044	0.05 to 3.55	87.8±3.3	0.063	-0.10 to 3.70
Biacromial distance (cm)	35.9±2.2	36.5±1.2	0.243	-0.41 to 1.59	36.9±1.6	0.063	-0.06 to 2.11
Biiliacal distance (cm)	27.9±1.8	27.6±2.7	0.537	-1.62 to 0.85	27.5±1.5	0.545	-1.76 to 0.94
Subischial leg length (cm)	70.7±2.8	71.2±4.1	0.641	-1.52 to 2.45	70.1±3.1	0.530	-2.84 to 1.48
Breast size and Sexual maturation							
Breast size (cm)[a]	13.5±3.6	13.9±4.3	0.730	-1.86 to 2.64	12.7±3.1	0.518	-3.45 to 1.76
Tanner breast stage	4.5±0.7	4.6±0.5	0.619	-0.26 to 0.44	4.7±0.4	0.398	-0.23 to 0.56
Tanner pubic hair stage	4.2±0.9	4.4±0.5	0.335	-0.22 to 0.64	4.5±0.6	0.185	-0.16 to 0.79
Tanner breast stage last pediatric visit	4.4±0.8	4.3±0.8	0.475	-0.65 to 0.31	4.5±0.6	0.663	-0.40 to 0.63
Tanner SDS last pediatric visit	-0.5±0.8	-0.6±0.9	0.552	-0.70 to 0.38	-0.3±0.8	0.712	-0.47 to 0.69

(continued)

TABLE 3 *Continued*

	GH+Pl (n=23)	GH+Ox 0.03 (n=25)	GH+Ox 0.03 vs GH+Pl P value	CI difference GH+Ox 0.03 vs GH+Pl	GH+Ox 0.06 (n=18)	GH+Ox 0.06 vs GH+Pl P value	CI difference GH+ Ox 0.06 vs GH+Pl
Subjective virilization							
Excessive hair growth	0/23=0.0%	9/25=36.0%	0.001		10/18=55.6%	0.000	
Acne	0/23=0.0%	4/25=16.0%	0.045		1/18=5.6%	0.252	
Greasy skin	0/23=0.0%	3/25=12.0%	0.086		2/18=11.1%	0.101	
Large clitoris	2/23=8.7%	0/25=0.0%	0.132		0/18=0.0%	0.200	
Low voice	6/22=27.3%	4/25=16.0%	0.346		5/18=27.8%	0.972	
One or more virilizing effects	8/23=34.8%	13/25=52.0%	0.230		15/18=83.3%	0.002	
Objective virilization							
Pubic hair (more than normal)	0/22=0%	1/23=4.4%	0.323		3/16=18.8%	0.034	
Ferriman & Gallweyscore >6[b]	0/23=0.0%	2/25=8.0%	0.166		1/18=5.6%	0.252	
Ferriman & Gallweyscore	0.09±0.3	3.5±8.8	0.039	0.17 to 6.61	1.7±2.2	0.372	-1.93 to 5.09
Acne	1/23=4.4%	5/25=20.0%	0.101		2/18=11.1%	0.409	
Greasy skin	0/23=0.0%	1/25=4.0%	0.332		2/18=11.1%	0.101	
Clitoromegaly	0/22=0.0%	1/23=4.4%	0.323		2/16=12.5%	0.088	
Voice frequency, counting (Hz)	219.1±27.3	212.1±26.1	0.368	-22.53 to 8.48	209.9±23.6	0.275	-26.04 to 7.54
Voice frequency, reading (Hz)	225.6±19.1	218.9±17.0	0.206	-17.09 to 3.77	212.7±16.6	0.028	-24.29 to -1.48
Laboratory results							
High density cholesterol	1.6±0.4	1.4±0.4	0.025	-0.5 to -0.03	1.4±0.4	0.017	-0.5 to -0.05
Low-density cholesterol	2.9±0.8	3.0±0.8	0.874	-0.4 to 0.5	2.9±0.8	1.000	-0.5 to 0.5
Triglycerides	1.1±0.5	1.8±3.8	0.305	-0.6 to 2.1	1.1±0.5	0.973	-1.5 to 1.5
HbA1c	5.2±0.3	5.2±0.4	0.597	-0.2 to 0.3	5.1±0.3	0.647	-0.3 to 0.2
HOMA	2.1±1.4	3.0±3.8	0.281	-0.8 to 2.5	2.4±2.7	0.759	-1.5 to 2.1
IGF-1	14.0±6.0	14.7±4.9	0.663	-2.5 to 3.8	15.4±5.2	0.410	-2.0 to 4.9
Creatinine	58.3±7.8	59.3±7.2	0.682	-3.9 to 6.0	60.5±11.1	0.423	-3.3 to 7.7
ALAT	32.4±13.1	38.4±38.0	0.530	-13.1 to 25.2	40.2±43.3	0.464	-13.4 to 29.1
Alkaline phosphatase	80.1±28.3	85.7±28.1	0.488	-10.4 to 21.6	84.2±26.3	0.650	-13.7 to 21.8

CI=confidence interval; BMI=body mass index; UAMA=upper arm muscle area; HOMA=homeostatic model assessment; IGF-1=insulin like growth factor-1; ALAT= alanine aminotransferase

[a] Six patients were excluded for breast size measurements: two patients refused measurement without underwear and four had a history of breast surgery.

[b] 3 girls had a Ferriman & Gallweyscore >6, all girls had a Mediterranean ethnicity.

[c] For height related items an intention to treat analysis was performed, without exclusion of the two girls in the Ox 0.03 group

DISCUSSION

We present the long-term outcome of a Dutch multi-center trial on the effects of Ox (besides GH and estrogen) in girls with TS. We have shown that the positive effect of Ox in a dosage of 0.03 mg/kg/day on adult height gain found in the pediatric study has been maintained. The initial study showed a mild deceleration of breast development, mild subjective virilization and a lower voice frequency due to Ox. Our current findings indicate that the deceleration of breast development during Ox treatment is transient. In the long-term study final breast size and Tanner stage were similar between the Ox and Pl groups. The Ox treated adult women still reported more subjective virilization, but actual virilization was only confirmed objectively in a few cases.

Besides our study, two placebo controlled trials have confirmed an increase in height gain with Ox.[8,12,14] The mechanism by which Ox influences growth is unclear. A positive effect on the growth plate as well as effects via other growth factors such as IGF-I have been suggested.[25,26] Data of our original study and the US study point towards a dose dependent effect of Ox on bone maturation.[8,12] However, others could not confirm a faster bone maturation during Ox treatment when using a dosage of 0.05 mg/kg/day, with a maximum Ox dose of 2.5 mg daily.[14]

Previous studies in TS girls using Ox dosages of ~ 1 mg/kg/day found that these dosages frequently needed to be lowered due to virilization.[9-11] In our study as well as in the US study virilization was more frequently seen in girls treated with Ox than placebo, particularly with Ox 0.06.[8,12] Gynecological examination revealed three patients with mild clitoromegaly, and although the numbers are too small to allow conclusions, it is noteworthy that all three patients had been treated with Ox. Furthermore, there were three other Ox treated women, all of Mediterranean origin, with overt (though mild) hirsutism. We speculate that this hirsutism was mainly related to ethnicity but we cannot rule out an effect of Ox. Even in the cases without hirsutism, we still found a small yet significant, dose-dependent effect of Ox on androgen dependent body hair. This is in line with increased subjective scores of hair growth. The lower voice frequency observed in girls treated with Ox in our pediatric study confirmed the data of a cross-sectional study on Ox from Sweden.[27] As expected the voice changes were irreversible and we confirmed the dose dependent lower mean voice frequencies in the Ox 0.06 treated group. However, mean voice frequencies of both Ox treated groups are normal. Remarkably, no virilizing effects were found in the UK study of 106 TS girls treated with Ox in a dosage of 0.05 mg/kg/day with a maximum of 2.5 mg/day.[14] A possible reason for this discrepancy with our study might be the use of a maximum Ox dose, and also the lack of systematic assessments of virilization in that study.

Although pubertal induction with low-dose estrogens starting at 12 years of age seems to be appropriate, half of the patients reported delayed breast development. Consequently, a further delay of breast development due to Ox (observed in the US study as well as in our pediatric

study, while not specifically assessed in the UK study) is undesirable.[8,12,14] Theoretically, Ox inhibits the estrogen effect on breast development.[28] Our follow-up study, however, showed no differences in subjective breast development between the treatment groups. Furthermore final breast size and Tanner stage were similar in both Ox and Pl groups, indicating that the effect of Ox on breast development is transient. This is in line with the improvement of breast stage SDS after discontinuation of Ox seen in our pediatric study.[12]

Theoretically, Ox could affect body composition in a beneficial way. In general women with TS tend to have an increase in BMI and fat mass including visceral fat.[29-31] In a pilot study in adult women with TS, androgen treatment increased lean body mass and decreased fat mass.[32] In our pediatric study we also showed a reduction in fat mass and an increment in muscle mass during Ox treatment.[15] In our current study, however, we did not find any differences in body composition. We found a small reduction of high density lipoprotein cholesterol in the Ox treated groups. This is in line with a recent study on the use of methyltestosterone in adult women with TS, although in that study the decrease of HDL during treatment was accompanied by a reduction of total cholesterol and triglycerides resulting in a more favourable lipid profile.[32] Also in the US study the decrease in HDL was accompanied by a decrease in triglycerides.[8] Since women with TS in general have an unfavorable cardiovascular risk profile, this may be an important issue.[33]

Regarding body proportions, TS women have relatively shorter legs, larger hands and feet, and broader shoulders and pelvis.[34] GH treatment has a beneficial effect on the disproportion between total height and sitting height.[34,35] Ox, however, seemed to negatively influence this disproportionality. In the pediatric study, we found a significant increase in sitting height/height ratio in the Ox treated groups, which was confirmed for the Ox 0.06 group at long-term follow-up. Furthermore the notion that Ox increases biacromial distance and decreases biiliacal distance is confirmed in our adult population, although the numbers did not reach statistical significance.

An important limitation of the current study is that only half of the original participants could be included for follow-up. We cannot rule out that certain differences between the treatment groups remained undetected due to lack of power. For example, the difference in height gain favoring Ox 0.03 observed in the full cohort failed to reach statistical significance (p=0.094) in the follow-up study. The number of drop-outs were equally distributed among the treatment arms, suggesting that the sample remains representative of the cohort. However, the participants of the follow-up study had a larger height gain at last pediatric follow-up as well as a longer duration of Ox treatment than the non-participants. This may have affected the outcome. Also, we cannot rule out that possible differences in spontaneous puberty, although not significant, have impacted long-term effects.

Our study is the first long-term follow-up study on the effects of Ox in TS patients. Based on our previous and current findings we conclude that Ox 0.06 mg/kg/day should not be used in girls with TS since there is no significant effect on adult height and it results in (non-

reversible) virilizing effects. Ox 0.03 mg/kg/day in addition to growth hormone and estrogen treatment has a beneficial effect on adult height gain in TS patients. Despite previously reported deceleration of breast development during Ox 0.03 treatment, eventual adult breast size is not affected. We conclude that Ox in this dosage can be safely used in girls with TS, although usually mild virilization can persist in a minority of patients. We therefore recommend careful counseling before the start of Ox. Currently Ox is not universally available in Europe. Our study might contribute to the reintroduction of Ox. Long-term follow-up, including assessment of cardiovascular and uterine status, is necessary to assess effects of Ox even on the longer term.

DISCLOSURE

Declaration of interest

A.R.M.M.H. and H.J.L.M.T. received a research grant from Pfizer for this research. T.C.J.S. received lecture fees from Novo Nordisk and Pfizer, and did advisory work for Novo Nordisk. J.M.W. has served on the advisory boards of Pfizer, Ipsen, Versartis, Prolor and Biopartners. J.M.W. received fees from Pfizer, Ipsen and Ferring. L.A.M. received honorarium for her thesis from Pfizer, Eli Lilly, ACE pharmaceuticals, Ferring, Novo Nordisk, Ipsen and Sandoz.
All other authors have no conflict of interest.

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ERRATUM

In the discussion section we incorrectly stated that the use of a maximum Ox dose in the UK study could explain the differences in virilization frequency: 'A possible reason for this discrepancy with our study might be the use of a maximum dose, and also the lack of systematic assessments of virilization in that study.' The correct phrase should be: 'A possible reason for this discrepancy with our study might be the use of a lower maximum Ox dose (2.5 mg in the UK study, versus 3.75 mg in the US and Dutch study), and also the lack of systematic assessments of virilization in that study.'

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Chapter 5

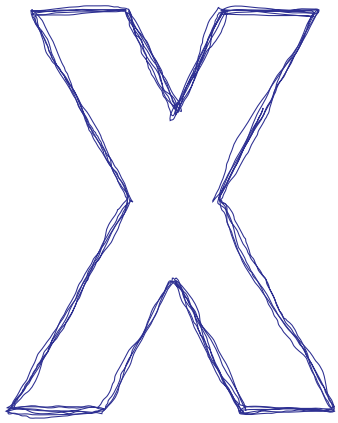
Long-term effects of oxandrolone treatment in childhood on neurocognition, quality of life and social-emotional functioning in young adults with Turner syndrome

Kim Freriks[1], Chris M Verhaak[2], Theo CJ Sas[3,4], Leonie A Menke[5], Jan M Wit[5], Barto J Otten[6], Sabine MPF de Muinck Keizer- Schrama[3], Dominique FCM Smeets[7], Romana T Netea-Maier[1], Ad RMM Hermus[1], Roy PC Kessels[2,8,9], Henri JLM Timmers[1]

Author affiliations

[1]Radboud university medical center, Nijmegen, Department of Internal Medicine, Division of Endocrinology, [2]Radboud university medical center, Nijmegen, Department of Medical Psychology, [3]Erasmus Medical Centre, Rotterdam, Department of Pediatrics, [4]Albert Schweitzer Hospital, Dordrecht, Department of Pediatrics, [5]Leiden University Medical Center, Leiden, Department of Pediatrics, [6]Radboud university medical center, Nijmegen, Department of Pediatrics, [7]Radboud university medical center, Nijmegen, Department of Human Genetics, [8]Vincent van Gogh Institute for Psychiatry, Venray, Centre of Excellence for Korsakoff and Alcohol-Related Cognitive Disorders, [9]Radboud university medical center, Nijmegen, Donders Institute for Brain, Cognition and Behaviour

Hormones and behavior, accepted



ABSTRACT

Turner syndrome (TS) is the result of (partial) absence of one X-chromosome. Besides short stature, gonadal dysgenesis and other physical aspects, TS women have typical psychological features. Since psychological effects of androgen exposure in childhood probably are long-lasting, we explored long-term psychological functioning after oxandrolone (Ox) therapy during childhood in adults with TS in terms of neurocognition, quality of life and social-emotional functioning. During the initial study, girls were treated with growth hormone (GH) combined with placebo (Pl), Ox 0.03 mg/kg/day, or Ox 0.06 mg/kg/day from the age of eight, and estrogen from the age of twelve. Sixty-eight women participated in the current double-blinded follow-up study (mean age 24.0y, mean time since stopping GH/Ox 8.7y). We found no effects on neurocognition. Concerning quality of life women treated with Ox had higher anxiety levels (STAI 37.4±8.4 vs 31.8±5.0, p0.002) and higher scores on the depression subscale of the SCL-90-R (25.7±10.7 vs 20.5±4.7, p0.01). Regarding social-emotional functioning, emotion perception for fearful faces was lower in the Ox-treated patients, without effect on interpersonal behavior. Our exploratory study is the first to suggest that androgen treatment in adolescence possibly has long-term effects on adult quality of life and social-emotional functioning. However, differences are small and clinical implications of our results seem limited. Therefore we would not recommend against the use of Ox in light of psychological consequences.

INTRODUCTION

Turner syndrome (TS) is the result of total or partial absence of one X chromosome and has an incidence of approximately 1:2000 in live born girls.[1] In addition to short stature, gonadal dysgenesis -with infertility in the majority of the women-, and dysmorphic features, TS is associated with a wide range of abnormalities affecting nearly every organ system. Apart from these physical aspects, psychological problems including neurocognitive dysfunction, diminished quality of life and social-emotional deficits have been reported.

