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# Differential influence of peripheral and systemic sex steroids on skeletal muscle quality in pre- and postmenopausal women

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## Summary

**Aging is associated with gradual decline of skeletal muscle strength and mass often leading to diminished muscle quality. This phenomenon is known as sarcopenia and affects about 30% of the over 60-year-old population. Androgens act as anabolic agents regulating muscle mass and improving muscle performance. The role of female sex steroids as well as the ability of skeletal muscle tissue to locally produce sex steroids has been less extensively studied. We show that despite the extensive systemic deficit of sex steroid hormones in postmenopausal compared to premenopausal women, the hormone content of skeletal muscle does not follow the same trend. In contrast to the systemic levels, muscle tissue of post- and premenopausal women had similar concentrations of dehydroepiandrosterone and androstenedione, while the concentrations of estradiol and testosterone were significantly higher in muscle of the postmenopausal women. The presence of steroidogenic enzymes in muscle tissue indicates that the elevated postmenopausal steroid levels in skeletal muscle are because of local steroidogenesis. The circulating sex steroids were associated with better**

**muscle quality while the muscle concentrations reflected the amount of infiltrated fat within muscle tissue. We conclude that systemically delivered and peripherally produced sex steroids have distinct roles in the regulation of neuromuscular characteristics during aging.**

**Key words: endocrine effects of steroid hormones; intracrinology of skeletal muscle; localization of steroidogenic enzymes; menopause; paracrine effects of steroid hormones; systemic and local steroidogenesis.**

## Introduction

Aging is associated with gradual decline of skeletal muscle strength, loss of muscle mass and changes in muscle composition because of infiltration of fat (Delmonico *et al.*, 2009). This phenomenon is known as sarcopenia (Cruz-Jentoft *et al.*, 2010). During perimenopause, ovarian function declines leading to irregular circulating levels of especially 17 $\beta$ -estradiol (E<sub>2</sub>) but also of other sex steroids. At the onset of perimenopause, both E<sub>2</sub> and follicle-stimulating hormone (FSH) levels may be increased even though women are still having regular menstrual flow, but towards menopausal transition E<sub>2</sub> levels decrease and eventually women stop menstruating (Prior, 2005). While menopause marks the end of fertility, it is also thought to lead to severe estrogen deficiency in peripheral tissues. The results of the cross-sectional studies suggest that age-induced changes in skeletal muscle accelerate in women postmenopausally compared to age-matched men or age-matched women on estrogen-containing hormone replacement therapy (HRT) (Phillips *et al.*, 1993; Maltais *et al.*, 2009). Recent systematic meta-analysis concluded that postmenopausal women on HRT had greater muscle strength than those who did not receive HRT (Greising *et al.*, 2009). Similar results were gained in our study showing that long-term HRT users had better lower-limb muscle power, mobility, and composition than their nonusing monozygotic co-twin sisters (Ronkainen *et al.*, 2009). Despite these results, the mechanism by which the loss of ovarian function at menopause and replacement of hormones by HRT influences muscle properties remains unclear.

Peripheral tissues, including skeletal muscle, can produce steroids locally by conversion of prehormones to estrogens and/or androgens involving a cascade of steroidogenic enzymes. Aizawa *et al.* (2007) showed that 3 $\beta$ -hydroxysteroid dehydrogenases (HSD3Bs), HSD17Bs, and aromatase cytochrome P450 (aromatase) are present in murine skeletal muscle and involved in catalyzing the local synthesis of sex steroid hormones from dehydroepiandrosterone (DHEA) or testosterone. They also

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showed that acute exercise activates muscle steroidogenesis, which however was gender dependent (Aizawa *et al.*, 2008, 2010). Even though aromatization of androgens by muscle and adipose tissue has been known since 1970s (Longcope *et al.*, 1978), to our knowledge, the only human study directly investigating the role of local steroidogenesis on the regulation of muscle properties has been reported by Vingren *et al.* (2008). They studied the effects of acute resistance exercise on steroid hormone synthesis in muscle tissue in young men and women but found no changes in muscle testosterone content or the protein expression of HSD17B or HSD3B.

