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Tamoxifen affects glucose and lipid metabolism parameters, causes browning of subcutaneous adipose tissue and transient body composition changes in C57BL/6NTac mice

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ABSTRACT

Tamoxifen is a selective estrogen receptor (ER) modulator which is widely used to generate inducible conditional transgenic mouse models. Activation of ER signaling plays an important role in the regulation of adipose tissue (AT) metabolism. We therefore tested the hypothesis that tamoxifen administration causes changes in AT biology *in vivo*. 12 weeks old male C57BL/6NTac mice were treated with either tamoxifen (n = 18) or vehicle (n = 18) for 5 consecutive days. Tamoxifen treatment effects on body composition, energy homeostasis, parameters of AT biology, glucose and lipid metabolism were investigated up to an age of 18 weeks.

We found that tamoxifen treatment causes: I) significantly increased HbA_{1c}, triglyceride and free fatty acid serum concentrations ($p < 0.01$), II) browning of subcutaneous AT and increased UCP-1 expression, III) increased AT proliferation marker *Ki67* mRNA expression, IV) changes in adipocyte size distribution, and V) transient body composition changes.

Tamoxifen may induce changes in body composition, whole body glucose and lipid metabolism and has significant effects on AT biology, which need to be considered when using Tamoxifen as a tool to induce conditional transgenic mouse models. Our data further suggest that tamoxifen-treated wildtype mice should be characterized in parallel to experimental transgenic models to control for tamoxifen administration effects.

1. Introduction

Conditional transgenic mouse models provide a powerful tool for functional analyses of genes expressed preferentially in a particular tissue. Efficient silencing of a specific gene can be achieved by the CreER-*lox*P recombination technology, which allows for a temporally and tissue-specifically (promoter dependent) regulated recombination induced by tamoxifen administration [1]. Tamoxifen is a selective estrogen receptor (ER) modulator, which may affect whole body metabolism, but also adipose tissue (AT) biology. In ovariectomized rats, tamoxifen mimicked the effects of estradiol and caused significant changes in food intake, body weight and composition [2].

Although expression of the CreER tamoxifen-induced system ($CreER(T)^2$) in adipocytes does not seem to affect AT biology itself [3,4], tamoxifen may contribute to the regulation of various AT processes. Therefore, we tested the hypothesis that tamoxifen administration causes changes in parameters of AT biology, whole body glucose and lipid metabolism in male C57BL/6NTac mice.

2. Material and methods

2.1. Animals

Animal experiments followed the 'Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985) as well as

Abbreviations: NMR, nuclear magnetic resonance.

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specific national laws approved by the local authorities of the state of Saxony, Germany as recommended by the responsible local animal ethics review board (Regierungspräsidium Leipzig, TVV21_23/12, Germany). Twenty 11weeks old C57BL/6NTac male mice were obtained from Taconic Laboratories (Taconic Europe, Denmark) and randomly assigned to either tamoxifen (n = 18) or vehicle (Miglyol, n = 18) administration groups. Tamoxifen (Sigma–Aldrich; #T5648-1G, St. Louis, MO, USA) was dissolved in Miglyol (Fagron, #700282-0001, Rotterdam, NL) at a concentration of 20 mg/ml. At an age of 12 weeks, 1 mg tamoxifen or 50 μl Miglyol was administered intraperitoneally for five consecutive days, changing the injection site daily. All mice were housed in pathogen-free facilities in groups of three to five at 22 ± 2 °C on a 12-h light/dark cycle. All animals had free access to water and were fed with standard chow (Sniff GmbH, Soest, Germany).

2.2. Phenotypic characterization

Mice were studied from 10 up to an age of 18 weeks. Body weight and food intake were recorded daily; naso-anal length and rectal body temperature (TH-5, Thermalert Monitoring Thermometer, Clifton, NJ, USA) were measured at the end at 18 weeks of age (n = 10 per experimental group). Intraperitoneal insulin tolerance tests (ITTs) were performed at the age of 11 and 16 weeks as described previously [5]. Whole body composition (fat mass, lean mass, water) was determined in awake mice by using NMR technology with EchoMRI700™ instrument (Houston, TX, USA) at 11, 13, 15 and 17 weeks of age. Indirect calorimetry was assessed by a Calorimetry Module (TSE Systems, Bad Homburg, Germany) at an age of 16 weeks as previously described [5].

