CLINICAL STUDY

Quantitative liver functions in Turner syndrome with and without hormone replacement therapy

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Abstract

Background: Studies have documented elevated levels of liver enzymes in many females with Turner syndrome (TS). Histology has shown a range of changes. Treatment with female hormone replacement therapy (HRT) reduces liver enzymes.
Aim: To study quantitative liver functions in TS in detail with and without HRT.
Design: Randomized crossover study with active treatment (HRT in TS and P-pill in controls) or no treatment.
Subjects: Women with TS ($n=8$, age 29.7 ± 5.6 (mean$\pm$S.D.) years), verified by karyotype, and age-matched controls (C; $n=8$, age 27.3 ± 4.9 years).
Methods: We determined liver enzymes in blood, used the galactose elimination capacity to assess hepatocyte cytosol activity, plasma clearance of indocyanine green to assess excretory function, antipyrine clearance to estimate microsomal activity, and the functional hepatic nitrogen clearance (FHNC) to assess mitochondrial-cytosolic metabolic capacity for conversion of amino-nitrogen.
Results: Liver enzymes were elevated in untreated TS and reduced by HRT. The hepatic capacities for conversion of galactose, indocyanine green, and antipyrine were normal and did not change by HRT. The FHNC was marginally reduced (untreated TS vs C: 19.4 ± 5.4 vs 25.2 ± 7.3 L/h, $P=0.1$). FHNC changed slightly with HRT in TS (19.4 ± 5.4 vs 24.4 ± 10.2 L/h, $P=0.2$).
Conclusions: The elevations of liver enzymes in untreated TS are readily suppressed by HRT. Quantitative liver functions in TS are comparable to controls and are not affected by HRT.

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Introduction

Turner syndrome (TS) is due to the absence of a part of or the entire X chromosome in females. Stature is short, and morbidity is increased due to risk of osteoporosis and fractures, type 2 diabetes, ischemic heart disease, hypertension, and stroke, but also the risk of cirrhosis is increased (1). Clinical studies have shown a frequent occurrence of elevated liver enzymes, primarily alanine aminotransferase (ALAT), γ-glutamyl-transferase (γGT), and alkaline phosphatase (AP), while bilirubin is normal (2–5).

We and others have shown a normalizing effect of hormone replacement therapy (HRT), containing 17β-estradiol and a gestagen, on liver enzymes (3–5), which may point towards a protective effect on hepatocyte integrity. Marked architectural changes, including nodular regenerative hyperplasia, multiple focal nodular hyperplasia and cirrhosis are observed in some patients and are associated with a risk of liver-related complications. These changes are frequently associated with vascular disorders such as obliterative portal venopathy, probably related to congenitally abnormal vessels. Steatosis, steatofibrosis, and steatohepatitis are seen and may be caused by metabolic disorders. In addition, bile duct alterations resembling small duct sclerosing cholangitis are observed in several patients (6). Presently, it is not known whether these perturbations in liver morphology and in liver-derived enzymes are related to functional defects in females with TS and whether this may change by HRT.

To further explore quantitative liver function in TS, we examined adult women with TS on and off HRT and compared them with a control group of age matched normal women. We used the galactose elimination capacity (GEC) to assess hepatocyte cytosol activity (7), the plasma clearance of indocyanine green (ICGCl) to assess hepatic blood flow and excretory liver cell function independently of hepatic blood flow (8), the antipyrine plasma clearance (ApCl) to estimate hepatic microsomal system activity (9), and the functional hepatic nitrogen clearance (FHNC) to assess mito-chondrial-cytosolic metabolic capacity for conversion of amino-nitrogen (10).

We assumed that one or more these metabolic liver functions would be diminished in untreated TS and normalized by HRT. Our principal objective was to understand mechanistically how HRT improves liver function in TS.

Subjects and methods

Subjects and design

We studied women with TS ($n=8$, age 29.7 ± 5.6 (mean $\pm$ s.d.) years), verified by karyotype, and age-matched controls ($n=8$, age 27.3 ± 4.9 years; Table 1). The study was a randomized cross-over study, with 2 month periods each completed by 2 study days. Study subjects were taken off their usual treatment 2 months (wash-out period) before entering the study. The treatment regimen was given in a random order to the individual participants. All women with TS were non-menstruating and had required HRT for years in order to menstruate regularly. Thyroid hormone levels were normal in all study individuals. Women with TS were treated with oral hormone substitution consisting of 2 mg 17β-estradiol/day for days 1–12, 2 mg 17β-estradiol/day and 1 mg norethisterone acetate/day for days 13–22 and 1 mg 17β-estradiol/day for days 23–28 (Trisekvens, Novo Nordisk, Bagsværd, Denmark), or no treatment for 2 months. Control subjects were treated with oral contraceptives or no treatment for 2 months. All subjects were studied in the early follicular stage (days 5–10) of the menstrual cycle, or during the corresponding phase of the HRT/contraceptive cycle. None of the participants were smokers or received medication other than HRT/contraceptives.