Regarding neurocognition, women with TS have a distinct profile characterized by a normal to high verbal intelligence quotient (VIQ) and a decreased performance IQ (PIQ).[2,3] Cognitive problems commonly persist into adulthood and adult women with TS are prone to impairments in visual-motor integration, attention, (working) memory, executive function and spatial cognition.[2,3]

Quality of life in TS is generally considered to be unaffected, although some studies reported diminished scores on especially physical functioning subscales.[4-7] Sexual functioning may be impacted and women with TS have a higher rate of lifetime depression.[6,8] Furthermore, women with TS report more social isolation, shyness, social anxiety and problems in relationships.[4,9,10]

Infertility may be one of the main contributing factors, since similar problems have been reported in women with premature ovarian failure.[6,8,10] In addition to health-related problems, social interaction is probably influenced by difficulties in interpreting non-verbal communication, that is, recognizing facial expression of certain emotions (especially fear).[11-13]

In general, girls with TS are treated with growth hormone (GH) to increase adult height.[14] In order to improve the growth-enhancing effect of GH in TS, the addition of the weak synthetic androgen oxandrolone (Ox) has recently been investigated in three placebo (Pl) controlled trials, including ours.[15-17] We investigated the additional growth-enhancing effect of Ox in two different dosages.[16] Compared with GH+Pl, GH+Ox in a dose of 0.03 mg/kg/day (Ox 0.03) significantly increased adult height gain (9.5 vs. 7.2 cm in Pl) at the cost of mild deceleration of breast development. At a higher dose of 0.06 mg/kg/day (GH+Ox 0.06), no significant increase in height gain was found and significantly more girls reported virilization.[16] In the Ox groups a decrease in fat mass, an increase in muscle mass and lowering of the voice was found.[18,19] During the same study a psychological survey (testing emotional and behavioral problems, sexual aspects of quality of life and gender role) revealed no differences between the Ox and Pl treated groups.[20]

Others found that during methyltestosterone treatment in TS quality of life, including general health, and sexual desire improved.[21] In the same study androgen treatment was associated with neuro-cognitive functioning in terms of improved selective attention and verbal episodic memory, and a decline in some of the executive functions including working memory.[21] Strikingly, other researchers treated TS girls with Ox for 2 years and found significant improvement of working memory and measures of immediate recall, but no effects on verbal abilities, spatial cognition, and executive function.[22] Four years of Ox treatment resulted in slight improvement of mathematical learning disabilities, but no effect was found on reading learning disability.[23]

Conventionally, the effects of androgens on psychological functioning are divided into activational (temporary, during exposure) and organizational (permanent).[24] While research on organizational effects has primarily focused on the prenatal and neonatal period, the timeframe in which cerebral function can be permanently influenced by external hormonal influences possibly last into puberty.[25]

Taking together the susceptibility to (neuro)psychological problems in TS and the potentially permanent psychological effects of exposure to exogenous androgens during childhood and adolescence, this raises important questions regarding the safety of growth-enhancing treatment with Ox in these patients. The aim of this exploratory follow-up study was to determine the long-term effects of Ox on neuro-cognitive functioning (i.e., 'traditional' cognitive functions related to information processing), quality of life and social-emotional functioning.

METHODS

Participants and previous treatment

The current study is a follow-up evaluation of the pediatric multi-center randomized, placebo-controlled, double-blind Turner Oxandrolone Study. The initial study started in 1991. In this study 133 girls with TS were treated with GH (1.33 mg/m^2 body surface/day) from baseline combined with Pl, Ox 0.03, or Ox 0.06 mg/kg body weight/day from the age of eight and estrogen from the age of twelve. More detailed participant information and treatment modalities, including inclusion and exclusion criteria, were reported previously.[16]

For the current study all patients and investigators remained blinded for the study medication and the patients who discontinued GH treatment at least six months before entry were invited. Additional exclusion criteria of this study were participation in another drug study within two months of entry, malignant or severely disabling disease, suspicion of major psychiatric disorder and pregnancy or current fertility treatment.

Assessments

All neuropsychological tests and psychological questionnaires were performed during a whole-day program, which included medical assessments as well. The questionnaires were computerized and set out in a quiet room without any company. All neuropsychological tests were performed by two well-trained assistant psychologists.

Neurocognition and intelligence

Intelligence (total IQ, verbal and performal IQ) was assessed using the abbreviated version of the Wechsler Adult Intelligence Scale (WAIS-III), consisting of the 7 subtests Arithmetic, Information, Digit Span, Similarities, Picture Completion, Block Design and Symbol Substitution.[26] Executive function was measured using the Brixton Spatial Anticipation Test (Brixton) as an index of rule detection and concept shifting and the Zoo Map subtest of the Behavioral Assessment of the Dysexecutive Syndrome (BADS) as a test for visuospatial planning.[27] Visuospatial working memory was addressed with the Box Task, a computerized paradigm to assess visuospatial efficiency and working memory.[28] The Box Task consists of different trials with increasing difficulty (4, 6, 8 and 10 boxes). Outcome measures are within-search errors (errors within a single search reflecting the ability to keep visuospatial information active), between-search errors (errors between several search trials, reflecting the ability to maintain visuospatial information over longer periods of time) and a strategy score (reflecting search efficiency).[28]

Quality of life

Health-related quality of life was assessed with the RAND 36 adapted from the MOS 36-item short-form health survey.[29] The original RAND consists of 8 subscales: Physical Functioning, Social Functioning, Limitations due to Physical Problems, Limitations due to Emotional Problems, Mental Health, Vitality, Bodily pain and General health. A ninth subscale 'Health change' was added.

The Dutch revised version of the Symptom Checklist (SCL-90-R) was performed to estimate general psychological, somatic and cognitive wellbeing.[30] The test consists of 90 items that have to be rated on a five-point scale. Eight subscales are defined as Agoraphobia, Somatization, Anger-Hostility, Depression, Interpersonal Sensitivity and Paranoid Ideation, Anxiety, Cognitive Performance Difficulty, and Sleep Disturbance.

More detailed information about depression and anxiety was collected by two additional questionnaires. The level of depressive symptoms was measured using the Beck Depression Inventory-2nd Edition (Dutch version, BDI-II-NL).[31,32] The scores for the 21 items range from 0-3 and are divided into three categories: Cognitive, Somatic and Affective. We considered a score above 16 as indicative for depression. Anxiety was measured using the Spielberger State Trait Anxiety Inventory (Dutch version, STAI).[33] The STAI measures the Trait Anxiety (a general tendency of an individual to be anxious) and the State Anxiety (the level of anxiety on a certain moment). Both measures include 20 items which scores range from 1-4.

To examine women's evaluation of the impact of TS in their life (cognitive coping), the Illness Cognition Questionnaire (ICQ) was used.[34] A score was computed for each of the three subscales: Helplessness, Acceptance and Disease Benefits.

Five items of the Inventory Social Involvement (ISI)- the social dimension of the Arthritis Impact Measurement Scales- were used to assess the participant's own perception of social support in the past six months.[35,36]

To assess the thoughts and emotions concerning sexuality we used the Dutch version adapted from the Women's Sexual Self-Concept Scale (WSSCS).[37] The three different subscales of the WSSCS are defined as Agentic Sexuality (women's interest and active role in sexuality), Negative Associations (sexual coercion, negative emotions and concerns about sexuality) and Reserved Approach (responsibility, carefulness and faithfulness).[37]

Social and emotional functioning

We assessed three different aspects of social-emotional functioning: understanding of emotions, interpersonal distress, and recognition of emotions.

To assess the ability to understand and process emotions the Bermond-Vorst Alexithymia Questionnaire (BVAQ) was administered.[38] The five sub-scales differentiate between cognitive (Identifying, Verbalizing and Analyzing) and affective (Emotionalizing, Fantasizing) dimensions of alexithymia.

As an index for social behavior and the associated experience of stress, the Scale for Interpersonal Behaviour (SIB) was administered.[39] The SIB consists of two different scales (50 items): one for the frequency of engagement in a specific social situation (SIB-F) and one for the degree of experienced discomfort (SIB-G). The index provides four subscales named Negative Assertion (disclosure of negative feelings), Insecurity (expression of and dealing with personal limitations), Initiating Assertiveness (social assertiveness and expressing one's own opinion) and Positive Assertion (praising others and the ability to deal with compliments).

To examine the ability to perceive and label emotional expressions the short form of the Emotion Recognition Test (ERT) was administered.[40] This is a computerized test for the recognition of the six basic facial emotional expressions: anger, disgust, fear, happiness, sadness and surprise. The emotions are mimicked by two male and two female actors. Morphed video clips show a neutral face gradually changing into different emotions at different levels of intensities (40, 60, 80 en 100%). After each video clip, the participant is asked to make a forced choice between the six emotional expressions. Each emotion in each intensity is presented four times, each by a different actor.

Statistical analyses

We performed a 'modified intention to treat analysis' including only those patients who took at least one dose of the study medication (Pl or Ox). For comparison between the three groups (Pl, Ox 0.03, Ox 0.06) ANOVA or Chi-square test were used where appropriate. Only in the case of significant main effects post-hoc comparisons were performed. ERT and Box Task were analyzed using a general linear model repeated-measures analysis. For comparison between Pl and the total Ox group (Ox 0.03 and Ox 0.06 together) we used ANOVA and unpaired T-tests. To correct for multiple testing, alpha was set at 0.01 for comparison of the three treatment modalities. We used Statistical Package for the Social Sciences version 16.0 (SPSS, Inc., Chicago, Illinois). Effect sizes have been calculated using the Effect Size Calculator (www.cognitiveflexibility.org/effectsize/).

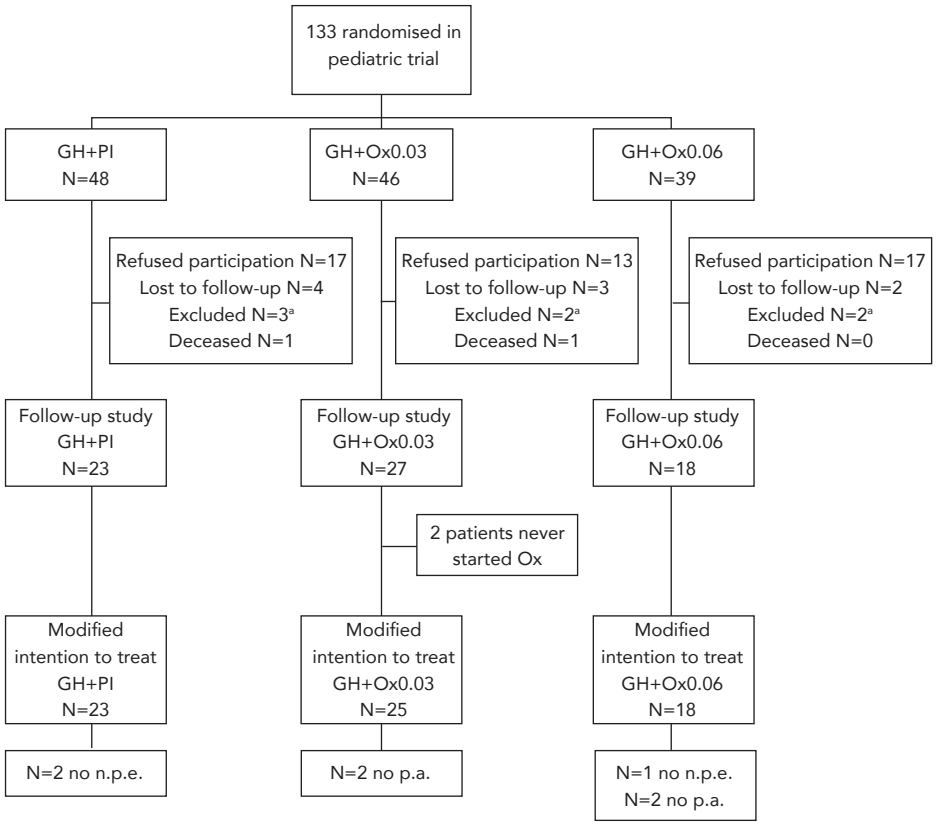
The study was performed in accordance with the declaration of Helsinki and approved by the ethics committee of the Radboud university medical center. Written informed consent was obtained from all participants.

RESULTS

Recruitment

Of the original 133 patients, 68 patients participated in the follow up study. For recruitment and participation details see Figure 1. Between the three groups there were no differences in education level, employment and living situation. One patient in the Ox 0.06 group was excluded because of a serious psychiatric disorder. Three patients were under regular care of a psychiatrist and/or psychologist, all three had been treated with Ox 0.03.

FIGURE 1
Flow chart of patient inclusion



n.p.e.=neuropsychological examination; p.a.=psychological assessment
[a] exclusion due to current GH treatment (n=5), multiple handicaps (n=1) or psychiatric illness (n=1). The patient with psychiatric illness was in the Ox 0.06 group.

Two patients did not complete the psychological questionnaires because their parents considered them incapable (IQ 63 and 74, respectively) and another patient because of anxiety to fail. The fourth patient was excluded because of too many missing values. Three other patients did not undergo neuropsychological examination: one patient refused to participate and in two patients data could not be collected due to logistic reasons. According to the modified intention to treat analysis, two patients were excluded in the Ox 0.03 group because they had never started Ox. Table 1 summarizes the main patient characteristics and physical outcome measures. More detailed information about growth, body composition and virilization has been reported in a previous paper.[41] The mean time±SD interval since the end of GH/Ox was 8.7±3.3 years. The timing and duration of GH/Ox was not different between the three treatment modalities. Estrogen therapy was equal in all groups.

TABLE 1
Patient characteristics modified intention to treat (n=66)

	GH+Pl (n=23)	GH+Ox 0.03 (n=25)	GH+Ox 0.03 vs GH+Pl P value	GH+Ox 0.06 (n=18)	GH+Ox 0.06 vs GH+Pl P value
Age	24.6±4.2	24.2±3.4	0.707	23.6±3.4	0.414
Percentage 45,X	14/23=60.9%	8/25=31.0%	0.045	8/18=44.4%	0.295
Age at start Ox/Pl	10.7±2.5	10.4±2.2	0.593	9.7±1.7	0.140
Duration of Ox/Pl therapy	5.1±1.6	4.7±1.5	0.315	5.0±1.3	0.709
Adult height (cm)	156.7±4.7	159.0±6.7	0.171	157.9±5.2	0.531
Adult height gain (cm)	8.0±4.6	10.0±4.9	0.144	9.7±4.4	0.258
BMI (kg/m^2)	26.9±5.2	28.0±6.9	0.487	26.6±4.2	0.879
Breast size (cm)[a]	13.5±3.6	13.9±4.3	0.730	12.7±3.1	0.518
Tanner breast stage	4.5±0.7	4.6±0.5	0.619	4.7±0.4	0.398
Ferriman & Gallwey score[b]	0.09±0.3	3.5±8.8	0.039	1.7±2.2	0.372
One or more virilizing complaints[c]	8/23=34.8%	13/25=52.0%	0.230	15/18=83.3%	0.002

[a] Breast size was estimated by subtraction of the widest chest circumference (at the level of the nipples) minus the smallest chest circumference (under the breasts) with the patient in supine position. Six patients were excluded for breast size measurements: two patients refused measurement without underwear and four had a history of breast surgery.

[b] Ferriman & Gallwey score is a score for excessive, androgen dependent, body hair

[c] Excessive hair growth, acne, greasy skin, large clitoral size and/or lowering of the voice

Neurocognition and Intelligence

All neurocognitive results are summarized in Table 2. We found no main effect of treatment modality on intellectual ability (TIQ, PIQ, VIQ: all F values < 0.8). No differences between the treatment groups were found on visuospatial planning (Zoo Map: all F values < 1.9) and rule detection and concept shifting (Brixton: $F_{2,62} = 1.8$). Repeated-measures analyses revealed no effect of Ox on visuospatial working memory (Box task between-search errors). No strategy differences were observed on this task either (all F values < 2.0).

TABLE 2
Neurocognition and intelligence

	Placebo (n=23) Mean±SD	Ox 0.03 (n=23) Mean±SD	Effect size Ox 0.03 Cohen's d	Ox 0.06 (n=17) Mean±SD	Effect size Ox 0.06 Cohen's d	Total Ox group (n=40) Mean±SD	Effect size Total Ox group Cohen's d
WAIS III							
Total IQ	87.1±12.0	90.5±13.6	-0.27	86.7±10.6	0.04	88.9±12.4	-0.15
Verbal IQ	91.7±12.6	95.0±14.5	-0.24	91.8±14.2	-0.01	93.7±14.3	-0.15
Performal IQ	83.3±12.2	86.6±12.5	-0.27	82.5±8.7	0.08	84.9±11.1	-0.14
Brixton	11.1±4.2	13.0±5.6	-0.39	14.2±6.1	-0.60	13.5±5.8	-0.48
Zoo Map (BADS)							
Score (condition 1)	3.5±4.9	3.7±4.4	-0.04	2.9±5.3	0.12	3.4±4.8	0.02
Planning time (sec)	111.6±163.1	79.5±64.1	0.28	110.2±119.6	0.01	92.6±91.8	0.15
Total time (sec)	209.4±153.1	186.0±85.4	0.20	252.3±162.5	-0.27	214.2±126.7	-0.03
Score (condition 2)	7.4±1.8	6.6±2.2	0.40	7.6±1.2	-0.13	7.0±1.9	0.22
Planning time (sec)	10.3±10.2	15.2±25.2	-0.28	14.2±19.6	-0.26	14.8±22.7	-0.27
Total time (sec)	63.8±24.1	69.4±28.9	-0.21	72.2±31.5	-0.30	70.6±29.7	-0.25
Box Task							
Between search error							
4 boxes	0.2±0.3	0.2±0.3	0.00	0.3±0.4	-0.29	0.2±0.4	0.00
6 boxes	0.8±1.0	0.8±1.2	0.00	0.4±0.7	0.47	0.7±1.0	0.10
8 boxes	3.6±3.9	3.2±2.5	0.13	4.0±3.6	-0.11	3.5±3.0	0.03
10 boxes	10.0±4.3	10.0±4.4	0.00	9.2±3.5	0.21	9.7±4.0	0.07
Strategy score							
4 boxes	1.9±0.7	1.8±0.6	0.15	2.1±0.6	-0.31	1.9±0.5	0.00
6 boxes	3.2±1.1	3.4±0.7	-0.22	3.5±1.0	-0.29	3.5±0.8	-0.32
8 boxes	4.4±1.3	4.7±1.1	-0.25	4.6±1.0	-0.17	4.7±1.1	-0.25
10 boxes	6.5±2.0	6.7±1.1	-0.13	6.8±1.6	-0.17	6.7±1.3	-0.12

WAIS=Wechsler Adult Intelligence Scale; IQ=intelligence quotient; BADS=Behavioral Assessment of the Dysexecutive Syndrome; SD=standard deviation. Higher scores on the WAIS indicate a higher IQ. More errors for the Brixton reflect more problems in executive function. For the Zoo Map higher scores correlate with better visuospatial planning. Concerning the Box Task an increasing number of errors reflects problems in visuospatial efficiency and working memory, where a lower score on strategy indicates more efficient use of strategy.