Mice were sacrificed at the age of 18 weeks by an overdose of isofluran (Baxter, Unterschleißheim, Germany). Liver, brown (BAT), subcutaneous (SC) and epigonadal (EPI) adipose tissue were immediately removed and weighed.

2.3. Analytical procedures

Blood glucose values were determined from whole venous blood samples using a glucose monitor (FreeStyle, Abbott GmbH, Ludwigshafen, Germany). Insulin, leptin and adiponectin serum concentrations were measured by ELISA (mInsulin/Leptin ELISA; CrystalChem Inc, Downers Grove, IL., Adiponectin ELISA; AdipoGen Inc, Incheon, Korea). Serum lipid profile and HbA_{1c} level were measured by an automated analyzer (COBAS8000, Roche, Basel, Switzerland).

2.4. Adipocyte size and AT histology

Adipocytes were isolated from (EPI) and (SC) fat pads by 1 mg/ml collagenase digestion and adipocyte size distribution was determined in 200 μl suspension in a Coulter Counter (Multisizer III; Beckman Coulter, Krefeld, Germany) as described [5]. Biopsies of SC and EPI AT were fixed in 10% buffered formalin and imbedded in paraffin. Multiple sections were obtained from EPI and SC fat pads and analyzed systematically with respect to adipocyte size and number. The sections were stained with hematoxylin/eosin and UCP-1 (1:200, Abcam, ab10983, Cambridge, UK) immunohistochemistry was performed as previously described [5].

2.5. mRNA expression

Total RNA was isolated from SC and EPI AT and cDNA was subsequently amplified as described [5]. Customized primers (Biomers, Ulm, Germany) were used for the detection of *Esr1*, *Ucp1*, *Ki67*, *18Sr* and *L19r* mRNA (Table 1). mRNA expression was measured in a fluorescence temperature cycler (ABI PRISM7500, Applied Biosystems, Darmstadt, Germany) and calculated relative to *18S* or *L19* rRNA.

2.6. Western blot analyses

SC and EPI AT was removed and homogenized with tissue-mill homogenizer (MM400Retsch GmbH, Haan, Germany) in sucrose buffer as previously described [5] with antibodies raised against Estrogen Receptor alpha (ERα, 1:500, Abcam, ab32063, Cambridge, UK) and Uncoupling protein 1 (UCP1, 1:200, ab10983, Cambridge, UK). D-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH, 1:3.000; Research Diagnostics, Flanders, Netherlands) served as loading control.

2.7. Statistical analyses

Data are given as means ± SEM. Data sets were analyzed for statistical significance using a two-tailed unpaired t test or ANOVA. P values less than 0.05 were considered significant.

3. Results

3.1. Tamoxifen administration transiently affects body composition

During the entire study course, tamoxifen administration did not affect body weight dynamics, food intake, energy expenditure (mean oxygen consumption), spontaneous activity (Fig. 1A–D), CO_2 production, respiratory quotient, water intake (data not shown). However, tamoxifen led to an acute decrease in body fat mass 1 week after initiation of the treatment, which converted into a significantly higher relative body fat mass 5 weeks after the treatment (Fig. 1E) with reciprocal effects on lean body mass (Fig. 1F). In contrast, there were no significant body composition changes in vehicle treated controls (Fig. 1E and F). At 18 weeks, there was only a trend for higher relative organ weights of both subcutaneous (SC) and epigonadal AT of mice treated with tamoxifen (Fig. 2A and B).

3.2. Tamoxifen administration induces browning and adipocyte proliferation in subcutaneous AT

In an automated adipocyte size analysis, we found that tamoxifen administration led to a bimodal adipocyte distribution curve (Fig. 2C). Analysis of histological AT slides confirmed a significant reduction of mean adipocyte size in SC AT (Table 2) and the appearance of multilocular and mitochondria-rich adipocytes. The two adipocyte size peaks may therefore represent distinct subclasses of adipocytes. We then tested the hypothesis that tamoxifen may induce browning of SC AT. Indeed, positive UCP-1 immunostaining in AT (SC > epigonadal) of tamoxifen treated mice, but not in controls suggests a change of a subgroup of adipocytes into a more brown-like phenotype (Fig. 2D). At the mRNA level, tamoxifen treated mice displayed significantly higher (in SC AT) *Ucp-1* expression 6 weeks after tamoxifen administration (Fig. 2E), which could be confirmed at least as a tendency for UCP-1 protein (Fig. 2E).