All subjects received oral and written information concerning the study prior to giving written informed consent. The protocol was approved by the Aarhus County Ethical Scientific Committee (no. 1996/3561). Analysis of beat-to-beat variation and 24 h ambulatory

Table 1 Baseline characteristics. Number and age of participants and mean $\pm$ s.d. levels of anthropometric data in Turner patients and in normal women.

	Turner syndrome	Control group	*P*
Number	8	8	
Age (years)	29±6	28±4	0.8
Height (cm)	148±9	170±5	<0.0005
Weight (kg)	58±9	67±8	0.05
BMI (kg/m²)	27±3	23±4	0.09
Total body water (%)	51±5	56±5	0.07
Glucose (mmol/l)	4.5±0.4	3.9±0.5	0.02
Insulin (pmol/l)	34±11	28±14	0.4
HOMA index	0.71±0.24	0.56±0.28	0.3
IGF-I (μg/l)	195±82	199±75	0.9
Glucagon (ng/l)	50±12	46±11	0.5
Albumin (μmol/l)	598±33	599±29	0.9

blood pressure measurements from this study has previously been presented (11).

Methods

The participants were admitted to the Clinical Research Center in the morning on the day of the examinations. The investigations were carried out in the postabsorptive state the morning after an overnight fast (10–12 h) without any caffeine consumption or cigarette smoking; only ingestion of tap water was allowed and the participants were placed in the supine position under thermo-neutral conditions.

After an initial bed rest of at least 45 min, resistance and impedance were measured, total body water (TBW) was determined employing bioeletrical impedance (BIA) using BIA – 101/S (RJL Systems, Detroit, MI, USA), and fat mass (FM) and fat-free mass (FFM) was determined (12). Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared.

GEC

GEC was determined after injection of galactose (500 mg/kg) intravenously, with blood sampling at $t=$ 20, 25, 30, 35, 40, or 45 min and a urine sample after 3 h. GEC was calculated as previously described (7).

ICG clearance

ICG (ICG, Cardio Green, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) was given as a single immediate i.v. dose (0.5 mg/kg) with blood sampling at $t=0$, 5, 10, 15, and 20 min (8). The samples were analyzed on the same day as the procedure, and the plasma concentration calculated after correction for blanks and using standard curves. Clearance and volume of distribution (V_d) was calculated from the decay curve of ICG.

ApCl

At 0800 h, a standard dose of antipyrin (18 mg/kg) was injected intravenously, with blood sampling at $t=0$, 3, and 24 h. ApCl (CL_{AP}) was calculated as follows: $CL_{AP}=k \times V_d$, where $k=dc/dt$ and $V_d=D/c0$, c is concentration (milligrams per liter), t is time (minutes), k is the elimination constant, estimated as the slope of the linear regression of ln c on time, V_d is the apparent volume of distribution (liters), D is the dose of antipyrine given in milligrams, and $c0$ is the extrapolated concentration at time zero.

FHNC

Two i.v. cannulas were inserted in an antecubital vein, one in each arm. These were used for blood sampling

and alanine infusion respectively. Baseline blood samples were taken before an i.v. alanine infusion (2 mmol/kg BW; Ajinomoto Co., Tokyo, Japan) was started for 3 h from time zero, given by a volumetric pump (Terufusion STC-503, Rodovre, Denmark). The urea–nitrogen synthesis rate and the corresponding average blood α-amino nitrogen concentration was determined hourly for 4 h. The hourly blood samples were obtained with exact time registration, immediately after voiding each 1-h urine sample. Each subject ingested a minimum of 200 ml tap water per hour to keep urine production above 120 ml/h.

The urea-nitrogen synthesis rate (UNSR; mmol/h) was calculated as urinary excretion rate (E), corrected for accumulation (A) in TBW and for the fractional intestinal loss (L):

$$UNSR = \frac{E + A}{1 - L}$$

where $E =$ (urine flow, l/h)$\times$(urinary urea-N, mmol/h), $A =$ (change in blood urea-N, mmol/$l \times h$)$\times$(TBW, liter). L was taken to be 0.14 (10).