Quality of life

All results are summarized in Table 3.

Concerning quality of life the RAND-36 showed no main effect of Ox (all F-values < 2.7). When comparing the Pl group with both Ox treated groups taken together no significant differences on the subscales were found. In addition, the SCL-90-R subscales did not reveal any significant differences when comparing the Ox 0.03 and Ox 0.06 groups with Pl separately (all F-values <2.4). When combining the two Ox groups, the patients using Ox reported significantly more complaints compared to Pl on the subscale Depression (25.7 vs 20.5, p = 0.01). None of the other subscales showed a treatment effect.

Focusing on depression and anxiety, no main effect of the study medication was found on the different subscales of the BDI-II (all F-values < 1.4). Mean values in all three groups indicate low levels of depressive symptoms. When comparing the Pl group with the Ox treated groups together no significant differences were found. Eight patients had a total score > 16: one in the Pl group, four in the Ox 0.03 group and three in the Ox 0.06 group, but no significant differences were found in frequency of occurrence across the three groups. Testing anxiety with the STAI, we demonstrated no main effect of Ox compared to Pl for both current anxiety and anxiety predisposition. When combining the Ox treated groups for both subscales a significant effect was found when compared to Pl: current anxiety (37.4 vs 31.8, p = 0.002) and anxiety predisposition (40.2 vs 33.4, p = 0.002), indicating higher levels of anxiety in the Ox treated groups.

Testing cognitive coping, no significant main effect of treatment condition on the ICQ was found (all three F values < 1.5). A comparison between the Pl group and both Ox groups together showed no significant differences as well.

We found no main effect for Ox on the total ISI score ($F_{2,61} = 2.41$, p = 0.1), meaning that self reported social support is comparable between the treatment modalities. Comparing the Ox groups together revealed no differences with Pl.

Concerning psychosexual wellbeing mean scores of the WSSCS subscales were 4.3±1.1 for Agentic Sexuality, 2.1±0.7 for Negative Associations and 5.4±1.2 for Reserved Approach, without any differences between the treatment modalities.

TABLE 3
Questionnaires concerning quality of life and social and emotional functioning

	Placebo (n=21) Mean±SD	Ox 0.03 (n=25) Mean±SD	Effect size Ox 0.03 Cohen's *d*	Ox 0.06 (n=16) Mean±SD	Effect size Ox 0.06 Cohen's *d*	Total Ox group (n=41) Mean±SD	Effect size Total Ox group Cohen's *d*
QUALITY OF LIFE							
RAND (mean)	84.8±8.9	79.0±15.7	0.47	74.4±17.7	0.78	77.2±16.4	0.60
Physical functioning	93.4±7.9	89.2±17.2	0.33	88.8±16.5	0.38	89.0±16.7	0.36
Social functioning	92.5±10.0	86.4±20.1	0.41	78.8±24.1	0.80	83.4±21.8	0.57
Role limitation – physical	88.6±22.8	87.0±29.0	0.06	68.8±41.3	0.62	79.9±35.0	0.30
Role limitation – emotional	90.8±18.6	77.2±38.2	0.48	79.1±36.3	0.43	78.0±37.0	0.46
Mental health	77.6±9.6	74.6±15.8	0.24	71.8±15.0	0.47	73.5±15.4	0.33
Vitality	66.4±12.6	58.4±20.6	0.48	56.6±22.9	0.55	57.7±21.2	0.51
Bodily pain	95.5±8.8	88.4±17.4	0.54	86.9±15.5	0.71	87.8±16.5	0.61
General health	73.4±15.3	70.4±18.1	0.18	64.7±17.4	0.53	68.2±17.8	0.31
SCL-90-R (total)	117.7±24.6	135.6±50.2	-0.48	135.2±38.9	-0.55	135.4±45.6	-0.50
Anxiety	12.0±2.7	14.5±6.3	-0.56	13.8±4.0	-0.54	14.2±5.5	-0.54
Agoraphobia	7.4±0.7	8.7±3.3	-0.65	8.4±2.4	-0.65	8.6±2.9	-0.67
Depression	20.5±4.7	26.3±12.4	-0.68	24.7±7.6	-0.68	25.7±10.7*	-0.68
Somatization	15.3±3.6	16.4±5.5	-0.24	17.0±4.6	-0.41	16.6±5.1	-0.30
Cognitive performance difficulty	13.7±4.8	16.3±7.0	-0.44	16.8±6.7	-0.54	16.5±6.8	-0.48
Interpersonal sensitivity	26.3±8.4	28.8±11.8	-0.25	29.8±11.2	-0.36	29.2±11.4	-0.29
Anger-Hostility	7.1±1.4	7.6±1.7	-0.32	7.6±2.1	-0.29	7.6±1.8	-0.31
Sleep disturbance	4.4±1.7	4.5±1.8	-0.06	4.8±1.9	-0.22	4.6±1.8	-0.11
BDI-II-NL (total)	4.4±5.4	7.0±8.3	-0.38	6.9±7.9	-0.38	7.0±8.0	-0.39
Affection	0.4±0.9	0.9±1.8	-0.37	1.3±2.1	-0.60	1.1±1.9	-0.50
Cognition	1.5±2.4	2.9±3.8	-0.45	2.3±2.7	-0.31	2.7±3.4	-0.41
Somatic	2.5±2.5	3.2±3.9	-0.22	3.4±3.9	-0.28	3.3±3.8	-0.25
STAI							
Current anxiety	31.8±5.0	37.4±9.1	-0.79	37.5±7.4	-0.92	37.4±8.4**	-0.84
Anxiety predisposition	33.4±5.6	40.3±11.9	-0.79	39.9±9.2	-0.88	40.2±10.8**	-0.83
ICQ							
Helplessness	7.6±2.5	8.6±2.7	-0.38	8.7±2.7	-0.42	8.6±2.7	-0.38
Acceptance	21.4±2.5	19.5±4.1	0.58	19.8±4.7	0.44	19.6±4.3	0.53
Perceived benefits	14.8±4.5	15.4±4.7	-0.13	15.1±4.7	-0.07	15.3±4.6	-0.11
ISI	17.2±3.6	16.8±3.2	0.12	14.8±3.9	0.64	16.0±3.6	0.33

Continued

TABLE 3
Continued

	Placebo (n=21) Mean±SD	Ox 0.03 (n=25) Mean±SD	Effect size Ox 0.03 Cohen's *d*	Ox 0.06 (n=16) Mean±SD	Effect size Ox 0.06 Cohen's *d*	Total Ox group (n=41) Mean±SD	Effect size Total Ox group Cohen's *d*
SOCIAL AND EMOTIONAL FUNCTIONING							
BVAQ							
Emotionalizing	26.5±3.3	29.8±3.9	-0.92	26.4±3.8	0.03	28.5±4.2	-0.53
Fantasizing	22.6±7.3	24.5±6.6	-0.27	23.4±7.3	-0.11	24.1±6.8	-0.21
Identifying	30.6±4.4	29.8±6.0	0.15	30.1±5.1	0.11	29.9±5.6	0.14
Analyzing	30.6±4.6	29.3±5.2	0.27	29.4±3.8	0.29	29.4±4.6	0.26
Verbalizing	24.8±5.8	24.6±6.6	0.03	24.8±5.3	0.00	24.7±6.0	0.02
SIB-G							
Express negative feelings	36.7±11.4	38.4±12.7	-0.14	37.4±9.2	-0.07	38.0±11.4	-0.11
Express uncertainty	26.0±9.5	26.3±8.5	-0.03	30.4±9.4	-0.47	27.9±9.0	-0.21
Make yourself noticed	20.4±7.2	22.2±7.7	-0.24	22.7±6.8	-0.33	22.4±7.3	-0.28
Express positive feelings	17.8±7.4	16.8±5.8	0.15	17.9±7.0	-0.01	17.2±6.2	0.09
SIB-F							
Express negative feelings	40.4±10.3	40.6±8.1	-0.02	38.7±7.7	0.19	39.8±7.9	0.07
Express uncertainty	51.8±10.4	51.4±6.5	0.05	47.8±5.1	0.52	50.0±6.2	0.22
Make yourself noticed	26.5±6.2	27.2±5.3	-0.12	25.1± 3.4	0.29	26.4±4.7	0.02
Express positive feelings	24.7±6.3	25.2±5.9	-0.08	25.1±4.8	-0.07	25.1±5.5	-0.07

* P value when compared to placebo ≤ 0.01, ** P value compared to placebo ≤ 0.002

BDI-II-NL=Beck Depression Inventory-2nd Edition; SCL-90-R=Symptom Checklist-90 Revised; STAI=Spielberger State Trait Anxiety Inventory; ICQ=Illness Cognition Questionnaire; ISI=Inventory Social Involvement; SD, standard deviation; BVAQ=Bermond-Vorst Alexithymia Questionnaire; SIB=Scale for Interpersonal Behaviour; SD=standard deviation. A higher score for the RAND corresponds with a better quality of life. Higher scores on the SCL-90-R reflect more complaints. Higher scores on the BDI and STAI correspond with more depressive symptoms and a higher anxiety level respectively. For the ICQ higher scores indicate more feelings of helplessness, higher levels of acceptance and more perceived benefits. The higher the score on the ISI, the better the perception of social support. Concerning BVAQ increased scores on the affective dimension reflect problems in the conscious experience of arousal (Emotionalizing, Fantasizing), while a high score on the cognitive dimension is supposed to refer to difficulties in Identifying, Verbalizing and Analyzing emotions. For the SIB higher scores correspond with more discomfort (SIB-G) and a higher frequency of complaints (SIB-F).

Social and emotional functioning

Table 3 shows the results of the various tests. Concerning alexithymia, we found a main effect of Ox on the BVAQ subscale Emotionalizing ($F_{2,61} = 6.29$, $p = 0.003$). Post-hoc analysis however, showed no significant differences between the three treatment modalities.

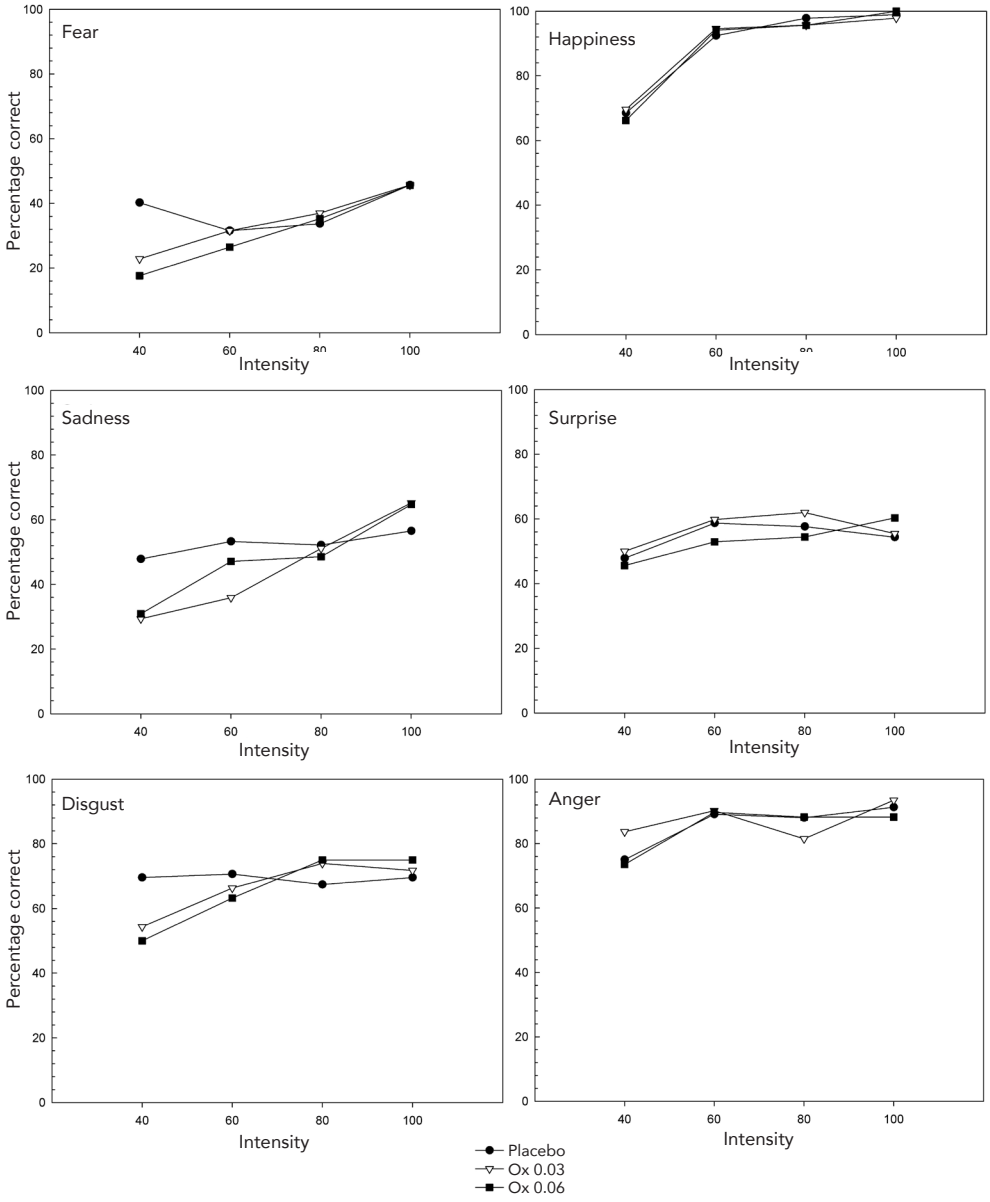
Scores on the SIB did not differ between the different treatment modalities on both SIB-F (all F values < 1.4) and SIB-G (all F values < 1.3). Comparing combined Ox groups with Pl revealed no significant differences for social behavior and associated stress.

Figure 2 shows the results of the ERT for the three treatment groups. Main effects for emotion type ($F_{5,300} = 103.5$, $p < 0.0001$) and intensity ($F_{3,180} = 68.1$, $p < 0.0001$) were found. ERT revealed no main effect of Ox ($F_{2,60} = 0.56$, $p = 0.57$). We found an interaction between treatment group and intensity ($F_{6,180} = 3.3$, $p = 0.004$). Subsequent analyses revealed a main effect of treatment condition only on intensity 40% ($F_{2,60} = 6.3$, $p = 0.003$). Combining both Ox groups, Ox treated patients performed worse on fear (0.83 vs 1.61, $p = 0.001$) for the 40% intensity trials.

Correlation physical aspects and quality of life

No significant correlations between adult height, adult height gain, Ferriman and Gallwey score, BMI, breast size and Tanner breast stage and quality of life were found (all Pearson correlation (r) ≤ 0.2). When comparing women with subjective virilization to women without subjective virilization no difference in quality of life was found (mean RAND score 77.6±14.3 and 82.5±14.9 respectively). Furthermore, no difference in quality of life was found when comparing women satisfied with adult height to women who were not satisfied (mean RAND score 80.3±15.1 and 77.9±13.2 respectively).

FIGURE 2
Emotion recognition task performance



Mean performance of the three different treatment modalities (Pl, Ox 0.03 and Ox 0.06) for the six emotions and the four intensities (percentage emotional expression 40, 60, 80 and 100 percent respectively).

DISCUSSION

We present the psychological and neuropsychological long-term follow-up data of the Dutch randomized double blind placebo controlled study on Ox in GH treated girls with TS at a mean of 8.7 years after discontinuation of Ox. Our exploratory study shows that women previously treated with Ox may have more feelings of anxiety and depression compared to those who had received Pl, although an overall effect on quality of life was not found. We found no long-term effect of Ox on neurocognition (i.e. intelligence, working memory and executive function). Regarding social-emotional functioning, only emotion perception for fearful faces was lower in the Ox groups, without affecting social functioning in daily life.

Our study is the first to explore quality of life using a follow-up assessment after discontinuation of Ox. We observed a trend towards lower quality of life in the Ox treated groups, which is likely related to the higher frequency of depressive complaints and the higher anxiety levels in the Ox groups. With respect to anxiety, the Ox treated groups taken together reported higher levels of anxiety (measured with the STAI) with a large effect size and a moderate, yet statistically not significant, effect on the anxiety subscale of the SCL-90-R. Considering depressive symptoms we observed a moderate significant effect on the depression subscale of the SCL-90-R, but depression measured with the BDI was not more common.

Studies on quality of life in relation to androgen use in TS are scarce. One previous study showed that *during* testosterone treatment in adult women with TS the quality of life improved.[21] Also in other patient groups androgen substitution has positive effects on well-being: for example in postmenopausal women after oophorectomy transdermal testosterone resulted in an improvement of well-being.[42] However, it is yet unclear if testosterone and Ox have comparable effects, since Ox is nonaromatizable.[43] Moreover, we do not know the exact effect of Ox on quality of life during treatment in our pediatric study, since quality of life was not measured in childhood. We cannot rule out that previous Ox treatment changed some aspects of quality of life with permanent effects years after treatment. A long-lasting effect of Ox on the endogenous androgen production is unlikely, because androgen levels during this follow-up were similar between the three treatment modalities (data not shown).

Additionally, we found no correlation between quality of life and certain physical effects of androgen treatment. First of all, objective and subjective virilizing effects were more frequently found in the Ox treated women.[41] We did neither find any differences in quality of life when comparing women with and without subjective virilization, nor a correlation between quality of life and the Ferriman and Gallwey score for hirsutism. Secondly, in our pediatric study we found that breast development was decelerated in the Ox treated groups.[16] One could hypothesize a negative effect of this experience on quality of life. However, the follow-up study after several years of treatment with adult estrogen dosages, did not reveal any differences in breast size. The current breast size did not correlate with quality of life and the women who

reported a subjective delay of breast development during puberty as compared to peers were equally distributed over the Pl and Ox groups. Thirdly, we speculated about a positive effect of height (gain) on quality of life, but in our study height was not correlated with quality of life. This is in line with previous studies in TS showing no positive effects of GH treatment and/or increased height on quality of life.[4,44,45] Previously Bannink *et al.* reported that patient satisfaction with adult height correlated positively with quality of life.[46] We could not confirm this observation, since we found no differences in quality of life between patients with and without satisfaction with their adult height.