Moreover, as a marker for increased proliferation, we found significantly higher *Ki67* expression in SC AT of mice treated with tamoxifen as compared to controls (Fig. 2F).

Tamoxifen effects on AT biology may be caused by alterations in ER expression. Supporting this hypothesis, we found higher Estrogen Receptor-α protein expression in tamoxifen treated compared to control mice (Fig. 2G).

Table 1
Primer sequences used for mRNA detection.

Gene	Forward	Backward	Accession no
Ki67	TGAAGTCAAAGAGCAAGAGGTATGA	TTCAAGTCCCCAAAGCCTGG	AC_000029
Ucp1	ACTGCCACACCTCCAGTCATT	TTGTCATCTACGGGCACAAAG	AC_000030.1
Esr1	TCTCTGGGCGACATTCTTCT	GCTTTGGTGTGAAGGGTCAT	AC_000032.1
L19	GGAAAAAGAAGGTCTGGTTGGA	TGCTGCTGTTCCTGTTTTC	NC_000077.6

3.3. Tamoxifen administration induces changes in glucose and lipid metabolism

6 weeks after tamoxifen administration, we found a significantly higher HbA1c in tamoxifen compared to vehicle treated mice (Fig. 2H). This significant difference could not be explained by group differences in insulin sensitivity as determined by ITTs at 16 weeks (data not shown), adiponectin or insulin serum concentrations at 18 weeks (Table 2). In addition, tamoxifen administration causes significantly higher serum triglyceride and free fatty acid