FHNC (l/h) was calculated as the slope of the linear regression analysis of UNSR on corresponding mean blood α-amino nitrogen concentrations. This measure standardizes the UNSR with regard to changes in blood α-amino nitrogen concentration (10). Insulin sensitivity was assessed by the homeostasis model assessment (HOMA) model, which is based on simultaneously sampled fasting levels of glucose and insulin.

Assays

Total α-amino nitrogen was measured by the dinitrofluorobenzene method (13) ICG (14) and antipyrine was analyzed in plasma by HPLC. Galactose was measured by galactose dehydrogenase. Urea was measured by the urease-Berthelot method (15). Glucose was measured by the glucose oxidase method. We used a two-site immunoassay ELISA to measure serum insulin. Plasma glucagon was measured by in-house RIA (16), serum insulin-like growth factor-I (IGF-I) with an in-house time-resolved fluoroimmunoassay. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by commercial time-resolved fluoroimmunoassays (DELFIA, Wallac Inc., Turku, Finland), with detection limits of 0.06 and 0.05 IU/l respectively, and intra- and interassay coefficients of variation of below 8%. Hepatic enzymes were determined on a Cobas INTEGRA (Roche).

Statistical analysis

All statistical calculations were performed with SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL, USA). Data were checked for parametric distribution. Data were examined by Student's two-tailed paired and unpaired t-tests where appropriate. Results are expressed as mean $\pm$ s.d. Significance levels $< 5\%$ were considered significant.

Results

None of the participants reported any side-effects to the examinations or the treatment.

Clinical characteristics: body composition, liver enzymes, and indirect calorimetry

TS women were shorter, lighter, but with a greater BMI, although this difference did not reach statistical significance (Table 1). Likewise, FM tended to be higher in TS, while FFM and TBW tended to be lower in TS. Weight, BMI, FM, FFM, and TBW did not change during the study period (results not shown). Glucose was slightly lower in controls, while serum insulin and HOMA index were similar among TS and controls. Glucagon, growth hormone (GH), and IGF-I were comparable among groups. FSH and LH were elevated and decreased significantly among TS (untreated versus treated TS: 51.5 (27.2–62.7) vs 18.4 (3.7–49.1) IU/l, $P = 0.01$ and 24.6 (6.5–36.2) vs 8.6 (1.5–35.7) IU/l, $P = 0.03$), while levels in controls were unchanged. There was no change in metabolic parameters due to treatment among TS or controls (results not shown). Energy expenditure was comparable among TS and controls and did not change during active treatment.

Liver enzymes

ALAT, γGT, AP and LDH were all elevated in TS and decreased during the 2 month treatment with HRT (Fig. 1), whilst there was no effect of contraceptive treatment in controls (Table 2). There was no difference between groups or due to treatment in the other measured analytes (Table 1).

Galactose elimination capacity

There was no difference in GEC between patients and controls and no effect of treatment in either group.

Indocyanine green infusion

ICG clearance and V_d was comparable in both untreated and treated TS and controls.