In this study, we found that Ox treated women only performed worse on the perception and labeling of fearful faces at low intensity with a large effect size for both the Ox 0.03 and 0.06 group. Previous studies showed that a typical female and male emotion perception profile can be defined: females typically recognized the emotions happiness, fear and sadness more easily, where male participants were more accurate in recognizing anger.[47] Although the effects of Ox in our study were small (besides the effect on the emotion fear, only a non-significant trend for the emotions sadness and disgust), Ox may induce a slight shift towards a less 'feminine' emotion perception.[47] An alternative explanation for the difference in emotion perception could be the higher levels of anxious and depressive symptoms in the Ox treated groups, since it is known that these factors negatively influence the emotion perception.[48] However, the differences in emotion perception found in the current study are small and these outcomes did not affect the interpersonal behavior as assessed with the SIB. Still, future studies using larger samples should include measure of emotion perception to examine these hypotheses in more detail.

In this follow-up study we found no effect on PIQ, nor on VIQ and TIQ. Furthermore, we found no effect on executive function and working memory, constructs that show conceptual overlap with performance intelligence tests that assess fluid intelligence.[49] Probably the effect of Ox and other androgens on fluid intelligence tasks is only apparent during treatment, since others found differences during or directly after treatment.[21-23]

A higher frequency of sexual problems in adult TS women was previously reported.[50] In this study, we found no differences in sexual self-concept between the treatment groups. Consequently, Ox treatment during childhood does not seem to have a long-lasting effect on sexuality in adulthood.

A limitation of this study is the relatively small sample of patients, since not all participants of the original study participated in the follow-up study. In this light, selection bias might have occurred towards 'psychologically better performing' women. However, loss to follow-up did not differ between the treatment groups.

This is the first study to explore the long-term (neuro)psychological effects of Ox treatment in childhood and the effects seem to be limited. Our findings suggest that during late childhood

and adolescence androgen treatment may influence cerebral function in an organizational way. Although even small differences may be of greatest interest from a scientific point of view, their clinical relevance is probably limited. As a result, we certainly would not advice against the use of Ox -in low doses- in clinical practice. However, awareness of potential psychological consequences of androgen use in girls with TS is encouraged. Future studies in larger cohorts should include evaluation of quality of life and social-emotional functioning. These studies will hopefully also shed light on the differential effects on aspects of quality of life in TS.

In conclusion, our exploratory data show that treatment with Ox in childhood and adolescence has only limited long-term effects on feelings of anxiety and depression, and emotion perception of fearful faces. No late effects on overall quality of life or neurocognition were present. Therefore, we would not advise against the use of Ox in girls with TS as a growth-enhancing drug. Future research during and after androgen treatment in larger samples is necessary to establish the exact effect of Ox and to improve the understanding of the underlying pathofysiology.

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Pfizer had no role in the study design, in the collection and analysis of the data, in the writing of the report and in the decision to submit the article for publication.

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Chapter 6

Karyotype-specific ear and hearing problems in young adults with Turner syndrome and the effects of oxandrolone treatment

Kim Freriks*[1], Eva JJ Verver*[2], Theo CJ Sas[3,4], Patrick LM Huygen[5], Ronald JE Pennings[5], Dominique FCM Smeets[6], Ad RMM Hermus[1], Leonie A Menke[7,8], Jan M Wit[7], Barto J Otten[9], Janiëlle AEM van Alfen-van der Velden[9], Sabine MPF de Muinck Keizer-Schrama[3], Vedat Topsakal[2], Ronald JC Admiraal[5], Henri JLM Timmers[1], Dirk HPM Kunst[5]

*contributed equally

Author Affiliations

[1]Radboud university medical center, Nijmegen, Department of Internal Medicine, Division of Endocrinology, [2]University Medical Center Utrecht, Department of Otorhinolaryngology, [3]Erasmus Medical Centre, Rotterdam, Department of Pediatrics, [4]Albert Schweitzer Hospital, Dordrecht, Department of Pediatrics, [5]Radboud university medical center, Nijmegen, Department of Otorhinolaryngology, [6]Radboud university medical center, Nijmegen, Department of Human Genetics, [7]Leiden University Medical Centre, Department of Pediactrics, [8]Haga Hospital The Hague, Department of Pediactrics, [9]Radboud university medical center, Nijmegen, Department of Pediatrics

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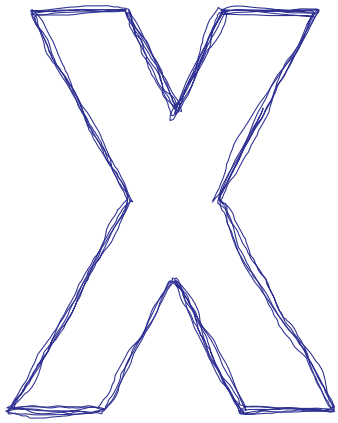


ABSTRACT

Objective

To evaluate karyotype-specific ear- and hearing problems in young adult patients with Turner syndrome (TS) and assess the effects of previous treatment with oxandrolone (Ox).

Study design

Double-blind follow-up study.

Setting

University hospital.

Patients

Sixty-five TS patients (mean age 24.3y) previously treated with growth hormone combined with placebo, Ox 0.03 mg/kg/day, or Ox 0.06 mg/kg/day from the age of eight and estrogen from the age of twelve years.

Intervention

Ear examination was performed according to standard clinical practice. Air- and bone conduction (AC and BC) thresholds were measured in dB hearing level (db HL).

Main outcome measures

We compared patients with total monosomy of the short arm of the X-chromosome (Xp) -monosomy 45,X and isochromosome 46,X,i(Xq)- to patients with a partial monosomy Xp -mosaicism or other structural X-chromosomal anomalies-. We assessed the effect of previous Ox treatment.

Results

Sixty-six percent of the patients had a history of recurrent otitis media. We found hearing loss in 66% of the ears including pure sensorineural hearing loss in 32%. Hearing thresholds in patients with a complete monosomy Xp were about 10 db worse compared to patients with a partial monosomy Xp. AC- and BC thresholds were not different between the placebo and Ox treatment groups.

Conclusions

Young-adult TS individuals frequently have structural ear pathology and many suffer from hearing loss. This indicates that careful follow-up to detect ear and hearing problems is necessary, especially for those with a monosomy 45,X or isochromosome 46,X,i(Xq). Ox does not seem to have an effect on hearing.

INTRODUCTION

Turner syndrome (TS), a chromosomal disorder with a prevalence of approximately 1 in 2000 live born girls, is caused by complete or partial absence of one X-chromosome. Prominent features are short stature, estrogen deficiency and dysmorphic abnormalities.[1] Monosomy 45,X, characterized by the presence of a single X-chromosome in all cells, is the most frequent karyotype. Other patients have a karyotype with a duplication of the long arm of the X-chromosome (isochromosome, 46,X,i(Xq)), mosaicism or another structural X-chromosomal anomaly. In general, women with monosomy 45,X have a higher disease burden than those with a mosaicism.[2,3]

Ear and hearing problems are frequently reported in TS. In childhood, middle ear infections resulting in conductive hearing loss are common.[4,5] Abnormal craniofacial morphology and Eustachian tube dysfunction are thought to be the main contributing factors. TS patients are also prone to develop sensorineural hearing loss.[6,7] A typical sensorineural mid-frequency dip is often already seen in adolescence. Progressive sensorineural high-frequency loss manifests earlier in TS women than in healthy women, especially in those patients already having a mid-frequency dip.[8]

TS patients with monosomy 45,X or isochromosome 46,X,i(Xq), *i.e.* those with monosomy of the complete short arm of the X-chromosome (Xp), are supposed to be more prone to hearing problems than those with mosaicism or other structural X-chromosomal anomalies, in which monosomy of Xp is only partial.[9,10] This suggests that the locus for hearing impairment may be located on Xp. Furthermore, estrogen deficiency is suggested to be a contributing factor in hearing problems.[7,11,12]

While growth hormone (GH) has been shown to improve final height, such treatment can only partially overcome the growth failure observed in TS. It is for this reason that adjunctive therapy has been tried, notably with oxandrolone (Ox), a synthetic anabolic steroid derived from dihydrotestosterone. In previous studies, we investigated the growth-enhancing effect of Ox, a weak androgen, in addition to GH in TS girls.[13] We, and others found that low-dose treatment of Ox increases growth velocity and adult height.[13-15] The effect of Ox on hearing has not been previously investigated. Theoretically, Ox may also positively affect the growth and development of the mastoid bone and middle ear. This in turn could lead to prevention of recurrent otitis media and conductive hearing loss. In addition, Ox could also positively affect inner ear function through a suspected decrease in sex hormone binding globulin and the resulting higher free estrogen levels.[16] However, negative effects of androgens on hearing are suggested as well.[17,18]

We recently carried out a follow-up study to determine the long-term effects of previous Ox treatment on growth and body composition in young adult women with TS.[19] This provided the unique opportunity to analyze the otologic and audiologic features of a well-characterized TS population. In addition to the evaluation of karyotype-specific ear- and hearing problems, the aim of the study was to investigate whether hearing is influenced by previous Ox treatment.

MATERIALS AND METHODS

Patients

All of the 133 participants of the original randomized double-blind placebo controlled trial were invited for a medical assessment including otologic and audiologic examination. Participants of the original trial were recruited between 1991 and 2003 in ten pediatric endocrine centers in the Netherlands and randomized to receive Ox in a dosage of 0.03 mg/kg/day (Ox 0.03), 0.06 mg/kg/day (Ox 0.06) or placebo (Pl) after reaching the age of 8 years.[13] Furthermore, girls were treated with GH (1.33 mg/m^2/day) from baseline and, when spontaneous puberty was absent, with estrogen from the age of twelve years. For more details see Menke *et al.*[13] and Freriks *et al.*[19] This follow-up study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the ethics committee of the Radboud University Nijmegen Medical Centre. Written informed consent was obtained for each participant. The study is registered at www.trialregister.nl (NTR1934).

Otologic and audiologic examination

All ears were macroscopically and micro-otoscopically examined according to standard clinical practice. Medical history was taken focusing on ear and hearing problems including questions about confounding factors such as meningitis, head injury and problems related to birth. Pure tone hearing thresholds were measured according to standard audiometric methods (ISO 389) in a sound proof room. Air conduction (AC) thresholds and bone conduction (BC) thresholds were measured in dB hearing level (db HL) at (0.25,) 0.5, 1, 2, 4, and 8 kHz.
We defined the following audiometric categories:

Normal hearing

The AC thresholds were equal to or better than 20 dB HL across the frequency range of 0.25-8 kHz.

Mild conductive hearing loss

The AC thresholds were never worse than 20 dB HL, though there was an air bone gap (ABG) of at least 10 dB at more than one frequency within normal range. By this classification we include these relevant ABGs in the analyses.

Conductive hearing loss (CHL)

AC thresholds were worse than 20 dB HL at one or more frequencies in the range of 0.25-8 kHz. There was an ABG of at least 10 dB at one or more frequencies at which the AC threshold was worse than 20 dB HL. BC thresholds were better than 20 dB HL at any frequency.

Mixed hearing loss (MHL)

The BC thresholds were worse than 20 dB HL at one or more frequencies in the range of 0.25-8 kHz and there was an ABG of at least 10 dB at one or more frequencies at which the BC threshold was worse than 20 dB HL.

Pure sensorineural hearing loss (SNHL)

The AC thresholds were worse than 20 dB HL at one or more frequencies in the range of 0.25-8 kHz and there was no ABG at the frequencies at which the AC threshold was worse than 20 dB HL.

Based on the BC thresholds of the MHL- and SNHL groups, we categorized the following subgroups:

Sensorineural mid-frequency dip

Audiograms showing a typical sensorineural mid-frequency dip at 1 and/or 2 kHz. The dip was defined as BC thresholds being at least 10 dB HL worse than all of the lower and higher frequencies.

Sensorineural mid- and high-frequency loss

Audiograms showing the typical sensorineural mid-frequency dip at 1 and/or 2 kHz in combination with a dip at 8 kHz. The dip at 8 kHz was only scored when worse or not more than 10 db better, as the maximum of the mid-frequency dip.

Sensorineural high-frequency loss

High-frequency SNHL was defined as BC thresholds worse than 20 dB HL at 4 kHz or higher frequencies or BC thresholds worse than 20 dB HL at frequencies below 4 kHz with an increasing loss with increasing frequency (down sloping).

Statistical analysis

We tested the hypothesis that patients with monosomy 45,X or isochromosome 46,X,i(Xq) karyotype (complete monosomy Xp) have a higher prevalence of hearing problems compared to those with a mosaicism or other structural X-chromosomal anomaly (partial monosomy Xp). Linear regression was used to analyze the influence of age and karyotype on hearing. An *F* test was used to compare between regression lines. Testing on the slopes between the regression lines was performed first and testing between the 'elevations' (measure that pertains to the group's 'offset' threshold) was only performed if there was no significant difference in

slope. The total effect of all frequencies was considered significant when the p value was <0.11 at three or more frequencies. Concerning the influence of previous Ox treatment we tested the differences in mean hearing thresholds between different dosage groups and placebo by testing linear regression using two dummies (for Ox 0.03 and Ox 0.06). We performed a modified intention to treat analysis, i.e. including only those patients who took at least one dose of the study medication. We used the Statistical Package for the Social Sciences version 16.0 (SPSS, Inc., Chicago, Illinois) and the Prism program (GraphPad, San Diego) where applicable.

RESULTS

Patients

Of the 133 original participants in the trial, 68 could be included in the general follow-up study. Besides lost to follow-up (n=9), excluded (n=7) and deceased (n=2), 47 women refused to participate. Main reasons for refusal were the burden of the investigations besides regular medical follow-up and travel distance. For the specific ear and hearing analyses we excluded one extra patient because of an age just under 18 years. Furthermore, in two patients no ear and hearing evaluation took place for logistic reasons. Table 1 shows the karyotype differentiation of the 65 patients who participated in the study (Pl n=22, Ox 0.03 n=26, Ox 0.06 n=17). The mean age was 24.3±3.6 years (range 18-32 years). One patient did not undergo otoscopic examination. For the analysis of the effects of previous Ox use, we excluded two more patients, who did not take any dose of the study medication.

Ear, Nose and Throat history

Data concerning history are presented in Table 1. Many patients had suffered from otologic disease. Two out of the five patients with previous surgery for cholesteatoma suffered from cholesteatoma in both ears. Recurrent cholesteatoma occurred in five ears and surgery finally resulted in a modified radical cavity in five out of seven ears.

External ear findings

Forty-two of the 65 patients (65%) had one or more external ear anomalies including dysmorphic auricles, posteriorly rotated ears, low set ears and/or abnormally protruding ears varying from only mild to more severe forms.

TABLE 1
Karyotype distribution in relation to ear, nose, and throat history, eardrum and middle ear findings and audiometric results

	Monosomy 45,X (N=32 patients, 49%)	Isochromosome[a] (N=10 patients, 15%)	Mosaicism/ structural anomaly[b] (N=23 patients, 35%)	Total (N=65 patients or 130 ears)
Ear, nose, and throat history (N=65 patients)				
Recurrent otitis media	25 (78%)	6 (60%)	12 (52%)	43 (66%)
Adenotomy/tonsillectomy/adenotonsillectomy	21 (66%)	7 (70%)	12 (52%)	40 (62%)
Ventilation tubes	24 (75%)	5 (50%)	10 (43%)	39 (60%)
Otologic surgery[c]	7 (22%)	1 (10%)	2 (9%)	10 (15%)
Cholesteatoma	3 (9%)	0 (0%)	2 (9%)	5 (8%)
Hearing aid/BAHA at present time	3 (9%)	0 (0%)	1 (4%)	4 (6%)
Eardrum and middle ear findings (N=128 ears)[d]				
Myringosclerosis	21 (34%)	5 (25%)	16 (35%)	42 (33%)
Retraction	10 (16%)	0 (0%)	6 (13%)	16 (13%)
Conservative radical cavity	3 (5%)	0 (0%)	2 (4%)	5 (4%)
Atelectasis	1 (2%)	2 (10%)	1 (2%)	4 (3%)
T-tube	2 (3%)	0 (0%)	1 (2%)	3 (2%)
Perforation	2 (3%)	0 (0%)	1 (2%)	3 (2%)
Otorrhoea	2 (3%)	1 (5%)	0 (0%)	3 (2%)
Postmyringoplasty	1 (2%)	0 (0%)	0 (0%)	1 (1%)
Erosion of malleus/incus	0 (0%)	0 (0%)	1 (2%)	1 (1%)
Audiometric results (N=130 ears)				
Normal hearing	18 (28%)	2 (10%)	24 (52%)	44 (34%)
Mild conductive hearing loss	1 (2%)	0 (0%)	2 (4%)	3 (2%)
Conductive hearing loss	5 (8%)	1 (5%)	4 (9%)	10 (8%)
Mixed hearing loss	21 (33%)	4 (20%)	6 (13%)	31 (24%)
Pure sensorineural hearing loss	19 (30%)	13 (65%)	10 (22%)	42 (32%)

[a] Pure 46,X,i(Xq) (N=4) or in combination with 45,X: 45,X/46,X,i(Xq) (N=6).
[b] 45,X/46,XX (N=5), 45,X/46,X,r(X) (N=4), 45,X/46,X,del(X) (N=3), 45,X/47,XXX (N=3), 45,X/46,X+mar (N=2), 46,X,del(X) (N=1), other (N=5, including 4 patients with mosaicisms containing more than two cell lines and one patient with a complex mosaicism: 46,X,Xp-/46,X,i(Xq)).
[c] Including mastoidectomy, tympanoplasty, myringoplasty and operations for cholesteatoma resulting in a modified radical cavity.
[d] Excluding one patient with monosomy 45,X who did not underwent otoscopic examination.
BAHA=bone anchored hearing aid

Eardrum and middle ear findings

Myringosclerosis and tympanic membrane retraction were the most common findings, occurring in 42/128 (33%) and 16/128 (13%) of the ears, respectively. An overview of all eardrum and middle ear findings is shown in Table 1. Some ears showed multiple pathology.