Steroid hormone signaling in target tissues is in general largely mediated by nuclear androgen and estrogen receptors (AR, ESR1 and ESR2), which act as transcription factors and are expressed also in skeletal muscle (Kadi *et al.*, 2000; Lemoine *et al.*, 2003; Sinha-Hikim *et al.*, 2004; Wiik *et al.*, 2009). Testosterone can directly activate androgen, but its conversion to dihydrotestosterone (DHT) by 5 $\alpha$ -reductases (SRD5A1 or SRD5A2) leads to much stronger androgen activation. Interestingly, testosterone can alternatively be converted to the most potent estrogen, E<sub>2</sub>, by aromatase. E<sub>2</sub> can also be formed from estrone (E<sub>1</sub>) by HSD17Bs. The majority of androgens and estrogens in circulation are bound to specific carrier proteins, including albumin, sex hormone-binding globulin (SHBG), and corticosteroid-binding globulin (Mazer, 2009). On the contrary, the precursor hormones DHEA or its sulfate (DHEAS) as well as androstenedione do not extensively bind to SHBG, which suggests different accessibility to peripheral tissues compared to SHBG-bound estrogens and androgens. Furthermore, the concentration of pre-hormones in circulation is 100–500 times higher than of testosterone and 1000–10 000 times higher than of E<sub>2</sub>, which further emphasize their significance as precursor molecules for the synthesis of more active hormones (Labrie, 1991). At the moment, it is still unclear how the uptake of estrogens, androgens, and pre-hormones into the skeletal muscle is regulated especially in women after menopause.

The loss of ovarian function at menopause leads to the reduction in steroid hormones in circulation, which hypothetically leads to hormonal deficiency in skeletal muscle. We hypothesized that to cope with systemic reduction in steroid hormones after menopause, the enzymatic machinery needed for local steroidogenesis is activated in the skeletal muscle. Accordingly, the aim of this study was to investigate the association between age and skeletal muscle steroidogenesis among healthy pre- and postmenopausal women and to assess whether systemic and/or local steroidogenesis is associated with skeletal muscle quality.

## Results

### Premenopausal women had better muscle composition and function than postmenopausal women

As shown in Table 1, pre- and postmenopausal women did not significantly differ in their body composition or physical activity

levels. Muscle composition and muscle force results are summarized in Table 2. Premenopausal women had somewhat larger cross-sectional area (CSA) of the *quadriceps femoris* (QF) muscle even though the difference was in the border line of significance ( $P = 0.052$ ). Premenopausal women had also larger relative muscle area within muscle compartment than postmenopausal women ( $P = 0.026$ ). The attenuation coefficient of QF was significantly higher ( $P < 0.001$ ) among premenopausal women when compared to the postmenopausal group, suggesting greater fat infiltration into the muscle of postmenopausal women. Furthermore, the knee extension force ( $P = 0.002$ ) and the knee extension force per CSA<sub>QF</sub> ( $P = 0.011$ ) were significantly greater in pre- than postmenopausal women.

### Premenopausal women had more steroid hormones in circulation, but not in skeletal muscle than postmenopausal women

As expected, there were large differences between pre- and postmenopausal women in serum hormone concentrations (Table 3): premenopausal women having significantly higher concentrations of total estrogens (E<sub>2</sub> and E<sub>1</sub>), androstenedione,

**Table 1** General characteristics of the study participants

Variable	Premenopausal women ( $n = 13$ )	Postmenopausal women ( $n = 13$ )	$P$ -value
Age	33 $\pm$ 2	64 $\pm$ 2	
Height, cm	164 $\pm$ 4	164 $\pm$ 5	0.944
Weight, kg	67 $\pm$ 15	68 $\pm$ 14	0.858
LBM, kg	47 $\pm$ 4	46 $\pm$ 5	0.745
Fat mass, kg	21 $\pm$ 12	22 $\pm$ 9	0.754
Physical activity level	3.8 $\pm$ 1.1	3.6 $\pm$ 0.8	0.681

Values are mean  $\pm$  standard deviation.  $P$ -value is obtained from independent samples  $t$ -test. LBM, lean body mass.