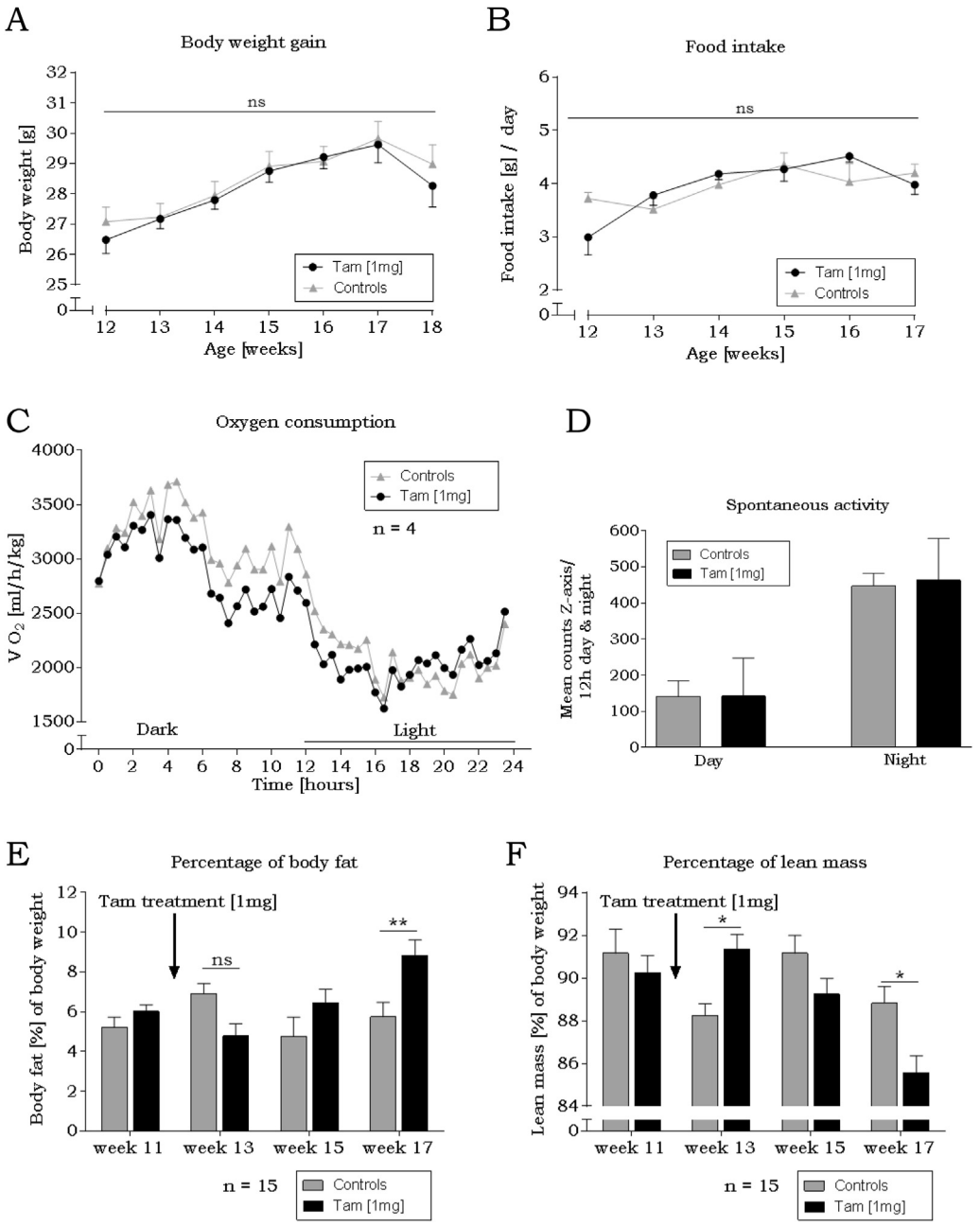

Fig. 1. Effects of tamoxifen on body composition and energy expenditure. (A) Body weight change and (B) food intake were indistinguishable between Tamoxifen (Tam) treated mice (N = 10) and controls (Con) (N = 10) over the entire study period. (C) Mean oxygen consumption (VO_2) in tamoxifen (Tam) treated (N = 4) and control mice (N = 4) at an age of 17 weeks. (D) Spontaneous activity was estimated as sit up of the mice on the z-axis and depicted as means over an observation period of overall 72 h. Whole body fat mass (E) and lean mass (F) was determined in awake mice by using NMR technology (EchoMRI700, Medical Systems, Houston, TX, USA) in control (N = 15) and tamoxifen-treated mice (N = 15) at 11, 13, 15 and 17 weeks of age. Data are given as means ± SEM. *, $p < 0.05$; **, $p < 0.01$).