Audiologic examination

Normal hearing was present in 44/130 (34%) ears. The other ears could be classified in one of the categories of abnormal audiometric results (Table 1). CHL was found in 10/130 (8%) ears. Figure 1A shows the hearing thresholds of the ears in which the mean AC was worse than 25 dB, counting 5/130 (4%) ears. The other 5 ears showed CHL at one or more frequencies, though the mean hearing threshold of all frequencies was better than 25 dB. MHL was present in 31/130 (24%) ears (Figure 1B). Pure SNHL was found in 42/130 (32%) ears (Figure 1C). Figure 1C only shows the ears in which the mean AC threshold was worse than 25 dB, counting 15/130 (12%) ears.

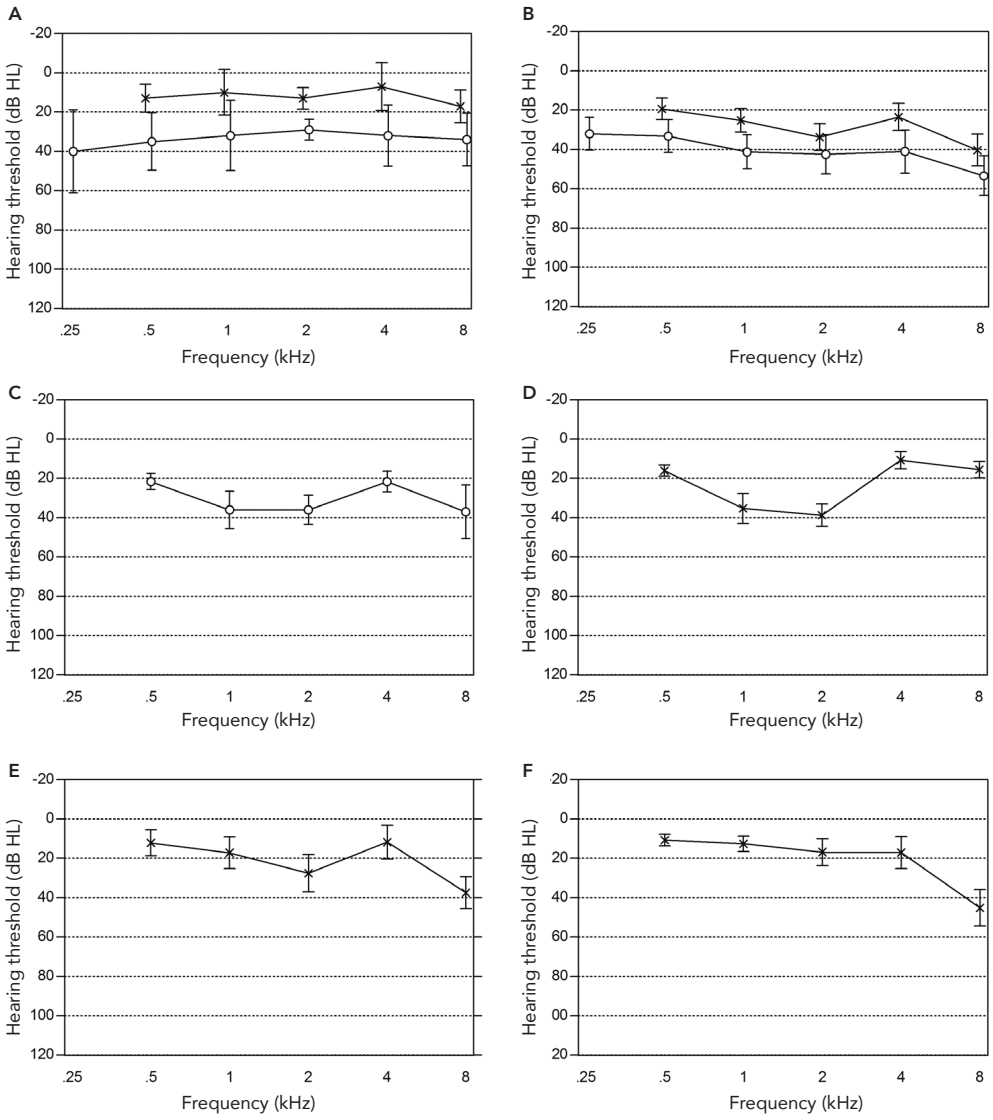
Concerning only the BC thresholds of the MHL-and SNHL group, the following audiogram configurations were seen (Figure 1D-E-F): a typical sensorineural mid-frequency dip (19/130 ears, 15%), a combined mid-and high-frequency sensorineural hearing loss (14/130 ears, 11%) and high-frequency sensorineural hearing loss (27/130 ears, 21%). The remaining 13/130 (10%) ears showed BC-threshold configurations that could not be classified in one of these categories.

Audiometric findings in relation to karyotype

Linear regression showed no significant deterioration in mean BC thresholds with increasing age of each patient, for both the patients with a complete monosomy Xp and those with a partial monosomy Xp. Furthermore, there was no significant difference in slopes between both groups. However, there was a significant difference in elevation indicating a substantial difference in hearing thresholds, being about 10 dB worse for patients with a complete monosomy Xp compared to those with a partial monosomy Xp. Figure 2 shows an example at 2 kHz.

Regression analysis for AC thresholds showed no progression with age. The differences in AC thresholds were similar, being about 10 dB worse in the patients with complete monosomy Xp compared to those with a partial monosomy Xp. Figure 3 shows an example at 1 kHz.

FIGURE 1
-O- : Air conduction, -X- : Bone conduction



A Mean air conduction (AC) and bone conduction (BC) threshold for the conductive hearing loss (CHL) group, only showing the ears with a mean AC threshold worse than 25 dB (n = 5 ears).

B Mean AC and BC threshold for the mixed hearing loss (MHL) group (n = 31 ears).

C Mean AC threshold for the sensorineural hearing loss (SNHL) group, only showing the ears with a mean AC threshold worse than 25 dB (n = 15 ears). Six audiograms of the SNHL group had an ABG of 10-20 dB at more than one frequency at which the BC was within normal range.

D Mean BC threshold for the sensorineural mid-frequency dip group (n = 19 ears).

E Mean BC threshold for the group showing combined mid-and high-frequency sensorineural hearing loss (n=14 ears).

F Mean BC threshold for the group with high-frequency sensorineural hearing loss (n=27 ears).

FIGURE 2

Mean bone conduction (BC) thresholds at 2 kHz plotted as a function of age for the patients with a monosomy/isochromosome (--- and o), and those with a mosaicism/structural anomaly (— and ●) (n = 65 patients). Linear regression analysis shows a significant difference in elevation between both groups.

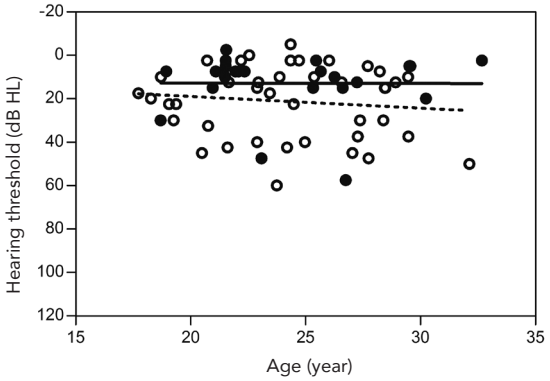
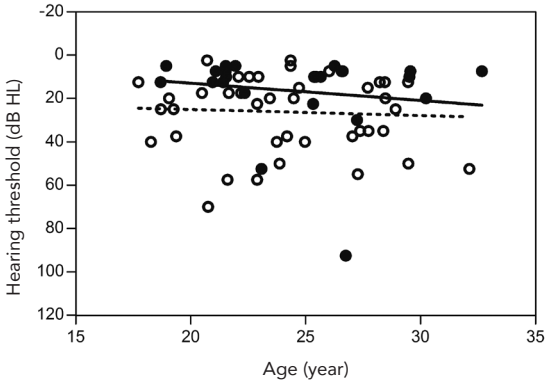


FIGURE 3

Mean air conduction (AC) thresholds at 1 kHz plotted as a function of age for the patients with a monosomy/isochromosome (--- and o), and those with a mosaicism/structural anomaly (— and ●) (n =65 patients). Linear regression analysis shows a significant difference in elevation between both groups.



Oxandrolone and hearing

Regarding the influence of Ox on hearing we found that mean AC- and BC thresholds were not significantly different in the three treatment groups (Pl, Ox 0.03 and Ox 0.06, see Table 2). All *p*-values were >0.11 at all frequencies. We found no relevant correlation with height, adult height gain and duration of Ox use (data not shown).

TABLE 2
Mean hearing thresholds related to oxandrolone use (n=63)[a]

	GH+Pl (n=22)	GH+Ox 0.03 (n=24)	GH+Ox 0.06 (n=17)
Air conduction[b]			
0.25 kHz	18.3±10.3	18.6±12.7	20.3±21.9
0.5 kHz	17.7±10.3	20.1±11.6	22.5±22.1
1.0 kHz	19.0±15.0	24.3±16.7	25.3±25.3
2.0 kHz	21.6±19.8	20.0±14.7	23.7±28.3
4.0 kHz	18.6±16.5	17.8±17.3	22.5±23.3
8.0 kHz	28.4±20.7	28.5±21.9	29.7±24.3
Bone conduction[b]			
0.5 kHz	12.2±9.4	14.0±9.7	12.6±11.4
1.0 kHz	12.7±13.6	18.8±14.9	15.9±15.1
2.0 kHz	19.7±18.0	17.2±13.5	18.2±18.7
4.0 kHz	11.8±13.4	8.5±14.0	10.9±11.3
8.0 kHz	21.9±16.8	23.8±21.7	22.6±17.2

[a] Excluding those two patients who never took any dose of the study medication.
[b] All P values for the comparison of GH+Ox 0.03 and GH+Ox 0.06 to Placebo are > 0.1.
GH=growth hormone; Pl=placebo; Ox 0.03=oxandrolone 0.03 mg/kg/day; Ox 0.06=oxandrolone 0.06 mg/kg/day

DISCUSSION

Middle ear disease

The prevalence of a history of recurrent otitis media of 66% is consistent with previous reports on TS.[4,6,9,10,20-25] Ventilation tubes were placed in 60% of the patients. Other studies showed lower percentages varying from 32 to 57.[4,6,21,26,27]

Middle ear infections in TS are thought to be related to an abnormal craniofacial morphology, including a high arched palate and an abnormal orientation of the Eustachian tube. Rizell *et al.* showed that TS patients have a short posterior and flattened cranial base, retrognathy, short and posteriorly rotated maxilla and mandible, increased ramus height, and relatively shorter posterior facial height.[28] These abnormalities are probably the result of a prolonged cell cycle in the brachial arches and neck region and a lack of transacting growth-regulating genes, such as the SHOX–gene.[29] A subnormal immune response could also be a contributing factor in recurrent infections.[4,20]

Audiometric findings

Normal hearing was found in only 34% of the ears. CHL was found in 8% of the ears. In other TS populations percentages of CHL vary between 0 and 44, depending on the age group included.[4,5,9,20,21,23,25,27,30,31] In our previous report on TS children (age 4.1-21.1 years) CHL was more common with a percentage of 33.[10] The relatively low percentage of CHL found in the current adult group might be explained by the lower prevalence of middle ear pathology at increasing age. Others found no pure CHL in an adult population.[23]

MHL was present in 24% of the ears, which is more common than in our pediatric cohort (14%).[10] The range of MHL described in literature varies from 3 to 23%.[5,20,21,23,24,27,30-32]

We found a high percentage of pure SNHL in 32% of the ears. The percentages of SNHL described in literature are highly variable in a range of 4 to 66.[5,9,10,20,21,25,27,30-33] The prevalence of pure SNHL was much higher than the four percent in our pediatric population.[10]

Regarding the bone conduction thresholds of the MHL- and SNHL group we found typical audiograms with a mid-frequency dip (15%), combined mid-and high-frequency loss (11%) and high-frequency loss (21%). In literature, percentages of a mid-frequency dip vary between 8 and 88.[4,6,8-10,21,24,31-33] This wide range might be caused by different definitions and age categories.[6,24] Our previous study on TS children showed a mid-frequency dip in 17% of the ears. The mean depth of the dip in the current adult patients is 38.7 dB, which is more pronounced compared to 25 dB reported in TS children.[10] The dip maximum exceeded 20 dB in all adult cases in contrast with the findings in TS children where the dip maximum was within normal range as well.[4,6,10] In the present study, 11% of the ears showed a combined mid-and high-frequency loss, which is more than was found in TS children (6%).[10] It is likely that the high-frequency loss develops after the dip in the same individual.[23] A high-frequency loss was the most common finding in

21% of the ears of the adult TS patients. Our study on TS children showed a high-frequency loss in 4% of the ears. This confirms that sensorineural hearing levels in TS decrease with age, as we and others demonstrated previously.[6,8,10,23]

The cause of SNHL in TS is not clear. Specific cochlear malformations have not yet been revealed.[21,34,35] In two TS patients with SNHL hypoplastic lateral semicircular canals were found.[36] Furthermore, the cell cycle delay hypothesis might be an explanation for SNHL.[29] In general, the middle turn of the cochlea has the highest hair cell density,[37] and this is where the cell cycle needs to be up-regulated the most during development to acquire a sufficient number of sensory hair cells for signal analysis. In TS it is speculated that this middle part of the cochlear duct is most prone to growth disturbances due to a delayed cell cycle which might cause a mid-frequency dip.[29] The early onset of the high-frequency hearing loss in TS is also suggested to be the result of a reduced number of sensory hair cells.[29]

Low estrogen levels in TS women could be a possible contributing factor to SNHL, since in women without TS more rapid hearing deterioration occurs after the menopause when circulating levels of endogenous estrogen drop rapidly.[38] Estrogen receptors have been shown to exist in human inner ear.[12] However, since almost all women used adequate estrogen replacement therapy at moment of audiometric measurement, this could not be an explanation for the differences found in this study.

Oxandrolone and hearing

We and others have shown that, when given at a low dose, the weak androgen steroid Ox has a growth-enhancing effect in girls with TS.[13-15] This is the first study to investigate whether there is an association between previous Ox use and hearing. We hypothesized that Ox would have a beneficial impact on hearing.

First, along with the growth stimulating effect of Ox on body height, Ox may also enhance the growth and development of the mastoid bone, middle ear cavity, eardrum and outer ear canal. This might occur through increase in insulin like growth factor 1 (IGF-1) observed in TS patients treated with Ox.[13,16,39] Low IGF-1 levels are associated with hearing loss.[40] Both middle ear infections and sensorineural hearing loss were related to low IGF-1 levels in TS.[29] Subtle improvement in growth and development of the ear resulting in a more physiological anatomy could to some extent prevent recurrent otitis media and hearing loss. Our current findings indicate, however, that there were no differences in mean hearing thresholds between the groups treated with Pl, Ox 0.03 and Ox 0.06. Unfortunately, audiologic examinations were not performed during treatment, so effects may have been transient.

Second, Ox could also positively influence hearing through estrogen-mediated effects on the inner ear. Estrogen deficiency may have deleterious effects on hearing, which was suggested to

be caused by lack of stimulation of the estrogen receptors ER- Ð and ER-Ð, which are abundantly present in the inner ear.[7,11,12] Ox treatment is expected to decrease estrogen protein binding and in turn increase free active estrogen levels, which could positively affect inner ear function. In our study, however, we did not discern any differences in hearing thresholds between the treatment groups. This might be related to the fact that all patients received proper estrogen replacement therapy, with no additional effects of Ox on the availability of estrogen in the ear.

In theory, beneficial effects might also be counterbalanced by disadvantageous effects of androgens. Previous studies suggested that prenatal exposure to androgens negatively influences the auditory system by weakening of the cochlear mechanisms that underlie the production of otoacoustic emissions.[17] In addition, there is some evidence that circulating androgen levels postnatally might negatively influence otoacoustic emissions as well.[41,42] In men higher levels of circulating testosterone were found to be associated with smaller click-evoked otoacoustic emission amplitudes[42] and male monkeys produced click-evoked otoacoustic emissions of lower amplitude during the breeding season, when testosterone levels are higher.[41] In human females, data are controversial. One study reported that females with polycystic ovary syndrome (PCOS) -having higher androgen levels- showed significantly more high frequency hearing loss compared to women without PCOS.[18] On the other hand, a recent study in women with PCOS showed that hyperandrogenism did not seem to influence otoacoustic emission levels in adult female subjects.[43]

Audiometric findings in relation to karyotype

We found that hearing thresholds in general were about 10 dB worse in adults with a complete monosomy Xp compared to those with a partial monosomy Xp. This agrees with previous findings in TS children and adults.[9,10] These results support the hypothesis that genes located on the short arm of the X-chromosome (Xp), are of importance in hearing.