Antipyrine plasma clearance

ApCl was similar in TS and controls, while V_d was lower in TS. There was no effect of treatment in either group.
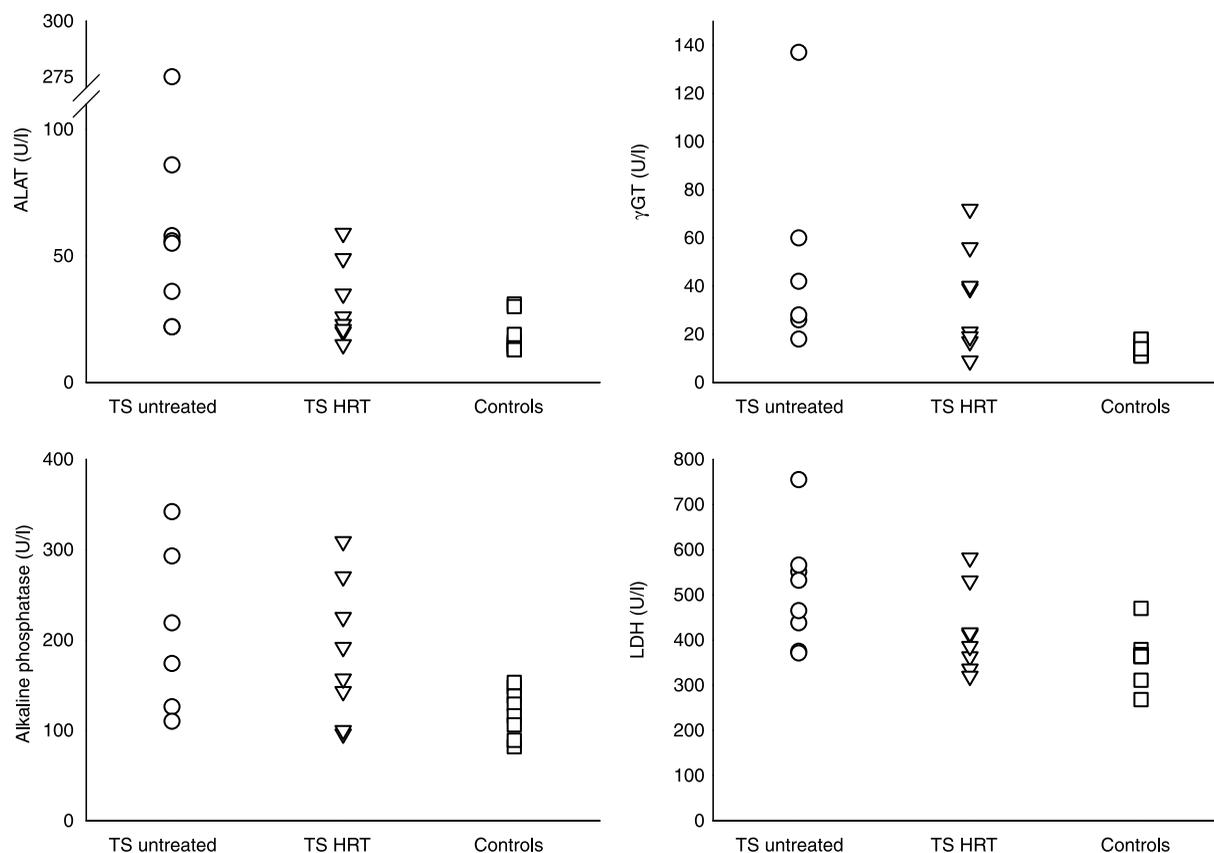
Figure 1 Levels of alanine amino transferase (ALAT), γ-glutamyl transferase (γGT), alkaline phosphatase, and lactate dehyrdogenase (LDH) in untreated TS, TS during HRT and in untreated controls.

Functional hepatic nitrogen clearance

The FHNC was statistically marginally decreased in TS (Fig. 2) and did not change during HRT (Table 3).

Discussion

The main finding of this quantitative mapping of essential hepatocellular metabolic functions in patients with TS was that such liver functions were neither compromised by the syndrome nor affected by HRT. Likewise, the same liver functions did not change by estrogen treatment in normal women.

As expected, we found elevated liver enzymes in young, healthy and active women with TS, and a normalizing effect of HRT in TS. The rationale behind the present study was a wish to understand mechanistically how HRT improves liver function, and the pathophysiology behind the elevated liver enzymes and the frequent histological changes seen in TS. These are quite variable and have mainly been described in case reports. They include minimal abnormalities (17), steatosis (12), steatohepatitis (18), biliary involvement, including primary sclerosing cholangitis (19–21), cirrhosis (22, 23), nodular regenerative hyperplasia, and portal hypertension (24–26). The only larger study reported a variety of histological changes which could be divided into three major groups comprising i)

Table 2 Fasting liver related enzymes in Turner syndrome (TS) and controls before and during treatment.

	TS	TS+HRT	Control	Control+pill	*P**	*P*†
ALAT (U/l)	56 (22–275)	25 (15–59)	16 (13–31)	16 (12–61)	0.002	0.07
γGT (U/l)	51 (18–254)	30 (9–72)	13 (11–18)	12 (9–20)	<0.0005	0.05
LDH (U/l)	499 (371–755)	399 (320–582)	365 (371–755)	305 (281–477)	0.005	0.01
Alkaline phosphatase (U/l)	288±162	187±77	120±26	101±20	0.01	0.03
Bilirubin (μmol/l)	12.4±2.9	13.0±6.8	11.3±2.9	10.3±2.3	0.6	0.6

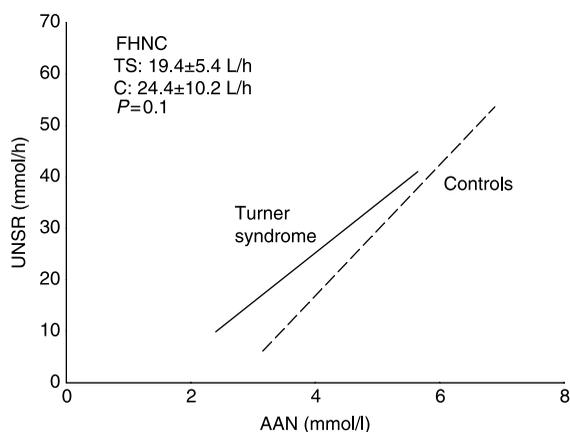*Untreated TS versus controls. †Untreated TS versus treated TS.