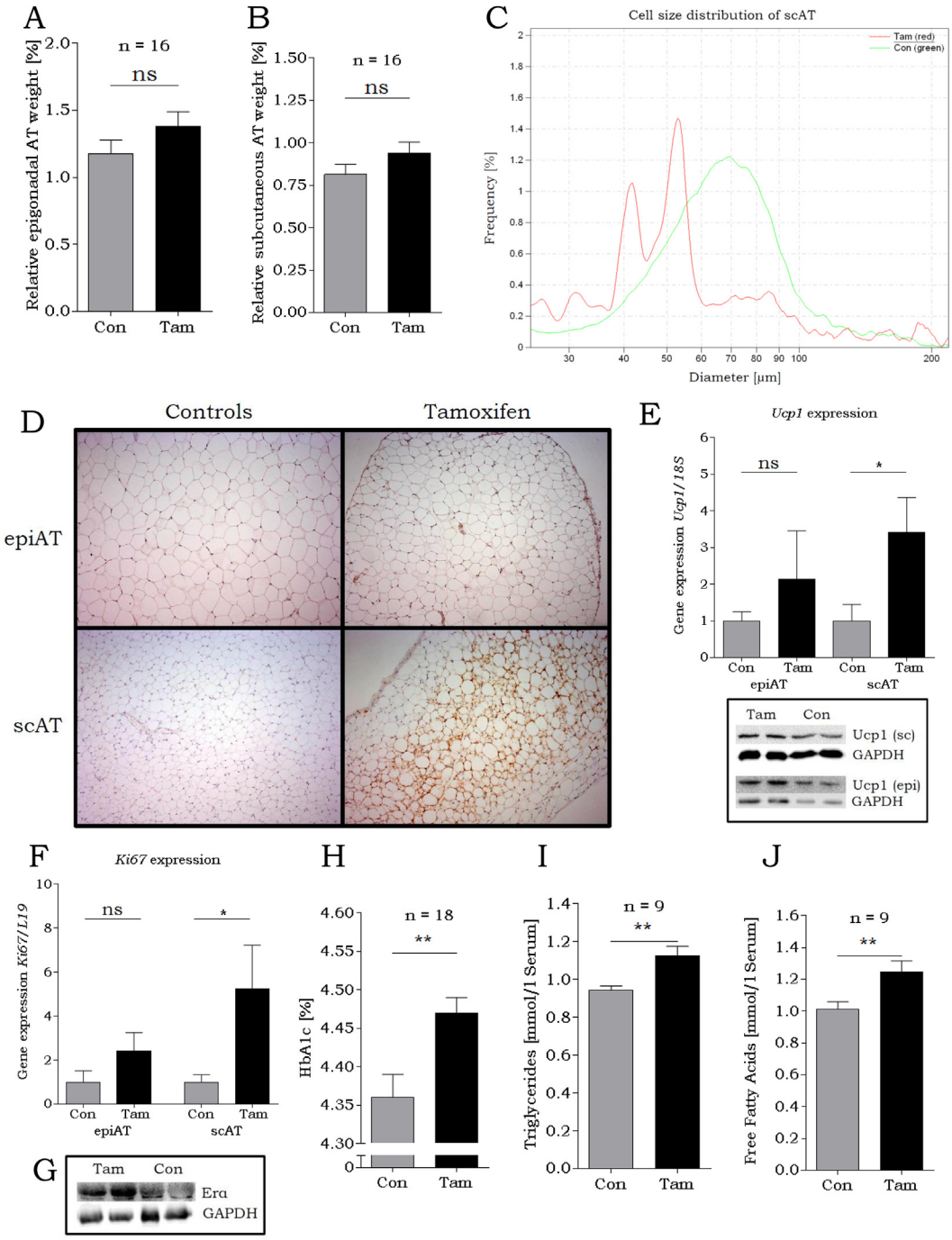

Fig. 2. Effects of tamoxifen on adipose tissue biology and parameters of glucose and lipid metabolism. Relative weight of epigonadal (A) and subcutaneous (B) adipose tissue (AT) are presented relative to total body weight from control (Con; N = 16) and tamoxifen treated (Tam; N = 16) mice. (C) Subcutaneous cell size (scAT) distribution of adipocytes measured in a Coulter Counter (Multisizer III; Beckman Coulter, Krefeld, Germany). 6 weeks after initiation of tamoxifen administration (red), adipocyte size distribution changes towards a bimodal curve and smaller mean adipocyte diameters as compared to controls (green). The two peaks of the adipocyte size distribution curve in tamoxifen treated mice may represent distinct cell types (beige/brite versus white adipocytes). (D) Representative images (original magnification ×200) of UCP-1 immunohistochemistry (positive staining of the UCP-1 protein with DAB as chromogen in brown) of epigonadal (epiAT) and subcutaneous (scAT) adipose tissue 5 µm paraffin sections of control mice (left panels) and tamoxifen treated mice (right panels) at an age of 18 weeks. SC adipose tissue of tamoxifen treated mice displays strongly positive UCP-1 staining (supporting a browning effect of tamoxifen), whereas control mice in both fat depots and epigonadal tissue of tamoxifen treated mice were UCP-1 negative. (E) *Ucp-1* mRNA analyses revealed a significantly higher expression in scAT of tamoxifen treated mice compared to controls, whereas in epiAT there was only a non-significant trend for higher *Ucp-1* expression in tamoxifen treated mice compared to controls (n = 6 per treatment group). Representative Western blots for UCP-1 (compared to GAPDH) of subcutaneous (sc) and epigonadal (epi) adipose tissue of control (Con) and tamoxifen treated (Tam) mice. (F) Expression of proliferation marker *Ki67* was significantly higher in scAT of tamoxifen treated (Tam; N = 6) mice compared to controls (Con; N = 6), whereas in epiAT no significant differences could be observed. (G) Representative Western blots for ERα compared to GAPDH of epigonadal adipose tissue of control (Con) and tamoxifen treated (Tam) mice. Tamoxifen administration caused significantly higher (H) HbA_{1c}, (I) triglyceride, and (J) free fatty acids serum concentrations at 18 weeks compared to controls (n = 18). Data are given as means ± SEM. (*, $p < 0.05$; ns, non-significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Characterization of treatment groups. Circulating parameters of lipid and glucose metabolism as well as parameters of adipose tissue biology at an age of 18 weeks (N = 16). FFA, free fatty acids, epiAT, epigonadal adipose tissue, scat, subcutaneous adipose tissue.