Otologic surgery

Cholesteatoma was found in as many as 8% of the patients (5 patients, 7 ears). A canal wall down procedure was necessary in 5 out of 7 ears. This can be problematic in the hearing aid dependent TS patient with a tendency to develop recurrent ear infections. In those patients a bone anchored hearing aid may offer a solution. A previous study in young adults reported cholesteatoma in 2.3%.[21] Previous studies in TS children showed percentages ranging from three to five percent.[10,34] Abnormal craniofacial morphology and Eustachian tube dysfunction could be contributing factors in developing cholesteatoma. Early insertion of ventilation tubes should be considered in TS patients who suffer from middle ear disease.

CONCLUSION

This systematic otologic and audiologic evaluation in a well-described young-adult TS population treated with Ox or placebo in addition to GH in childhood shows that TS women frequently have eardrum- and middle ear pathology and that many of them suffer from hearing loss. Sensorineural hearing loss, in contrast to conductive hearing loss, is more pronounced in young-adult TS women than in TS children. Hearing thresholds in patients with a monosomy 45,X or isochromosome 46,X,i(Xq) (complete monosomy Xp) are significantly worse compared with those having a mosaicism or other structural X-chromosomal anomaly (partial monosomy Xp). These findings indicate that careful follow-up is necessary to detect ear and hearing problems, especially for those with monosomy 45,X or isochromosome 46,X,i(Xq). Ox, which at a low dose has a positive effect on growth, has no long-term effects on hearing.

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Chapter 7

Summary and perspective

Partly based on 'Safety and efficacy of oxandrolone in growth hormone-treated girls with Turner syndrome: evidence from recent studies and recommendations for use'

Theo CJ Sas[1,2], Emma J Gault[3], Martha Zeger Bardsley[4], Leonie A Menke[5], Kim Freriks[6], Rebecca J Perry[3], Barto J Otten[7], Sabine MPF de Muinck Keizer-Schrama[2], Henri JLM Timmers[6], Jan M Wit[5], Judith L Ross[4], Malcolm DC Donaldson[3]

Author affiliations

[1]Albert Schweitzer Hospital, Dordrecht, Department of Pediatrics, [2]Erasmus Medical Centre, Rotterdam, Department of Pediatrics, [3]University of Glasgow, Department of Child Health, Royal Hospital for Sick Children, Glasgow, United Kingdom, [4]Thomas Jefferson University, Department of Pediatrics, Philadelphia, United States, [5]Leiden University Medical Center, Department of Pediatrics, [6]Radboudumc, Nijmegen, Department of Internal Medicine, Division of Endocrinology,[7]Radboudumc, Nijmegen, Department of Pediatrics

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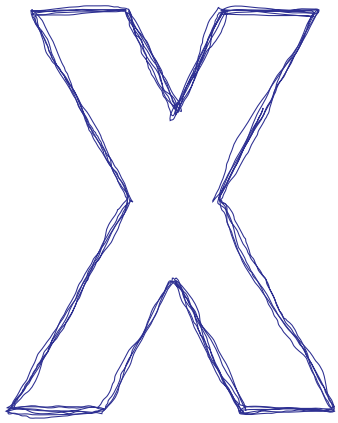

Summary

This thesis focuses on several aspects of health care in adult women with Turner syndrome (TS), dealing with diagnostic strategies and the long-term effects of oxandrolone (Ox) treatment.

In **chapter 2** we report the yield of an initial standardized multidisciplinary screening in adult TS patients in our multidisciplinary care unit. All 150 adult women with a mean age of 31 years old (47% karyotype 45,X) were consulted by an endocrinologist, a gynecologist, a cardiologist, an otorhinolaryngologist, and when indicated, a psychologist. The screening included magnetic resonance imaging of the heart and aorta, echocardiography, electrocardiography, dual-energy x-ray absorptiometry, renal ultrasound, audiogram, and laboratory investigations. We showed that medical care according to international expert opinion based guidelines yields significant morbidity. Furthermore we found that 30% of the patients currently lacked medical follow-up, and 15% lacked estrogen replacement therapy in recent years. We concluded that standardized multidisciplinary evaluation of adult women with TS as advocated by expert opinion is effective and that girls with TS probably benefit from a careful transition to ongoing adult medical care.

Chapter 3 describes the results of genetic analysis of a second cell line in TS. In general, the diagnosis is based on karyotyping of 30 blood lymphocytes. This technique, however, does not rule out tissue mosaicism or low grade mosaicism in the blood. Because of the associated risk of gonadoblastoma, mosaicism is especially important in case this involves a Y chromosome. We investigated different approaches to improve the detection of mosaicisms in 162 adult women with TS (mean age 29.9±10.3). Standard karyotyping identified 75 patients (46.3%) with a non-mosaic monosomy 45,X. Of these 75 patients, 63 underwent additional investigations including FISH on buccal cells with X- and Y-specific probes and PCR-Y on blood. FISH analysis of buccal cells revealed a mosaicism in almost one third of the patients. In five patients the additional cell lines contained a (derivative) Y chromosome. With sensitive real-time PCR we confirmed the presence of this Y chromosome in blood in three of the five cases. Although Y chromosome material was established in ovarian tissue in two patients, no gonadoblastoma was found. Our results confirm the notion that TS patients with 45,X on conventional karyotyping often have tissue specific mosaicisms, some of which include a Y chromosome. Although further investigations are needed to estimate the risk of gonadoblastoma in patients with Y chromosome material in buccal cells, we conclude that FISH or realtime PCR on buccal cells should be considered in TS patients with 45,X on standard karyotyping.

In **chapter 4, 5 and 6** we discuss the long-term effects of Ox in a population of young adult TS patients. During the pediatric randomized controlled trial, 133 girls were treated with GH combined with placebo (Pl), Ox in a dose of 0.03 mg/kg/day (Ox 0.03), or Ox in a dose of 0.06 mg/kg/day (Ox 0.06) from 8 years of age and estrogen from 12 years. The pediatric study showed that Ox 0.03 significantly increased adult height gain, whereas Ox 0.06 did not, at the cost of deceleration of breast development and mild virilization. Sixty-eight women (Pl, n=23;

Ox 0.03, n=27; and Ox 0.06, n=18) participated in the double-blind follow-up study (mean age, 24.0 years; mean time since stopping GH, 8.7 years; and mean time of Ox/Pl use,4.9 years).
In **chapter 4** we assessed height, body proportions, breast size, virilization and body composition. We found that height gain (final minus predicted adult height) was maintained at follow-up (Ox 0.03 10.2±4.9 cm, Ox 0.06 9.7±4.4 cm vs Pl 8.0±4.6 cm). Breast size, Tanner breast stage, and body composition were not different between groups. Ox-treated women reported more subjective virilization and had a lower voice frequency.
In **chapter 5** neurocognition, quality of life and social-emotional functioning were assessed. We found no effects on neurocognition. Concerning quality of life women treated with Ox had higher anxiety levels when measured with the STAI and higher scores on the depression subscale of the SCL-90-R, without differences in actual depression measured with the BDI. We found no effect on overall quality of life. Regarding social-emotional functioning, only emotion perception for fearful faces was lower in the Ox-treated patients, without effect on interpersonal behavior. In **chapter 6** we describe ear examination according to standard clinical practice and air- and bone conduction (AC and BC) thresholds in dB hearing level (db HL). For the total group we showed that 66% of the patients had a history of recurrent otitis media. We found hearing loss in 66% of the ears including pure sensorineural hearing loss in 32%. Hearing thresholds in patients with a complete monosomy Xp were about 10 db worse compared to patients with a partial monosomy Xp. Concerning ear and hearing we found no effect of Ox.

In conclusion, based on the results of **chapter 4, 5 and 6,** we show that Ox 0.03 mg/kg/day has a beneficial effect on adult height gain in TS patients. Despite previously reported deceleration of breast development during Ox 0.03 treatment, adult breast Tanner score and breast size are not affected. Mild virilization (including lower voice frequency) persists in only a small minority of patients. Our exploratory data on Ox suggest that androgen treatment in adolescence has long-term effects on certain aspects of adult quality of life and social-emotional functioning, although differences are small and clinical impact probably limited. Ox does not seem to have an effect on hearing. We conclude that the use of Ox in a dosage of 0.03 mg/kg/day is effective and safe.

Perspective

Diagnostic strategy

Adult women with TS potentially deal with a wide range of morbidities. This has been increasingly recognized and for several years now, devoted multidisciplinary care units are being advocated, as well as the use of expert opinion based guidelines.[1] We have evaluated the implementation of a standardized diagnostic screening, as advocated in these guidelines, and we have shown that the initial visits to our multidisciplinary unit yield significant previously undiagnosed morbidity.[2] TS patients are therefore likely to benefit from a careful transition from pediatric into adult medical care and a screening program similar to ours has now been implemented in the recent guidelines of the Dutch-Flemish Turner platform.[3] Beyond optimal and timely diagnosis of TS related morbidities, the ultimate goal of health care in TS patients is to improve long-term outcome, improve health related quality of life and reduce mortality. The approach should be cost-effective and outweigh the benefits of medical screening against the burden of repetitive examinations in individual patients.

Since we have moved into the era of personalized medicine, the need for an individualized approach to the patient is increasingly recognized. This seems particularly important in disorders with highly variable phenotypes, such as TS. For example, genotype and age are factors that likely need to be taken into account for tailor-made personalized care in TS. It has been well established that complete monosomy 45,X results in a higher disease burden than 45,X/46,XX mosaicism.[4,5] For example, in our study we have shown that the chance for structural cardiovascular abnormalities is higher in the 45,X group than in patients with mosaicism.[2] Furthermore, women with 45,X are more prone to infertility given the fact that only 8-9% develops (temporary) menstrual cycles compared to 40-47% of the girls in the group with 45,X/46,XX.[6,7] Also, patients with isochromosome Xq have a distinctive profile with a higher prevalence of diabetes mellitus and probably a higher frequency of auto-immune diseases and increased levels of anti-TPO and anti-GAD antibodies.[8-10] Additionally, it is shown that patients with a total loss of one of the short arms of the X-chromosome are more likely to develop hearing loss.[11-13] Taking these genotype-specific differences into account could result in a more personalized health care for TS patients. Besides genotype, age appears to be an important determinant of TS associated morbidity. The frequency of aortic dilation, autoimmune disease, cardiovascular risk factors and hearing loss increases with age.[2,9,13-15] This suggests that the attention for these health issues should be intensified with increasing age as a step towards more personalized medicine.

Another issue that needs to be resolved in the diagnostic strategy in TS patients is the lack of disease-specific reference values for several physical parameters. We know that the reference values for bone mineral density and aortic diameters of the general population are not directly applicable in TS patients. This lack of TS specific values hampers decision making in clinical

practice. Although the actual frequency of osteoporosis in TS is not well established, fracture risk in adult women with TS is assumed to be increased, which makes adequate measurement of bone mineral density important.[16] We know that conventional two-dimensional 'dual-energy X-ray absorptiometry' (DEXA) measurements underestimate the bone mineral density in smaller persons, especially in persons shorter than 150 cm.[17] It is therefore recommended to use volumetric instead of areal bone mineral density.[17,18] However in clinical practice reference values for volumetric bone mineral density are currently lacking.

For aortic diameters current opinion is to use aorta size index (aortic diameter/body surface are) instead of aortic diameters. Stricter follow-up is necessary in case of an aorta size index ≥ 2.0 cm/m^2 and evaluation for prophylactic intervention is advised when the aorta size index exceeds 2.5 cm/m^2 in combination with an absolute aorta diameter above 3.5 cm.[19] However, aortic diameters probably need to be put in the context of other risk factors for aortic dilation and dissection. A nice example of individualized medicine is the use of a dynamic predictive model based on subsequent MRI images of the aorta and other characteristics such as the presence of a bicuspid aortic valve and/or coarctation, diastolic blood pressure, age, body surface area and the use of antihypertensive medication (http://www.biostat.au.dk/MERL/Aorta_Prediction_model.htm).[20] Although not yet validated, this model seems more reliable in identifying high risk patients than static data alone and enables a more specified follow-up.

Treatment

The decreased life expectancy of TS patients is largely determined by excess cardiovascular morbidity related to structural anomalies and cardiovascular risk factors.[21,22] Therefore, a TS specific approach to cardiovascular risk management is probably warranted. Unfortunately, there is a lack of randomized controlled trials regarding optimal timing of surgical interventions of the aorta and the management of hypertension, hypercholesterolemia and diabetes. It is unknown whether the guidelines for the general population are applicable. Considering hypertension no uniform treatment goals are defined and it is assumed that TS is an additional risk factor in the cardiovascular risk assessment, as is for example diabetes.[23] Additional cardiovascular abnormalities urge for stricter antihypertensive treatment. Regarding optimal drug treatment for hypertension in the context of TS related anomalies of the thoracic vessels (i.e. bicuspid aortic valve, coarctation of the aorta, aortic dilation) many uncertainties exist. Because of the lack of randomized controlled trials in TS, currently the use of betablockers, in line with Marfan syndrome, is advocated.[1] However, it is not unlikely that other antihypertensive drugs, especially ACE inhibitors, are superior in this context.[23]

Also for optimal hormone replacement therapy (HRT) in adult women with TS no randomized controlled trials comparing different regimens exist. For induction of puberty transdermal applied estrogens have shown a physiological response with the possibility of

achieving a diurnal rhythm.[24,25] Omitting the first-pass effect of the liver will result in less effect on liver proteins. However, large trials comparing oral and transdermal applications are lacking. Small studies suggest that transdermal patches result in more physiological estrogen levels with faster bone accrual, increased uterine growth and probably increased lean body mass.[26-28] However, transdermal low-dose patches for induction of puberty are lacking. But also in adulthood, although theoretically superior, transdermal estrogens are not used as frequent as expected. The guideline advocates to personalize hormone replacement therapy and generally continue HRT till normal age of menopause.[1]

Optimal fertility treatment and counseling is a subject of debate as well. Since only approximately 2-5 percent of the TS have spontaneous pregnancies,[29,30] assisted pregnancy is frequently discussed. Until recently, assisted pregnancy with oocyte donation was the only option for TS women with infertility and still, possibilities of having a child of their own are limited. Since most of the TS women have developed ovarian failure at the time of family planning in vitro fertilization is often not possible. Preservation of oocytes and ovarian tissue in adolescence is therefore upcoming, but harbors many medical and ethical challenges. In the international literature it is suggested that ovarian tissue preservation could be considered in patients older than 14 years with spontaneous puberty, a follicle stimulating hormone (FSH) below 40 IU/l, a measurable anti mullerian hormone, a normal cardiac status in combination with informed consent and research ethics approval.[31] And although clinical practice in some Scandinavian countries, in the Netherlands cryopreservation of oocytes and ovarian tissue of TS women is still considered experimental.[3] A survey in the dedicated TS clinics in the Netherlands revealed that three academic centers offer such a procedure to adult (at least older than 16) mosaic TS women with regular menstrual cycles. Regardless of the technique used, pregnancies in TS women are considered high risk and preconceptive cardiovascular screening is necessary.

Impact of novel karyotyping

The approach to karyotyping in TS and its implications are a subject of ongoing debate. The diagnosis of TS is based on cytogenetic analysis of 30 cultured blood lymphocytes.[1,32,33] Currently, the international guidelines do not comment upon the investigation of other tissues besides lymphocytes in patients with proven TS without clinical virilization or marker chromosomes. Our study is one among others that shows that tissue mosaicisms in patients with apparent 45,X are rather common.[34-37] This is in line with the theory that for a TS fetus to survive to term an additional cell line besides 45,X is needed.[38,39] The question remains which technique is superior to detect additional cell lines in different tissues. Several studies, including ours, showed that FISH is very helpful in identifying additional cell lines in tissues different from lymphocytes,[34,36,37] while others advocate the use of PCR.[35] Finding additional cell lines is especially important when it involves a (derivative) Y chromosome, since a Y chromosome in

patients with gonadal dysgenesis is linked to an increased risk of gonadoblastoma and associated malignant dysgerminoma.[40] The exact risk is difficult to estimate and dependent on the existence of undifferentiated gonadal tissue, but likely lower than 15%, especially when no signs of virilization are present.[41] The tumor risk associated with a Y chromosome found with other techniques than conventional karyotyping and in other tissues besides lymphocytes should be a focus for future research. At this moment, we see no reason to assume that Y chromosomal derivates found in other tissues than lymphocytes, harbor another chance for Y chromosomal tissue in the gonads than Y chromosome derivates found with conventional karyotyping of lymphocytes only. However, even when the risk is small, gonadectomy seems appropriate in light of non functional gonads in most of the women with TS and relatively low operation risks. Therefore, in TS patients with apparent 45,X we would recommend routine screening for tissue mosaicisms by karyotyping an additional tissue, such as easy obtainable buccal mucosa cells. It is of importance to investigate the incidence of gonadoblastoma in case of the finding of a Y chromosome in any tissue in large groups of patients in order to improve the knowledge about Y chromosome material in women with TS and to prevent the development of gonadoblastoma on the one hand and prevent unnecessary gonadectomies on the other hand.