**Table 2** Muscle composition and strength

Variable	Premenopausal women ( $n = 12$ )	Postmenopausal women ( $n = 13$ )	$P$ -value
Total CSA <sub>QF</sub> , cm <sup>2</sup>	56 $\pm$ 8	50 $\pm$ 8	0.091
Muscle CSA <sub>QF</sub> , cm <sup>2</sup>	53 $\pm$ 8	47 $\pm$ 7	0.052
Fat CSA <sub>QF</sub> , cm <sup>2</sup>	2.5 $\pm$ 1.4	3.1 $\pm$ 1.2	0.304
Relative muscle area within muscle compartment, %	95 $\pm$ 3	94 $\pm$ 2	<b>0.026</b>
QF attenuation, HU	61 $\pm$ 4	55 $\pm$ 4	<b>&lt; 0.001</b>
Knee extension force, N	514 $\pm$ 115 <sup>†</sup>	386 $\pm$ 58	<b>0.002</b>
Muscle force per CSA <sub>QF</sub> , N cm <sup>-2</sup>	9.9 $\pm$ 1.6	8.3 $\pm$ 0.9	<b>0.011</b>

$n = 13$ .

Values are mean  $\pm$  standard deviation.  $P$ -value is obtained from independent samples  $t$ -test except that for relative muscle area within muscle compartment and for muscle force per CSA<sub>QF</sub> for which Mann–Whitney  $U$  test was used.

CSA<sub>QF</sub>, cross-sectional area of *quadriceps femoris* (QF); HU, hounsfield unit. Statistically significant  $P$ -values are bolded.

**Table 3** Serum and muscle hormone concentrations

	Premenopausal women (n = 13)	Postmenopausal women (n = 13)	P-value
<b>Serum hormones</b>			
E <sub>2</sub> , pM	400 ± 313	23 ± 8	< <b>0.001</b>
FE <sub>2</sub> , pM	6.7 ± 4.1	0.4 ± 0.1	< <b>0.001</b>
E <sub>1</sub> , pM	372 ± 215	96 ± 27	< <b>0.001</b>
FE <sub>1</sub> , pM	13.2 ± 8.0	3.2 ± 0.9	< <b>0.001</b>
Testosterone, pM	1094 ± 430	921 ± 312	0.293
FT, pM	15.5 ± 8.9	10.3 ± 3.7	<b>0.022</b>
DHT, pM	432 ± 258	348 ± 279	0.165
FDHT, pM	2.4 ± 1.2	1.7 ± 1.3	0.064
Androstenedione, pM	4808 ± 1750	2205 ± 937	< <b>0.001</b>
DHEAS, μM	4.8 ± 1.3	2.2 ± 1.0	< <b>0.001</b>
SHBG, nM	54.7 ± 30.0	69.0 ± 17.6	0.149
IGF-1, nM	23.7 ± 5.9	13.2 ± 2.9	< <b>0.001</b>
LH, IU L <sup>-1</sup>	8.6 ± 5.0	29.5 ± 11.7	< <b>0.001</b>
FSH, IU L <sup>-1</sup>	5.8 ± 2.2	78.4 ± 22.6	< <b>0.001</b>
<b>Muscle hormones</b>			
E <sub>2</sub> , nmol μg <sup>-1</sup> protein	1.3 ± 0.2	1.5 ± 0.2	<b>0.030</b>
Testosterone, nmol μg <sup>-1</sup> protein	10.7 ± 2.6	13.2 ± 1.8	<b>0.010</b>
DHT, nmol μg <sup>-1</sup> protein	0.5 ± 0.1	0.6 ± 0.1	0.140
DHEA, nmol μg <sup>-1</sup> protein	66.4 ± 12.4	70.2 ± 7.7	0.356

Values are mean ± standard deviation. P-value is obtained from Mann-Whitney U test for all serum hormones except for SHBG. Independent samples t-test was used for SHBG and muscle hormones.