Figure 2 The relation between blood concentration of α-amino-N and urea nitrogen synthesis rates (UNSR) in TS (solid line) and controls (broken line). FHNC (l/h) was calculated as the slope of the linear regression analysis of UNSR on corresponding mean blood α-amino nitrogen concentrations. This measure standardizes the UNSR with regard to changes in blood α-amino nitrogen concentration.

non-alcoholic fatty liver disease, linked to metabolic disease, ii) liver architectural changes and vascular lesions, and finally and iii) biliary lesions (6).

Our results do not indicate that the liver enzyme elevations or the morphological liver lesions in TS are due to functional metabolic hepatocyte deficits. On the other hand, that such lesions are associated with functional deficits cannot be excluded from our data. We saw that a short 2 month course without HRT lead to elevation of liver enzymes, readily suppressible by standard HRT, as also documented previously (3–5). Recently, the same response to HRT, albeit less pronounced, has also been found among postmenopausal women (27). Thus, lack of estrogens in some way seems to affect the liver. The liver does express the estrogen receptor α (ERα), which can only be detected after the onset of puberty but later ovariectomy does not change the number of ERα (28). Exogenous estradiol suppresses fibrosis of the liver induced by dimethyl-nitrosamine in ovariectomized rat (29), and specifically estradiol reduced the deposition of type I and III procollagen (PINP, PIIINP) and the level of tissue inhibitor of metalloproteinase-1 (29). In hepatitis C

infected women, higher exposure to endogenous or exogenous estrogens protects against the development of liver fibrosis progression (30), while postmenopausal status is related to accelerated liver fibrosis, and in other conditions of liver fibrosis females are relatively protected compared with males (31). Here, we did not assess the circulating levels of PINP and PIIINP, but previously we have found normal levels in hormone substituted TS (32). Estradiol, through ERα and ERβ, is also a direct transcriptional regulator of vascular endothelial growth factor (VEGF; (33)), which activates receptor 1 and 2 (VEGFR-1 and -2) of liver sinusoidal endothelial cells (34), leading to paracrine release of various growth and survival factors that protects hepatocytes from toxins (35), and promote hepatocyte proliferation. It is thus possible that such effects of estrogen in protecting hepatocyte integrity and prolonging hepatocyte life-span are responsible for the hepatocyte leakage of liver enzymes during estrogen deficiency and the reversal by estrogen supplementation. This mechanism would be in line with the effect of experimental hypothyroidism in rats in accelerating hepatocyte apoptosis, and its prevention by thyroid hormones (36). Previously, however, we tested other aspects of premature ageing examining telomere length in lymphocytes with and without HRT, and did not find evidence of such processes in TS (37).

During the study, we saw a significant, albeit small, decrease in fasting glucose, but no change in fasting insulin, in line with previous studies (38, 39), although Elsheikh *et al.* (40) also found a slight decrease in fasting insulin during HRT. But we did not see a change in the HOMA-index, in accordance with previous studies being unable to document a positive effect of HRT on insulin sensitivity in TS or in other populations of premature ovarian failure (reviewed in (41)). Thus, in light of the fact that liver enzymes dropped precipitously during the study along with only minimal changes in glucose homeostasis, it seems less likely that the observed changes are due to an improved metabolic status.

By GEC, we tested the cytosolic enzymatic efficacy of the galactose-kinase in the hepatocyte, and found this to be normal and unaffected by HRT in TS and by contraceptive pills in controls. This is in agreement with the galacto-kinase enzyme being a phylogenetically old

Table 3 Liver function tests from the galactose elimination capacity (GEC) and indocyanine green (ICG), antipyrin clearance and functional hepatic nitrogen clearance (FHNC) examinations in Turner syndrome (TS) and controls before and during treatment. Results are presented as means and standard deviation.