Treatment with oxandrolone in growth hormone and estrogens treated girls: long-term effects

Short stature is one of the most prominent features of TS and related to haploinsufficiency of the 'short-stature homeobox-containing' (SHOX) gene.[42] Although patients with TS are not GH deficient, suprafysiological GH doses increase adult height by 5-12cm.[43-45] The effect of GH treatment on adult height in TS girls is not always satisfying. Differences in outcome can be explained by age at initiation and duration of therapy, compliance, estrogen therapy regimen, and several, partly unknown, genetic factors.[44,46,47] In order to improve adult height alternative therapies were suggested, and besides higher doses of GH the addition of oxandrolone (Ox) was investigated. Initial studies on Ox were not controlled and did not report on long-term effects of Ox.[48-51] Our studies on Ox are among two other randomized controlled trials on the growth-enhancing effect of Ox. Our initial study in the Netherlands investigated the effect of Ox in a low dose of 0.03 mg/kg/day (Ox 0.03) and a previously conventional dose of 0.06 mg/kg/day (Ox 0.06) compared to placebo (Pl) in standard growth hormone and estrogen treated girls.[52] The second study is from the US and addressed the effect of Ox at a dosage of 0.06 mg/kg/day in addition to GH. The third study from the UK investigated Ox in a dosage of 0.05 mg/kg/day.[53,54]

Although outcome measures differed, taking these three randomized controlled trials together, the average effect of Ox on adult height gain is between the 2.3 en 4.6 cm.[55] Differences in the effect of Ox probably are explained by differences in patient characteristics and dosage

regimens. For study characteristics and an overview of the main results of the three different studies see Table 1 and 2 respectively. In our pediatric study we found that compared with Pl, Ox 0.03 significantly increased adult height gain (9.5 vs. 7.2cm in Pl).[52] At a higher dose (Ox 0.06) no significant increase in height gain was found.[52] In our follow-up study at young adult age we see that the differences in adult height gain are maintained.[56]

TABLE 1
Characteristics of three placebo-controlled studies on the effect of oxandrolone in girls with Turner syndrome treated with growth hormone 1.33 - 1.43 mg/m^2/d. Adapted from Sas et al.[55]

	Dutch study Menke et al.,2010	**US study Zeger et al.,2011**	**UK study Gault et al.,2011**
Inclusion criteria			
Karyotype	Karyotype associated with TS except Y chromosomal material	Karyotype associated with TS except Y chromosomal material	Karyotype associated with TS
Age (yr)	2-16	10-15	7-13
Bone age (yr)	< 12	≤ 12	Open epiphysis
Exclusion criteria			
Previous treatment	No Ox, No E2, No GH	No Ox,No E2 No prior GH exceeding 12 months or in the preceding 3 months	No Ox, No E2, No GH or only GH range 8.3-11.7 mg/m2/week
Study design			
Pl/Ox dose (mg/kg/d)	Pl vs 0.03 vs 0.06	Pl vs 0.06	Pl vs 0.05
Maximal Ox dose (mg/d)	3.75	3.75	2.5
Minimum age of starting Ox (yr)	8	10	9
GH dose	1.33 mg/m2/d at 1 m2 ~ 0.046 mg/kg/d	0.05 mg/kg/d	1.43 mg/m2/d= 10mg/m2/week~ 0.049 mg/kg/d
'Discontinuation' criteria	GH+Ox/Pl were stopped when height velocity < 1 cm in previous 6 months or because patients were satisfied with their height.	Near-adult height defined as height obtained when bone age was ≥ 13.5 years.	Treatment until adult height was reached. Final height defined as height velocity < 1 cm/yr and bone age ≥ 15.5 years
Start estrogen therapy	From the age of 12 years onwards	After 2 yrs (12-17 yr) of treatment with Ox	Randomized to starting at 12 yr or 14 yr
Estrogen therapy (oral)	17 ß estradiol : 1stand 2ndyrs 5 ug/kg/d; 3rd yr 10 ug/kg/d or ethinylestradiol : 1st and 2ndyrs 50; 3rd 100 ng/kg/d	Ethinyl estradiol : 50 ng/kg/d , during year 3 and 100 ng/kg/d, during year 4.	Ethinyl estradiol: year 1: 2 µg/d; year 2, 4 µg/d; year 3, 4 months each of 6, 8, and 10 µg/d

TABLE 2
Results of 3 placebo-controlled studies on the effect of oxandrolone in girls with Turner syndrome treated with growth hormone 1.33 - 1.43 mg/m^2/d. Adapted from Sas et al.[55]

	Dutch study Menke *et al.*, 2010			US study Zeger *et al.*, 2011		UK study Gault *et al.*, 2011	
Pl/ Ox Number of patients	**Pl n=42**	**0.03 n=42**	**0.06 n=36**	**Pl n=20**	**0.06 n=21**	**Pl n=43**	**0.05 n=39**
Mean age of starting GH (yr)	9.4	8.5	9.1	12.0	11.9	7.1	7.2
Mean age of starting Ox (yr)	10.9	10.2	10.2	12.0	11.9	10.6	10.8
Height SDS at start GH (Turner ref)	0.5[a]	0.3[a]	0.5[a]	0.1[b]	0.0[b]	NA	NA
Height SDS at start GH (healthy ref)[c]	-3.0	-3.0	-2.9	-2.9	-3.1	NA	NA
Mean ending oxandrolone after dose adjustments (mg/kg/d)[i]	Pl	0.030	0.059	Pl	0.049	Pl	0.040
Adult height (cm)	155.6	156.7	156.5	150.5[d]	152.6[d]	149.6	154.2
Adult height SDS (Turner ref)	1.4[a]	1.5[a]	1.5[a]	2.2[b,e]	2.4[b,e]	1.0[b]	1.7[b]
Adult height SDS (healthy ref)	-2.3	-2.1	-2.2	-1.6[e]	-1.4[e]	-2.3	-1.6
Delta height SDS (Turner ref)	0.8[a]	1.2[a]	1.0[a]	1.6[b,e]	2.3[b,e]	NA	NA
Delta height SDS (healthy ref)	0.7	0.9	0.7	1.2[e]	1.8[e]	-0.1	0.4
Height increase from baseline (cm)	25.5	29.3	28.3	22.2	26.2	23.6	26.1
Adult height minus predicted adult height (cm)	7.2[f]	9.5[f]	8.3[f]	6.9[g]	9.9[g]	NA	NA
Differences in height gain vs Pl (cm)[j]		2.3	1.1		3	NA	NA

Note that height at enrolment, rather than height at start of GH, were recorded in the UK study, so that data are not available for the latter.

[a] Turner specific references – Dutch Swedish Danish data

[b] Turner specific references – Lyon data

[c] healthy references: using reference data of healthy children of their own country

[d] near adult height

[e] height SDS using actual chronological age rather than 20 or 21 year of age

[f] Teunenbroek modified projected adult height

[g] Bayley-Pinneau method

[i] Ox dose adjustments: in Dutch study ceiling dose of 3.75 mg, in UK study ceiling dose of 3.75 mg, in US study ceiling dose of 3.75 mg and dose reduction in case of virilization.

[j] (near) adult height minus predicted adult height

Besides efficacy, of course safety parameters were an important subject of investigation in our follow-up study. First of all, when using an androgen like Ox, virilizing effects such as hirsutism, acne, excessive muscular development, deepening of the voice, and clitoromegaly are of concern.[48-50] Although all three studies used dosages not higher than 0.06 mg/kg/day both dose reduction and premature discontinuation of Ox due to virilization were reported in two out of the three studies.[52,53] In our pediatric Dutch study one girl in the Ox 0.03 (2.4%) and seven in the Ox 0.06 group (19.4%) discontinued Ox due to virilization.[52] In the US study dose reduction was effected in 40% of the women.[53] During follow-up, we report that even years after treatment patients treated with Ox have more subjective virilization, although objective virilization is mild.[56] We found that objective virilization is only present in a small minority of the patients. Furthermore we observed that hirsutism and acne regress while clitoromegaly and voice deepening, most of the time to a normal voice pitch, appear to be irreversible. Secondly, besides virilizing effects, two out of the three placebo controlled studies reported a delay in breast development during treatment with Ox.[52,53] Our pediatric study showed a catch-up in breast development after discontinuation of Ox which was confirmed in our follow-up study with equal breast sizes in both the Pl and Ox treated groups. A third concern is the possible increased cardiovascular risk with disturbances in glucose metabolism and cholesterol levels. Taking the three studies together we see a modest effect on cholesterol levels with a lowering of HDL-cholesterol. No effect on glucose metabolism was found. Fourth, during treatment no psychological problems due to the androgen treatment itself or related to physical aspects were reported. The US study found a positive side effect with a small decrease in frequency of severe arithmetic learning disability after four years of treatment.[57] In our pediatric study we found no differences in total and internalizing problem behavior. Our follow-up study suggests that Ox has long-term effects on feelings of depression and anxiety. However, we found no effect on overall quality of life. Fifth, Ox could negatively influence hearing. Previous studies suggest that prenatal exposure to androgens negatively influences the auditory system.[58] Also, postnatal androgen levels might negatively influence otoacoustic emissions.[59,60] Nevertheless, we found no long-term effect of previous androgen use during childhood and adolescence on hearing. The sixth matter of importance is the effect on bone mineral density (BMD). We know that in non-TS women androgen levels during adolescence predict peak bone mass.[61] In the TS population we see that girls with spontaneous menstruation, probably with coinciding physiological androgen levels, have a normal BMD.[62] The US study showed no effect of Ox on the BMD of the wrist and spine after four years of treatment with Ox 0.06 mg/kg/day versus Pl.[53] Zuckerman-Levin et al showed that subjects who received oral methyl testosterone for 1 year had a significant increment of BMD and bone mineral content.[63] Our own preliminary data during treatment suggest an improvement of BMD during Ox treatment.[64] Preliminary data of our follow-up study show that especially the women treated with Ox 0.06 have higher BMD,

even years after treatment (unpublished results).

Although the three randomized controlled trials add valuable information on the use of Ox in TS, two main questions still have to be further addressed: the exact optimal dose of Ox and the timing of use. Based on the results of the three randomized controlled trials we know that lower than previously used conventional dosages are efficient. And although Ox 0.06 increases height during therapy and shortens the duration of GH therapy, we would not recommend this dose since it does not lead to increased adult height gain and has several adverse effects. However the other way around, it is not unthinkable that even lower doses than Ox 0.03 are effective with a better safety profile. Based on the currently available data, we would advise to use Ox in a dose of 0.03-0.05 mg/kg/d with a pragmatic ceiling dose of 2.5 mg per day to prevent overdosing. Under ideal circumstances the Ox dose should probably be adjusted to the individual (pretreatment) androgen levels. Concerning the timing, Ox appears to be effective in the age range of 8-16 years. The findings of our pediatric study, showing no differences in the effect of Ox between the three age groups (age group 1 started at age 8, age group 2 started at age 8-11 and age group 3 started at age 12-16), suggest that starting Ox before the age of 8 is not better than starting in the early pubertal age range. One could hypothesize that Ox has the most optimal effect from the age of 10 to 13 when natural androgen values are lower in untreated TS girls compared to healthy controls.[65] Postponing the use of androgen until after the start of puberty (or start of estrogen treatment) could be of benefit to prevent the delay in breast development.

Concerning cost-effectiveness, we know that the costs of five years of Ox treatment at a maximum dose of 2.5mg are estimated at 6,000-7,000 US dollars (~ 4800 euro). Our pediatric study showed that Ox shortens the duration of GH treatment and with that the GH related costs.[52] The reduction of GH related costs was only significant for the Ox 0.06 group (-13.500±6.300 euro – total cumulative costs of GH use in the Pl group 161.200±59.500 euro). For the Ox 0.03 group a non-significant reduction of 10.100±6.100 euro was found.

In conclusion, low dose Ox has a growth-enhancing effect in GH treated TS girls with a good benefit to risk ratio. We would advise adjunctive treatment with Ox at a dosage of 0.03 to 0.05 mg/kg/day from the age of 8-10 years onwards. When using Ox prescribers are advised to monitor subjective and objective signs of virilization, breast development, and blood lipids. We hope that the recent publications of the randomized controlled trials and the results of the follow-up study help to re-implement Ox, since at current time Ox is not available in the Netherlands. It is an important option in the treatment for girls with TS, especially in those anticipated a very short adult stature or in whom the growth rate is modest despite good compliance with GH.

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Nederlandse samenvatting

Syndroom van Turner

Het syndroom van Turner (SvT) is het gevolg van complete of partiële afwezigheid van een X-chromosoom. In Nederland is de prevalentie onder levend geboren meisjes 1:2000-5000.[1] Tot de klassieke symptomen van dit syndroom behoren een gestoorde lichaamsgroei, gonadale dysgenesie –meestal leidend tot onvruchtbaarheid- en uiterlijke dysmorfieën. Echter, het fenotype is sterk wisselend en varieert van een presentatie met ernstige congenitale (hart) afwijkingen tot een milder fenotype gekenmerkt door secundaire amenorroe of onverklaarde infertiliteit op volwassen leeftijd.

De aandoening wordt vastgesteld door middel van karyotypering van minimaal 30 lymfocyten. Gebruikmakend van deze methode heeft ongeveer de helft van de patiënten een complete monosomie 45,X en 30-40% een mozaïcisme van verschillende karyotypen. De overige 5-15% van de patiënten heeft structurele afwijkingen aan één van beide X-chromosomen.[2-4]

De belangrijkste kindergeneeskundige aspecten van het SvT zijn, naast de opsporing van congenitale afwijkingen, groeistimulatie met groeihormoon en puberteitsinductie met oestrogenen. Zonder behandeling met groeihormoon worden vrouwen met het SvT in Noord-Europa gemiddeld 147 cm lang. Behandeling met groeihormoon geeft een lengtewinst van gemiddeld 5-12 cm.[5-7] Als gevolg van versnelde regressie van de ovaria komen de meeste meisjes met het SvT niet spontaan in de puberteit en heeft slechts 8-9% van de meisjes met monosomie 45,X en 40-47% van de meisjes met 45,X/46,XX, al dan niet tijdelijke, menstruele cycli.[8,9] Derhalve wordt een aanzienlijk deel van de meisjes bij het ontbreken van secundaire geslachtskenmerken behandeld met oestrogenen.

Ter voorkoming van oestrogeendeficiëntie op volwassen leeftijd is er na de inductie van de puberteit, of later bij het wegvallen van de spontane menstruele cyclus, een indicatie voor hormoonsuppletietherapie. Deze moet in principe gecontinueerd worden tot de normale leeftijd van de menopauze (gemiddeld 51 jaar).[10] Spontane zwangerschappen bij vrouwen met het SvT zijn zeer zeldzaam en vrijwel alleen beschreven bij vrouwen met 45,X/46,XX.[11-13] Bij een kinderwens zijn geassisteerde voortplanting, naast adoptie en pleegzorg, dan de enige optie. Zowel spontane als geïnduceerde zwangerschappen bij het SvT behoren tot de hoog-

risico zwangerschappen en het in kaart brengen van met name cardiovasculaire problemen vóór de zwangerschap is essentieel.[14-16] Andere kenmerken van het SvT op volwassen leeftijd zijn divers. Structurele hart- en vaatafwijkingen en een ongunstig cardiovasculair risicoprofiel -als gevolg van een hogere incidentie van hypertensie, hypercholesterolemie en diabetes- komen vaker voor dan in de algemene populatie.[17-19] Andere belangrijke kenmerken zijn een verminderde botdichtheid en daaraan gekoppeld een verhoogd fractuurrisico,[20,21] auto-immuunziekten waaronder hypothyreoïdie en coeliakie,[22-24] gehoorsverlies[25-27] en problemen op het psychologische vlak.[28-30]

Multidisciplinaire zorg bij vrouwen met het syndroom van Turner

Jarenlang werden patiënten met het SvT alleen op de kinderleeftijd behandeld door een medisch specialist. De kinderarts(-endocrinoloog) richtte zich met name op de groeihormoonbehandeling en puberteitsinductie, en coördineerde de controles bij eventuele andere behandelaars. Veel vrouwen werden na het bereiken van de volwassen leeftijd en ontslag uit de kindergeneeskundige controles niet meer regulier gecontroleerd.[31-35] De laatste jaren wordt de ernst van de morbiditeit van het SvT op volwassen leeftijd steeds duidelijker en zijn er richtlijnen gepubliceerd.[2,10,36] Vooral op volwassen leeftijd vereist de zorg voor vrouwen met het SvT expertise uit verschillende vakgebieden. Samenwerking tussen deze verschillende disciplines is van belang in de dagelijkse zorg, maar zeker ook in het geval van een zwangerschapwens.[14-16] Sinds de invoering van richtlijnen is de zorg voor volwassen vrouwen met het SvT verbeterd en zijn speciale Turnerklinieken voor volwassen vrouwen opgericht.[2,10,36]

In 2005 opende het Radboud universitair medisch centrum een multidisciplinaire poli voor volwassen vrouwen met het SvT. Binnen dit centrum wordt gestreefd naar een vloeiende transitie van de kindergeneeskundige naar de volwassen zorg, naar tijdige onderkenning en gecoördineerde behandeling van aandoeningen behorend tot het SvT en naar optimale hormoonsuppletietherapie en fertiliteitscounseling. In **hoofdstuk 2** van dit proefschrift beschrijven we onze studie naar de effectiviteit van het eerste bezoek aan de multidisciplinaire polikliniek. Het doel van deze studie was om vast te stellen welke morbiditeit bij een eerste screening volgens internationale richtlijnen wordt aangetoond. Alle 150 vrouwen (met een gemiddelde leeftijd van 31 jaar oud, 47% 45,X) werden gezien door een endocrinoloog, een gynaecoloog, een cardioloog, een KNO-arts en wanneer nodig een psycholoog. De screening bestond uit anamnese, lichamelijk onderzoek en laboratoriumonderzoek aangevuld met een MRI van het hart en de aorta, echocardiografie, electrocardiogram, botdensitometrie (DEXA), echo van de nieren en een gehoorstest. Wij stelden de volgende nieuwe diagnosen vast: bicuspide aortaklep (n=13), coarctatie van de aorta (n=9), elongatie van het transversale deel van de aorta

(n=27), dilatatie van de aorta (n=34), osteoporose (n=8), osteopenie (n=56), nierafwijkingen (n=7), subklinische hypothyreoïdie (n=33), coeliakie (n=3), glucose intolerantie (n=12), hypercholesterolemie (n=52), hypertensie (n=39), en gehoorsverlies met een indicatie voor een gehoorapparaat (n=8). Drieëntwintig patiënten werden verwezen voor een psychologisch consult. Naast deze nieuwe morbiditeit lieten we zien dat dertig procent van de vrouwen op het moment van bezoek en de jaren daarvoor geen medische zorg had genoten. Vijftien procent had op het moment van eerste screening geen enkele vorm van hormoonsuppletietherapie. Wij concluderen op basis van deze gegevens dat een initiële screening een belangrijke hoeveelheid nieuwe morbiditeit aantoont. Het lijkt er dan ook op dat vrouwen met het SvT belang hebben bij een adequate transitie. In hoeverre een goede transitie en daaropvolgende controles ook daadwerkelijk de kwaliteit van leven verbeteren en mortaliteit verminderen, is niet duidelijk. Een belangrijk discussiepunt blijft dan ook of de zorg zoals nu voorgesteld in de richtlijnen kosteneffectief is en of de medische screening opweegt tegen de belasting van de medische onderzoeken voor de individuele patiënt.