E<sub>2</sub>, 17β-estradiol; FE<sub>2</sub>, free 17β-estradiol; E<sub>1</sub>, estrone; FE<sub>1</sub>, free estrone; FT, free testosterone; DHT, dihydrotestosterone; FDHT, free dihydrotestosterone; DHEAS, dehydroepiandrosterone sulfate; SHBG, sex hormone-binding globulin; IGF-1, insulin-like growth hormone 1; LH, luteinizing hormone; FSH, follicle-stimulating hormone; DHEA, dehydroepiandrosterone.

Statistically significant P-values are bolded.

and DHEAS as well as free fractions of E<sub>2</sub>, E<sub>1</sub>, and testosterone [free 17β-estradiol (FE<sub>2</sub>), free estrone (FE<sub>1</sub>), and free testosterone (FT), respectively], whereas concentrations of luteinizing hormone (LH) and FSH were significantly lower compared to the postmenopausal group. The concentrations of serum testosterone, DHT, and free dihydrotestosterone (FDHT) did not differ between the study groups. The serum concentration of insulin-like growth hormone 1 (IGF-1) was significantly higher in premenopausal than in postmenopausal women. On the contrary, the muscle concentration of E<sub>2</sub> (-13%, P = 0.030) and testosterone (-19%, P = 0.010) was slightly but significantly lower in the premenopausal women compared to the postmenopausal women while the amount of muscle DHEA and DHT did not differ between groups (Table 3).

### Steroidogenesis-related genes are expressed in skeletal muscle

Muscle gene expression of steroidogenic enzymes *steroid sulfatase (STS)*, *HSD3B1*, *HSD17B5*, *aromatase*, *SRD5A1*, and *SRD5A2* as well as steroid hormone receptors *ESR1*, *AR*, and *G-protein-coupled ESR1 (GPER)* was measured by quantitative PCR (qPCR, Table 4). The expression levels of steroidogenic enzymes were relatively low and not detectable in all

**Table 4** mRNA expression levels of steroidogenic enzymes and hormone receptors in skeletal muscle tissue

Gene	Premenopausal women	Postmenopausal women	P-value
STS	1.2 ± 0.9	2.0 ± 1.4†	0.173
HSD3B1	0.01 ± 0.01‡	0.2 ± 0.4§	<b>0.007</b>
HSD17B5	8.7 ± 6.1	8.5 ± 6.6	0.913
Aromatase	0.04 ± 0.01¶	0.07 ± 0.03††	<b>0.018</b>
SRD5A1	1.0 ± 1.0‡‡	1.0 ± 0.7¶	0.831
SRD5A2	0.2 ± 0.1†	0.3 ± 0.2¶	0.305
ESR1	82.5 ± 45.1	152.8 ± 137.3	0.057
Androgen	74.1 ± 30.7	68.1 ± 21.1	0.786
GPER	2.6 ± 0.9	2.0 ± 1.4†	0.125

Values are mean ± standard deviation. Unit is arbitrary unit obtained by normalizing the expression of the gene of interest by the expression of GAPDH. Mann-Whitney U test was used to obtain P-values. Samples were excluded from the analysis if standard deviation between quantification cycles (C<sub>q</sub>) of the replicates were > 0.4 or no expression could be detected (C<sub>q</sub> > 38). Unless otherwise stated (†n = 11, ‡n = 5, §n = 7, ¶n = 9, ††n = 10, ‡‡n = 12), the sample sizes were 13 for the premenopausal group and 12 for the postmenopausal group.