Parameter	Controls	Tamoxifen	p-value
Triglycerides [mmol/l]	0.94 ± 0.02	1.13 ± 0.05	**
Total cholesterol [mmol/l]	2.42 ± 0.10	2.35 ± 0.20	ns
HDL-cholesterol [mmol/l]	2.20 ± 0.10	2.06 ± 0.18	ns
LDL-cholesterol [mmol/l]	0.27 ± 0.02	0.30 ± 0.04	ns
FFA [mmol/l]	1.01 ± 0.05	1.25 ± 0.07	*
Leptin [ng/ml]	42.9 ± 8.28	36.5 ± 5.73	ns
Adiponectin [μg/ml]	68.5 ± 3.67	64.9 ± 4.22	ns
Insulin [mg/l]	1.03 ± 0.16	1.10 ± 0.11	ns
HbA1c [%]	4.36 ± 0.03	4.47 ± 0.02	**
Relative liver weight [%]	4.85 ± 0.19	4.65 ± 0.16	ns
Relative BAT weight [%]	0.37 ± 0.03	0.33 ± 0.06	ns
Mean cell size scAT [μm]	71.3 ± 1.89	62.9 ± 2.82	*
Mean cell size epiAT [μm]	84.8 ± 4.62	88.9 ± 4.11	ns
Adipocytes/mg scat (n)	341 ± 37	357 ± 50	ns
Adipocytes/mg epiAT (n)	337 ± 21	445 ± 56	ns

Data are given as means ± SEM. Data sets were analyzed for statistical significance using a two-tailed unpaired t test (*p-value <0.05; **p-value <0.01).

concentrations (Fig. 2I and J) at 18 weeks. In contrast, total cholesterol, HDL- and LDL-cholesterol were not different between tamoxifen and vehicle treated mice (Table 2).

4. Discussion

Tamoxifen has been widely used to activate Cre-recombinase that spatiotemporally controls target gene expression in animal models. Recently, it has been demonstrated that tamoxifen itself may affect adipose tissue accumulation and function by inducing reactive oxygen species production, apoptosis and autophagy [6]. We confirm the observed reduction in fat mass [6], which was transient and followed by an over-compensation resulting in increased fat mass. The higher percentage of fat mass in Tam treated animals compared to controls is consistent with previous research works, which suggest a negative impact of Tamoxifen on body composition, translating into increased fat content in women who undergo this treatment [7,8].

In addition, we identified previously unrecognized effects of tamoxifen on browning of subcutaneous AT, adipocyte proliferation, but also tamoxifen-associated changes in glucose and lipid metabolism. Together with the notion, that tamoxifen may induce apoptosis in AT followed by a recovery phase [6], additional changes in AT biology have to be considered which may affect whole body glucose and lipid homeostasis even after normalization of AT function. To check if cell proliferation is influenced we performed gene expression analysis of proliferation marker *Ki67* [9]. Expression levels of *Ki67* were significantly increased in subcutaneous AT of Tam treated animals compared to controls. This finding suggests that Tamoxifen injection promotes cellular division in subcutaneous AT, which could be another evidence of brown adipocyte proliferation.

We could confirm that Tamoxifen induced up-regulation of *ERα* which was obtained by previous studies on the reproductive system: Tam treatment was associated with up-regulation of *ERα* in seminal vesicles of neonatal male rats [10] and in the uterus and vagina of neonatal and ovariectomized adult mice [11]. These results suggest that Tamoxifen up-regulates *ERα* expression by increasing promoter activity of the *ERα* gene (Ers1) at the transcriptional level [12].

Furthermore, Tam treated animals showed increased serum levels of triglycerides and free fatty acids compared to control animals. Serum HDL, LDL and total cholesterol levels were comparable between the two groups, in spite of what was shown by previous investigations [13,14]. Higher levels of serum triglycerides and free fatty acids might be a direct consequence of the conversion from white to beige adipocytes, because as known from literature lipid droplets of brown and beige adipocytes are smaller and scattered throughout. An important limitation of our study is that data presented herein only reflect the time up to 6 weeks after tamoxifen administration and long-term effects have not been evaluated.

Taken together, the observed effects of tamoxifen on AT biology and circulating parameters of glucose and lipid metabolism may confound the functional study of target genes in adipose tissue. Therefore, we propose that experiments using the CreER tamoxifen-induced system to generate transgenic mice should include appropriate tamoxifen-treated wildtype controls.