Karyotypering bij het syndroom van Turner

In de eerdere richtlijnen wordt aangegeven dat bij klinische verdenking op het SvT, het syndroom gediagnosticeerd wordt met standaard karyotypering van 30 bloed lymfocyten.[10,37,38] De richtlijnen adviseerden om alleen aanvullende cellijnen te onderzoeken als er sprake is van klinische virilisatie of een marker chromosoom.[39-42] De vraag is echter of standaard karyotypering een adequate weerspiegeling geeft van het genotype. Een laag-gradig mozaïcisme in het bloed wordt op deze manier niet uitgesloten en er wordt met deze methode ook geen rekening gehouden met weefselmozaïcisme: het vóórkomen van andere karyotypen in andere weefsels in het lichaam. Meerdere studies bij patiënten met het SvT bevestigen het bestaan van weefselmozaïcisme.[39-41] Het onderkennen van weefselmozaïcisme is niet alleen belangrijk om meer inzicht te krijgen in de genotype fenotype correlatie, maar met name ook als er sprake is van een Y-chromosoom. De aanwezigheid van een Y-chromosoom is geassocieerd met de ontwikkeling van gonadoblastomen in de ovaria en over het algemeen wordt geadviseerd om een preventieve gonadectomie te verrichten om de ontwikkeling naar een maligne dysgerminoom te voorkomen.[43] In **hoofdstuk 3** beschrijven we onze studie waarin we bij vrouwen met het SvT naast de standaard karyotypering ook 'polymerase chain reaction' (PCR) en 'fluorescence in situ hybridization'(FISH) verrichtten. Naast lymfocyten in het bloed onderzochten we ook een tweede cellijn: wangslijmvlies. Het doel van deze studie was om te onderzoeken welke additionele waarde deze aanvullende technieken hebben. Aan deze studie deden 162 vrouwen met het SvT mee (gemiddelde leeftijd 29,9±10,3 jaar). Ongeveer de helft van hen, 75 patiënten, had middels standaard karyotypering een monosomie 45,X. Van

deze 75 patiënten, ondergingen er 63 aanvullende onderzoeken met FISH op wangslijmvlies en PCR-Y op bloed. FISH op wangslijmvlies liet zien dan 19 van de 63 (30,2%) een mozaïek bleek te hebben. Bij het grootste deel van de patiënten bleek het te gaan om mozaïcisme met 46,XX, maar bij 5 patiënten ging het om een mozaïek met een Y-chromosoom. Conventionele PCR-Y op bloed was negatief in alle gevallen. Een sensitievere 'real-time' PCR kon de aanwezigheid van Y-chromosomaal weefsel bevestigen in 3 van de 5 patiënten. Vier patiënten hebben naar aanleiding van het vaststellen van de aanwezigheid van een Y-chromosoom een gonadectomie ondergaan. Bij pathologisch onderzoek bleek er in geen van de gevallen sprake te zijn van een gonadoblastoom. Bij twee patiënten is FISH onderzoek op het ovariumweefsel uitgevoerd, waarbij in beide gevallen Y-chromosomen werden aangetoond. Onze resultaten bevestigen dat standaard karyotypering in vrouwen met 45,X onvoldoende is om een volledig beeld te krijgen van het genotype. Het vinden van een additioneel Y-chromosoom lijkt van belang, al is het exacte risico op het ontwikkelen van een gonadoblastoom bij een Y-chromosoom gevonden met deze additionele technieken niet bekend. Het is echter aannemelijk dat een Y-chromosoom gevonden met deze additionele technieken een vergelijkbaar risico op gonadoblastoom met zich meebrengt als een Y-chromosoom vastgesteld met standaard karyotypering. Het risico op een gonadoblastoom bij vrouwen met het SvT en een Y-chromosoom lijkt relatief laag, zeker wanneer virilisatie ontbreekt.[44] Desalniettemin, lijkt ook een laag risico op het ontwikkelen van een gonadoblastoom een gonadectomie te rechtvaardigen, zeker wanneer de ovaria reeds in regressie zijn. Het advies is dan ook om bij vrouwen met het SvT en monosomie 45,X een tweede cellijn te onderzoeken. Wangslijmvlies is hiervoor, gezien de gemakkelijke verkrijgwijze, uitermate geschikt. Een dergelijk advies is opgenomen in de Vlaams-Nederlandse klinische richtlijn Turner syndroom.[45]

Lange termijn effecten van oxandrolon behandeling in de kinderjaren

Hoewel meisjes met het SvT niet groeihormoondeficiënt zijn, wordt groeihormoon (GH) routinematig voorgeschreven om de eindlengte van meisjes met het SvT te verbeteren. In de zoektocht naar betere behandelmogelijkheden zijn niet alleen hogere doseringen GH, maar ook de toevoeging van androgenen als oxandrolon (Ox) aan GH onderzocht. Bij eerste studies naar het effect van Ox, waarin doseringen tot 0,1 mg/kg/dag werden gebruikt, moest de dosering vaak naar beneden worden aangepast in verband met virilisatie en versnelde botrijping.[46-48] Recent zijn drie gerandomiseerde studies naar het groeibevorderende effect van lagere doseringen Ox naast GH gepubliceerd.[49-51] Hieronder valt ook de Nederlandse Turner Oxandrolon Studie, een samenwerking van 10 kinderendocrinologische centra in Nederland. Inclusie van deze studie vond plaats tussen 1991 en 2003.[49] In totaal zijn 133 meisjes geïncludeerd en gerandomiseerd om

naast GH behandeling (1,33mg/m^2/dag) vanaf achtjarige leeftijd òf Ox in een dosering van 0,03 mg/kg/dag (GH+Ox 0,03), òf Ox in een dosering van 0,06 mg/kg/dag (GH+Ox 0,06), òf een placebo (GH+Pl) te krijgen. Daarnaast werden de meisjes, als spontane puberteit afwezig was, behandeld met oestrogenen vanaf 12 jarige leeftijd.

De belangrijkste uitkomstmaat was de lengtewinst op volwassen leeftijd, gedefinieerd als volwassen lengte minus de voorspelde lengte. Vergeleken met GH+Pl gaf GH+Ox 0,03 een significante lengtewinst (9,5 vs. 7,2 cm in GH+Pl). Deze lengtewinst ging gepaard met een vertraagde borstontwikkeling in de GH+Ox 0,03 groep. In de groep met de hogere dosering Ox (GH+Ox 0,06) was er geen sprake van significante lengtewinst (8,3 vs. 7,2 cm in GH+Pl). Mogelijke verklaringen hiervoor zijn een snellere botrijping en eerder staken van Ox door virilisatie in de GH+Ox 0,06 groep. In deze groep gaven meer meisjes aan last te hebben van virilisatie. In beide met Ox behandelde groepen was er sprake van een afname van de vetmassa en een toename van de spiermassa. Er trad een verlaging van de stemfrequentie op, in de meeste gevallen naar een normale stemhoogte. [52,53] Er werd geen effect van Ox op gedrag, agressie, seksuele ontwikkeling, stemming en 'gender role' gevonden.[54] Ook waren er geen effecten van Ox op het glucose metabolisme.[55]

De genoemde resultaten betreffen de korte termijn effecten bij kinderen en adolescenten. In **hoofdstuk 4, 5 en 6** van dit proefschrift bespreken we de resultaten van de follow-up studie van de Nederlandse Turner Oxandrolon Studie. Het doel van de follow-up studie was om de lange termijn resultaten van de drie genoemde regimes in kaart te brengen. De vraag is of de resultaten van de kinderstudie op volwassen leeftijd blijven bestaan en of het positieve effect op de volwassen eindlengte opweegt tegen de potentieel negatieve bijwerkingen. Alle deelneemsters aan de kinderstudie werden gevraagd deel te nemen aan de follow-up studie. Achtenzestig volwassen vrouwen (GH+Pl, n=23; GH+Ox 0,03, n=27; and GH+Ox 0,06, n=18) participeerden in de dubbelblinde follow-up studie (gemiddelde leeftijd 24 jaar oud, gemiddelde tijd sinds stop GH 8,7 jaren; gemiddelde duur van GH/Ox behandeling 4,9 jaren).

In **hoofdstuk 4** wordt het effect van Ox op lengte, lichaamsproporties, borstgrootte, virilisatie en lichaamssamenstelling op volwassen leeftijd beschreven. De lengtewinst zoals gevonden in de kinderstudie blijft op volwassen leeftijd bestaan (GH+Ox 0,03 10,2±4,9 cm,GH+Ox 0,06 9,7±4,4 cm vergeleken met GH+Pl 8,0±4,6 cm). Borstgrootte, Tanner stadium en lichaamssamenstelling op volwassen leeftijd verschilden niet tussen de drie groepen. De vrouwen die in het verleden met Ox behandeld waren gaven meer klachten aan van virilisatie dan de vrouwen behandeld met Pl. Objectieve virilisatie persisteerde slechts in enkele gevallen. Wel werd er een lagere stemfrequentie gevonden bij met Ox behandelde vrouwen.

In **hoofdstuk 5** wordt het effect van Ox op neurocognitie, kwaliteit van leven en sociaal-emotioneel functioneren beschreven. We vonden geen effect op het neurocognitief functioneren. De vrouwen die in het verleden behandeld waren met Ox scoorden hoger op de angst vragenlijst (STAI; 37,4±8,4 vs 31,8±5,0, p0,002) en hadden hogere scores op de subschaal depressie van de SCL-90-R (25,7±10,7 vs 20,5±4,7, p0,01), echter er was geen effect op de totale kwaliteit van leven. Wat betreft sociaal-emotioneel functioneren, vonden we alleen een klein effect van Ox op emotie perceptie: vrouwen met Ox behandeling in het verleden herkenden de emotie angst minder goed in lage intensiteit. Overigens leidde dit niet tot andere scores op de sociale interactie vragenlijsten. Over het algemeen lijken de effecten van Ox op later (neuro) psychologisch functioneren beperkt.

In **hoofdstuk 6** beschrijven we het gehoor bij jong volwassenen met het SvT en de effecten van Ox behandeling in het verleden hierop. We verrichtten conventionele audiometrie met het meten van lucht- en botgeleiding in decibel gehoorsniveau. Tweederde van de vrouwen had een voorgeschiedenis van otitis media. De onderzoeken laten zien dat er bij 66% van deze jongvolwassen patiënten sprake is van gehoorsverlies. Dit betreft puur perceptief gehoorsverlies bij 32% van de vrouwen. De gehoorsdrempels waren ongeveer 10 decibel slechter bij de patiënten met een complete monosomie van de korte arm van het X chromosoom (monosomie 45,X en isochromosoom) vergeleken met de groep met slechts een partiële monosomie van de korte arm van het X-chromosoom (mozaiek en deleties). We vonden geen effect op oor en gehoor van Ox behandeling in het verleden.

Uit de studies beschreven in **hoofdstuk 4, 5 en 6** kunnen we concluderen dat Ox in een lage dosering van 0,03 mg/kg/dag een gunstig effect heeft op de volwassen eindlengte. Ondanks het feit dat er tijdens de kinderstudie sprake was van een vertraagde ontwikkeling van de borsten, is de borstgrootte op volwassen leeftijd gelijk. Terwijl subjectieve beleving van viriliserende kenmerken veelvuldig voorkomt, persisteert objectieve virilisatie slechts in een kleine minderheid van de patiënten. Uit onze onderzoeken kunnen we concluderen dat hirsutisme en acne met de tijd verdwijnen, terwijl stemverlaging en clitoromegalie persisteren. Op het gebied van de psychologische effecten op de volwassen leeftijd zijn de verschillen tussen de Ox groepen en de Pl groep slechts klein. In de kinderstudie werd reeds gemeld dat de positieve effecten van Ox in een dosering van 0,06 mg/kg/dag niet in balans zijn met de negatieve effecten. Wij onderschrijven de conclusie dat Ox in een dosering van 0,03 mg/kg/dag ook op de lange termijn effectief en veilig is. Het gebruik van Ox in een dosering van 0,06 mg/kg/dag wordt ontraden.

Onze studies naar het effect van Ox werden vrijwel gelijktijdig gepubliceerd met twee andere gerandomiseerde studies naar het groeibevorderende effect van Ox bij het SvT. De eerste is een studie uit de Verenigde Staten waarin het effect van Ox 0,06 mg/kg/dag onderzocht wordt en de tweede is een studie uit Groot-Brittannië waarin de effecten van Ox 0,05 mg/kg/dag worden beschreven.[50,51] Als we onze data samenvoegen lijkt het gemiddelde additionele effect van Ox toegevoegd aan GH tussen de 2,3 en 4,6 cm te zijn.[56] Alle drie studies laten zien dat lage doseringen van Ox effectief zijn. Het blijft een interessante vraag of zelfs nog lagere doseringen eveneens effectief zijn met een mogelijk nog gunstiger bijwerkingenprofiel. Wellicht is het zelfs fysiologischer, en daarmee mogelijk effectiever, als doseringen individueel aangepast worden aan de androgeenspiegels voor behandeling en een stijgende dosering toegepast wordt met het ouder worden. Wat betreft de timing van behandeling lijkt Ox effectief te zijn in de leeftijdsrange van 8 tot 16 jaar. In onze kinderstudie hebben we laten zien dat er geen verschillen in effectiviteit zijn tussen de verschillende leeftijdsgroepen (leeftijdsgroep 1 begon op 8 jarige leeftijd, leeftijdsgroep 2 begon op 8-11 jarige leeftijd, leeftijdsgroep 3 startte op 12-16 jarige leeftijd). Dit maakt het aannemelijk dat het niet van toegevoegde waarde is om vóór het 8ste levensjaar te starten. De behandeling uitstellen tot bijvoorbeeld na het begin van de puberteit of één tot twee jaar na start van oestrogeen behandeling zou eventueel nog een optie kunnen zijn om de negatieve gevolgen voor borstontwikkeling te omzeilen, al is het de vraag of de behandelduur dan niet teveel bekort wordt door het al deels sluiten van de epifysairschijven.

Concluderend is Ox een groeibevorderend medicijn dat van toegevoegde waarde kan zijn bij meisjes met het SvT. Ox is effectief en veilig en geeft weinig bijwerkingen als het in een lage dosering wordt gebruikt. Op dit moment is het advies om Ox vanaf het 8ste levensjaar te gebruiken in een dosis van 0,03-0,05 mg/kg/dag met een pragmatische maximale dosering van 2,5 mg per dag om overdosering te voorkomen. Bij gebruik van Ox dient er aandacht te zijn voor subjectieve en objectieve virilisatie, borstontwikkeling en lipiden. We hopen dat onze studies, samen met de andere twee gerandomiseerde trials, bijdragen aan het opnieuw beschikbaar komen van Ox op de markt aangezien het een belangrijke optie kan zijn voor meisjes met het SvT, vooral bij diegene met een opvallend lage voorspelde eindlengte of een tegenvallende groei ondanks adequaat GH gebruik.

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Curriculum Vitae

Kim Freriks werd geboren op 30 juni 1980 in Winterswijk. Aldaar behaalde zij in 1998 haar VWO diploma aan de 'Driemark'. In 1998 heeft zij een jaar Hoger Laboratorium Onderwijs gestudeerd, om vervolgens in 1999 aan de Radboud Universiteit de studie geneeskunde te starten. In maart 2006 resulteerde dit in haar artsexamen. In het jaar na haar afstuderen heeft zij als arts-onderzoeker op de afdeling Endocriene Ziekten (prof. dr. A.R.M.M. Hermus) gewerkt. In 2007 startte zij met de opleiding tot internist. De eerste twee jaar werkte zij in het Jeroen Bosch Ziekenhuis te Den Bosch (opleider dr. P.M. Netten). Het vervolg van de opleiding vond plaats in het Radboud universitair medisch centrum (hoofdopleiders prof.dr. J. de Graaf, prof. dr. J.W.M. van der Meer). Tijdens haar promotieonderzoek participeerde zij in de multidisciplinaire Turnerpolikliniek voor volwassen vrouwen in het Radboud universitair medisch centrum. Daarnaast schreef zij mee aan de klinische richtlijn Turner syndroom van het Nederlands-Vlaamse Multidisciplinair Netwerk Turner syndroom en aan een uitgebreide patiëntenfolder voor meisjes en vrouwen met het syndroom van Turner. Momenteel werkt zij als internist-endocrinoloog in opleiding op de afdeling algemeen interne geneeskunde (prof. dr. A.R.M.M. Hermus, prof. dr. C.J.J. Tack).