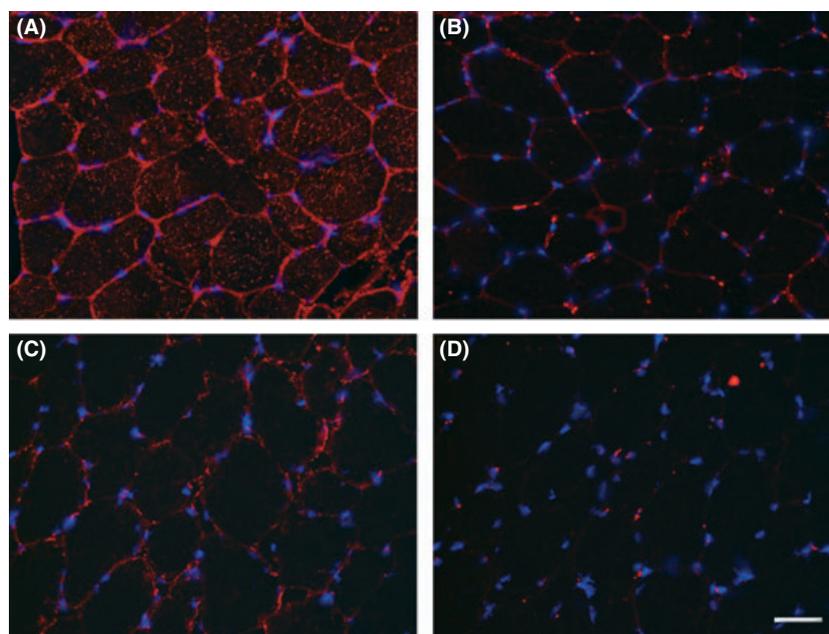
ESR, estrogen receptor; STS, steroid sulfatase.

Statistically significant P-values are bolded.

samples. Nevertheless, all measured genes were expressed in muscle tissue with a trend of the expression being higher in postmenopausal women except for *SRD5A1*. The difference in the gene expression between groups was statistically significant for *HSD3B1* (P = 0.007) and *aromatase* (P = 0.018). From the studied hormone receptors, the mRNA expression of *AR* did not differ between study groups. The mRNA expression of *GPER* tended to be higher in muscle tissue of pre- than postmenopausal women, but the differences did not reach statistical significance. The opposite trend of being more abundant in muscle tissue of post- than premenopausal women was observed for *ESR1*. We also investigated potential role of endocytosis in the uptake of steroid hormones by measuring the mRNA expression of the endocytic receptor called *megalyn* (official name: *low-density lipoprotein receptor-related protein 2; LRP2*), but found no detectable signals in neither pre- nor postmenopausal samples.

### Protein expression of steroidogenic enzymes in skeletal muscle

The localization of key enzymes in steroidogenesis, namely STS, aromatase, and SRD5A1, was studied from cryosectioned muscle samples by immunofluorescence staining (Fig. 1). Because each muscle cell contains tightly packed myofibrils almost entirely fulfilling the interior of the cells, there is only a narrow free space between myofibrils and muscle cell membrane (sarcolemma). This subsarcolemmal zone is occupied by most of the organelles like nuclei and mitochondria. The immunofluorescence analysis showed intense staining with STS antibody at or close to sarcolemma and also some sarcoplasmic staining between myofibrils. Both aromatase and SRD5A1 antibodies stained either the sarcolemma or the subsarcolemmal zone.



**Fig. 1** Immunofluorescence detection of STS, aromatase, and SRD5A1 proteins in the skeletal muscle. (A) Representative image of immunofluorescence for STS (red) shows intensive staining at or close to sarcolemma of muscle cells as well as some sarcoplasmic staining. (B) Representative image of immunofluorescence for aromatase (red) shows some staining at thin subsarcolemmal zone. (C) Representative image of immunofluorescence for SRD5A1 (red) shows staining at subsarcolemmal zone at close proximity to sarcolemma. (D) Omission of primary antibody showed no immunoreactivity in the skeletal muscle. Nuclei are stained blue in all figures. Bar = 50  $\mu\text{m}$ .

None of the antibodies showed muscle cell type-specific staining. On the contrary, they evenly stained the whole cross-sections of the studied muscle sample.

### Association of systemic and local hormone levels with muscle quality

The associations between systemic and local hormone levels with muscle quality variables were studied using Pearson's correlation (Table 5, Fig. 2). Serum concentration of  $E_2$ ,  $E_1$ , DHEAS, and IGF-1 correlated positively with indicators of muscle quality, i.e. muscle force per  $CSA_{QF}$  and QF attenuation. In addition, androstenedione was positively and SHBG negatively associated with QF attenuation. We found no significant correlation between serum testosterone and DHT to muscle quality. Muscle  $E_2$  and testosterone concentrations were negatively associated with QF attenuation, indicating that the high amount of infiltrated fat within muscle tissue is associated with increased muscle steroidogenesis.