Contribution statement

All authors made substantial contribution to the concept and design of this study, acquisition of data or analyses and interpretation of data and to drafting of the article. All authors gave final approval of the version to be published. NH and NK are the guarantors of this work, had full access to all data and take fully responsibility for integrity of data and the accuracy of data analysis.

Duality of interest

The authors declare that there is no duality interest associated with the manuscript.

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References

[1] V. Vasioukhin, L. Degenstein, B. Wise, E. Fuchs, The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8551–8556.
[2] G.N. Wade, H.W. Heller, Tamoxifen mimics the effects of estradiol on food intake, body weight, and body composition in rats, Am. J. Physiol. 264 (1993) R1219–R1223.
[3] K.Y. Lee, S.J. Russell, S. Ussar, J. Boucher, C. Vernochet, M.A. Mori, G. Smyth, M. Rourk, C. Cederquist, E.D. Rosen, B.B. Kahn, C.R. Kahn, Lessons on conditional gene targeting in mouse adipose tissue, Diabetes 62 (2013) 864–874.
[4] A. Sassmann, S. Offermanns, N. Wettschureck, Tamoxifen-inducible cre-mediated recombination in adipocytes, Genesis 48 (2010) 618–625 (New York, N.Y. 2000).
[5] M. Kern, J. Kosacka, N. Hesselbarth, J. Brückner, J.T. Heiker, G. Flehmig, I. Klöting, P. Kovacs, M. Matz-Soja, R. Gebhardt, K. Krohn, S. Sales, K. Abshagen, A. Shevchenko, M. Stumvoll, M. Blüher, N. Klöting, Liver-restricted Repin1 deficiency improves whole-body insulin sensitivity, alters lipid metabolism, and causes secondary changes in adipose tissue in mice, Diabetes 63 (2014) 3295–3309.
[6] L. Liu, P. Zou, L. Zheng, L.E. Linarelli, S. Amarell, A. Passaro, D. Liu, Z. Cheng, Tamoxifen reduces fat mass by boosting reactive oxygen species, Cell Death Dis. 6 (2015) e1586.
[7] P.A. Ali, F.H. al-Ghorabie, C.J. Evans, A.M. el-Sharkawi, D.A. Hancock, Body composition measurements using DXA and other techniques in tamoxifen-

treated patients, Appl. Radiat. Isotopes Incl. Data, Instrum. Methods Use Agric. Indus. Med. 49 (1998) 643–645.
[8] P.M. Sheean, K. Hoskins, M. Stolley, Body composition changes in females treated for breast cancer: a review of the evidence, Breast Cancer Res. Treat. 135 (2012) 663–680.
[9] T. Scholzen, J. Gerdes, The Ki-67 protein: from the known and the unknown, J. Cell Physiol. 182 (2000) 311–322.
[10] K. Williams, J.S. Fisher, K.J. Turner, C. McKinnell, P.T. Saunders, R.M. Sharpe, Relationship between expression of sex steroid receptors and structure of the seminal vesicles after neonatal treatment of rats with potent or weak estrogens, Environ. Health Perspect. 109 (2001) 1227–1235.
[11] T. Sato, Y. Ohta, H. Okamura, S. Hayashi, T. Iguchi, Estrogen receptor (ER) and its messenger ribonucleic acid expression in the genital tract of female mice exposed neonatally to tamoxifen and diethylstilbestrol, Anat. Rec. 244 (1996) 374–385.
[12] Y. Miyoshi, K. Murase, M. Saito, M. Imamura, K. Oh, Mechanisms of estrogen receptor-α upregulation in breast cancers, Med. Mol. Morphol. 43 (2010) 193–196.
[13] A.M. Dnistrian, M.K. Schwartz, E.J. Greenberg, C.A. Smith, D.C. Schwartz, Effect of tamoxifen on serum cholesterol and lipoproteins during chemohormonal therapy, Clin. Chim. Acta Int. J. Clin. Chem. 223 (1993) 43–52.
[14] A.L. Holleran, B. Lindenthal, T.A. Aldaghlas, J.K. Kelleher, Effect of tamoxifen on cholesterol synthesis in HepG2 cells and cultured rat hepatocytes, Metabolism: Clin. Exp. 47 (1998) 1504–1513.