	TS	TS+HRT	Control	Control+pill	P*	P†
GEC (mmol/min)	2.14±0.40	2.14±0.47	2.03±0.38	2.23±0.50	0.6	1.0
ICG clearance (l/min)	2.06±0.39	2.06±0.31	2.28±0.29	2.26±0.31	0.1	0.5
Antipyrin clearance (ml/min×kg)	5.0±2.0	6.0±4.0	7.0±4.0	5.0±2.0	0.4	0.6
FHNC (L/h)	19.4±5.4	24.4±10.2	25.2±7.3	27.2±7.9	0.1	0.2

*Untreated TS versus Controls. †Untreated TS versus treated TS.

enzyme and non-inducible. Correspondingly, the GEC has proven to be a robust measure of liver function during a variety of pathophysiological events such as dietary, hormonal, and stress-related changes. The present data show that the GEC is not sensitive to changes in estradiol, which may be valuable in the diagnostic work-up of women with transaminasemia.

By ICG bolus infusion, we quantitatively examined the hepatocyte carrier system, since ICG is bound to a putative hepatocyte carrier, taken up and transported through the hepatocyte and subsequently excreted irreversibly and un-metabolized into the bile (42). In normal subjects, the liver extracts almost all incoming ICG and, therefore, the clearance of ICG is close to the hepatic blood flow. ICG clearance is reduced if either hepatic blood flow or hepatic secretory function is reduced. This hepatocyte bile excretory system consists of several distinct transporters, some of which are responsible for biliary elimination of bile acids, drugs, electrolytes, and carcinogens. Thus, since ICG clearance was comparable among the groups studied, neither hepatic blood flow nor hepatic excretory function is likely to be affected by estrogenic status.

Antipyrin clearance was normal and unaffected by HRT, and we conclude that also the microsomal system activity is non-estrogen dependent. The microsomal system includes elements of the cytochrome P-450 system, responsible for detoxification of a large variety of xenobiotics, including carcinogens and drugs. The system is highly inducible by several drugs and by dietary changes, which may have consequences for rational pharmacotherapy. The present data indicate that such considerations are not warranted during estrogen treatment.

Urea synthesis is regulated by factors such as functional liver mass and different hormones (10, 43, 44) which plays an active role in whole body nitrogen homeostasis and body composition. The process is the final and irreversible step in amino acid N degradation – nitrogen not used for protein synthesis is converted into urea in the mitochondria and cytosol of hepatocytes. Glucagon and corticosteroids increase FHNC (45, 46) and GH decreases it (47, 48). Hypoglycemia induces a marked increase in FHNC and net release of amino-acids from muscle, an effect possibly mediated by glucagon (49), similar to that seen during long-term fasting (50). Our data show that in untreated TS the FHNC was quantitatively reduced to 80% but variable and thus not statistically decreased. If anything, this was normalized by HRT, thus supporting the notion that urea synthesis may be regulated by lack of estrogen. We have not been able to identify earlier reports on such a relationship. It should be noted that the reported reduction in FHNC, although being numerically small, may still be functionally important for regulation of body composition. It implies that 20% of whole-body nitrogen balance is diverted away from loss of amino-nitrogen towards nitrogen conservation, possibly favoring protein build-up. This might indicate a distinct nitrogen economy

in TS. If so, this was probably not due to changes in other hormones that influence FHNC, since we found similar levels of glucagon in all groups, normal insulin sensitivity, and normal levels of circulating hormones known to influence FHNC. Studies of similar sample size as the present of glucagon, corticosteroids and GH versus placebo have shown distinct excursions in FHNC (45–48).

The sample size in the present study is small and this obviously hampers our ability to making firm conclusions. In any case, the lack of difference in GEC, ICGCl, and ApCl between TS and controls both with and without treatment, and the small variation in data, do not indicate a large risk of type II errors. Furthermore, previously GEC, ICGCl, and ApCl have been used to show significant results in similarly small studies as the present. However, the results from the FHNC study may well be inconclusive due to a type I error. Furthermore, it would have been desirable to have access to *in situ* hybridization of liver biopsy tissue for apoptotic markers.

In conclusion, liver enzymes are elevated in TS and readily suppressed by HRT. During the detailed quantitative liver metabolic studies, we found a slight and uncertain change in liver function with lower set-point of urea nitrogen synthesis, while other liver functions were normal, both with and without HRT. Evidently, estradiol has no marked importance for hepatocyte function, and patients with TS have no serious deficit in liver function. The explanation to transaminasemia in TS should probably rather be sought among factors reflecting hepatocyte longevity.

Acknowledgements

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