### Discussion

Our study showed for the first time that despite the extensive systemic deficit of estrogens  $E_2$  and  $E_1$  as well as prehormones DHEAS and androstenedione in the postmenopausal compared to the premenopausal women, the levels of steroid hormones in skeletal muscle do not follow the same trend. In postmenopausal women, the total serum concentration of  $E_2$  was only 6% and  $E_1$  about one-third of the premenopausal concentrations. Further-

**Table 5** Association of systemic and local hormone levels with indicators of muscle quality

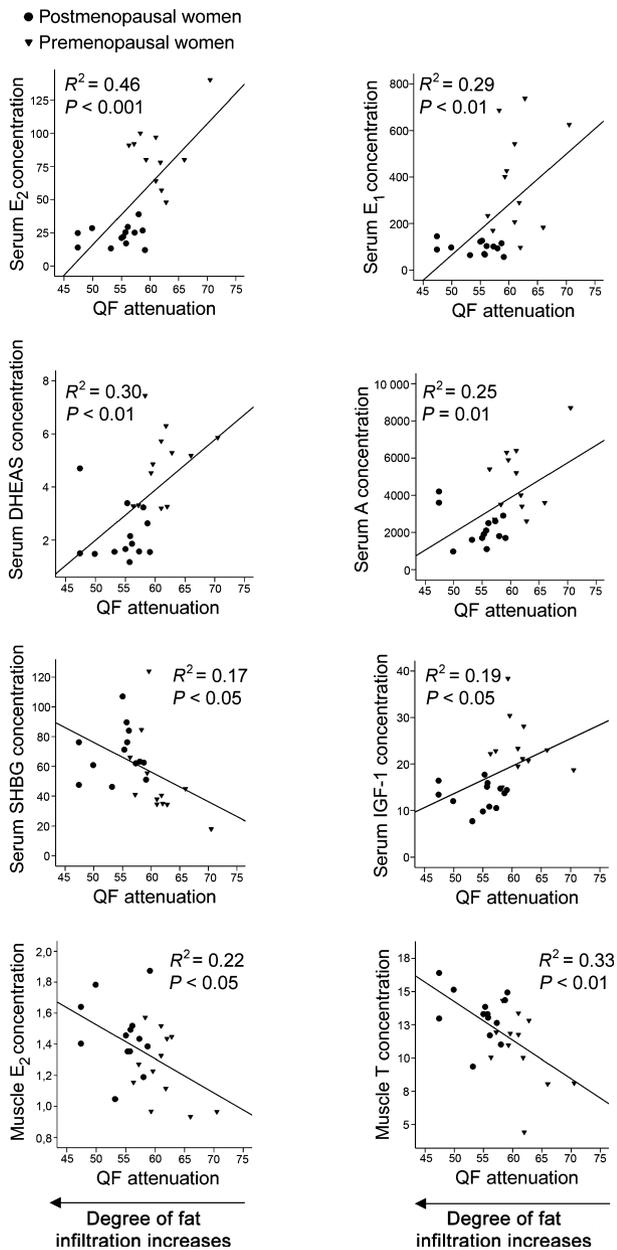
	Muscle force per $CSA_{QF}$ ( $n = 25$ )		QF attenuation ( $n = 25$ )	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Serum hormones				
$E_2$ †	0.411	<b>0.046</b>	0.674	< <b>0.001</b>
$E_1$	0.596	<b>0.002</b>	0.535	<b>0.006</b>
Testosterone	0.143	0.496	0.062	0.769
DHT	0.327	0.119	-0.214	0.316
Androstenedione	0.360	0.077	0.503	<b>0.010</b>
DHEAS	0.508	<b>0.009</b>	0.545	<b>0.005</b>
SHBG	-0.037	0.859	-0.417	<b>0.038</b>
IGF-1	0.451	<b>0.024</b>	0.437	<b>0.029</b>
Muscle hormones				
$E_2$	-0.142	0.499	-0.469	<b>0.018</b>
Testosterone	-0.050	0.811	-0.573	<b>0.003</b>
DHT	-0.130	0.537	-0.337	0.099
DHEA	0.228	0.273	-0.381	0.060

†For the premenopausal women, the normal basal levels of  $E_2$  were used in the analysis. The basal levels, i.e. the lowest levels during the menstrual cycle, were determined from the serum samples obtained during the early follicular phase of menstrual cycle.

QF, *quadriceps femoris*;  $CSA_{QF}$ , lean cross-sectional area of QF muscle; DHT, dihydrotestosterone; DHEAS, dehydroepiandrosterone sulfate; DHEA, dehydroepiandrosterone; IGF-1, insulin-like growth hormone 1; SHBG, sex hormone-binding globulin.

Statistically significant *P*-values are bolded.

more, the total serum concentration of DHEAS and androstenedione in postmenopausal women was about half of the levels in premenopausal women, while the amount of testosterone



**Fig. 2** Significant associations of serum and muscle hormone concentrations with muscle composition. Muscle quality was defined by attenuation characteristics of computed tomography images from *quadriceps femoris* (QF) muscle. Lower attenuation values indicate higher amounts of fat infiltration within the muscle. E<sub>2</sub>, 17 $\beta$ -estradiol; E<sub>1</sub>, estrone; DHEAS, dehydroepiandrosterone sulfate; A, androstenedione; SHBG, sex hormone-binding globulin; IGF-1, insulin-like growth hormone 1; T, testosterone.

and DHT did not differ between the groups. In contrast to the lower systemic levels, skeletal muscle tissue of postmenopausal women had higher concentration of E<sub>2</sub> and testosterone as well as similar concentration of DHT and DHEA than premenopausal women.

According to the free hormone hypothesis, only the unbound fractions of hormones in circulation are readily accessible to peripheral tissues while hormones bound to SHBG are considered inactive and need to be dissociated from their carriers

before they can enter target cells (Mendel, 1989). The precursor hormones DHEAS, DHEA, and androstenedione, which are only weakly or not at all bound to the SHBG, are considered to be freely accessible to the cells. The alternative pathway for the uptake of steroid hormones was introduced by Hammes *et al.* (2005), who reported that SHBG-bound steroid hormones can be actively internalized by endocytic megalin receptor (also known as LRP2 or GP330), removed from SHBG in lysosomes and act thereafter as inducers of steroid target genes. However, the role of megalin in the delivery of sex steroids to target tissues has faced some criticism (Rosner, 2006; Rosner *et al.*, 2010), but also some supportive studies have been published (König *et al.*, 2008). Yet, another possible mode of action, which does not necessarily involve internalization of sex steroid hormones at all, has been proposed and reviewed by Rosner *et al.* (2010). Accordingly, first SHBG itself binds to a membrane receptor, which may be distinct to megalin, and this binding is followed by activation of the receptor through interaction with steroid hormones, which leads to G-protein-coupled signal transduction inside the target cell (Nakhla *et al.*, 1999). Nevertheless, it is still unclear whether megalin or equivalent membrane receptor for SHBG exists in skeletal muscle tissue. We used qPCR to assess the mRNA expression of *megalyn* in the muscle samples of pre- and postmenopausal women, but found no detectable signal. Further studies are, however, needed to examine the protein level expression as well as to dissect the possible cell type-specific expression pattern before ruling out the existence of megalin within muscle tissue.

Only a small fraction of serum estrogens and androgens are in free form (Mazer, 2009). The proportions of free estrogens and androgens in our data were 1.7–3.5% and 0.5–1.4%, respectively. If skeletal muscle relies totally on free hormones for entrance into muscle tissue instead of producing hormones itself, the muscle sex steroid content should follow the abundance of free hormones in serum, and therefore, according to our data, the concentration of E<sub>2</sub>, E<sub>1</sub>, and testosterone should be substantially lower in postmenopausal compared to premenopausal women. Similarly, the muscle content of DHT should be similar in post- and premenopausal women because FDHT in serum did not differ between these groups. This was the case only for DHT. Even though the expression of megalin was not detected in our study, it does not rule out the possibility that the above-mentioned active endocytosis of SHBG-bound sex steroids occurs in muscle tissue. However, also in that case, the positive association between the hormone levels in serum and in muscle tissue should be evident unless the endocytosis is strongly enhanced in the postmenopausal compared to the premenopausal women. What we found out was that the muscle concentrations of DHEA (not considered to be bound with SHBG in sera) and DHT (considered to be mostly in SHBG-bound form) were similar between the study groups, but the amount of E<sub>2</sub> and testosterone (both are considered to be mostly in SHBG-bound form) was slightly higher in the postmenopausal women compared to the premenopausal women. Explanation for this observation would require active and up-regulated endocytosis

of E<sub>2</sub> and testosterone and/or up-regulated steroid synthesis in muscle tissue.

The synthesis route from DHEAS to testosterone/DHT and E<sub>2</sub> requires several steroidogenesis-related enzymes. We studied the gene and protein expression of these enzymes by qPCR and immunofluorescence microscopy and found out that at mRNA level, most of them were more abundant in muscle tissue of postmenopausal than premenopausal women. However, as stated by also other investigators before us (Bhasin *et al.*, 2003), we noticed that they were expressed at quite low level in muscle tissue making accurate qPCR determination difficult for some of the enzymes. Nevertheless, statistically significant differences were found in the mRNA expression of *HSD3B1* and *aromatase*. The immunofluorescence staining of skeletal muscle samples with antibodies specific to aromatase, SRD5A1, and STS revealed staining at or close to the sarcolemma. Staining was evenly distributed across the sections without muscle cell type specificity or obvious differences between age-groups. The studied enzymes are needed for first and last steps of steroidogenesis, indicating that steroidogenesis occurs in muscle tissue, which could also explain the higher concentrations of testosterone and E<sub>2</sub> observed in muscle of postmenopausal women. However, based on the current experiments, it is not possible to exclude a confounding effect via actively regulated endocytosis of steroid hormones from circulation or from the surrounding tissues.

At the phenotype level, the postmenopausal women were weaker and had smaller thigh muscles than premenopausal women. The amount of intramuscular fat within QF did not differ between the study groups, but the muscle quality, which was measured both as muscle attenuation and as muscle force per CSA<sub>QF</sub>, was lower in postmenopausal women than in premenopausal women. Plausible explanation for the impaired muscle phenotype of the postmenopausal women, in spite of higher sex steroid levels in muscle tissue, is the inability of sex steroids to compensate for the aging-related decrements in other factors impairing neuromuscular properties. One of such factors could be the diminished levels of IGF-1, which is either systemically secreted by liver or peripherally produced in muscle tissue. Several recent studies have shown that testosterone, DHT, and E<sub>2</sub> interact with IGF-1 signaling (Yin *et al.*, 2009; Gentile *et al.*, 2010; Hamzeh & Robaire, 2010; Hewitt *et al.*, 2010; Svensson *et al.*, 2010). Gentile *et al.* (2010) showed that in rats, orchietomy increases fat mass and decreases lean body mass (LBM), bone mineral content, and muscle strength. Both testosterone and DHT administration reversed the changes in fat mass, LBM, and strength, but only testosterone, possibly through conversion to E<sub>2</sub>, had a protective effect on bone. The same study demonstrated that DHT increased the gene expression of *IGF-1* splice variants *Ea* and *Eb* (equivalent to human *IGF-1Ec*), which led the authors to conclude that androgen-mediated induction of IGF-1 participates in the promotion of muscle function. In the current study, we observed lower serum levels of IGF-1 in postmenopausal than in premenopausal women. In addition, our earlier studies have shown that postmenopausal women not on

HRT have 28% lower expression of *IGF-1Ea* and 40% lower expression of *IGF-1Ec* in muscle when compared to premenopausal women (Ahtiainen *et al.*; unpublished data) and that one-year administration of HRT significantly increased the expression of *IGF-1Ec* compared to the decrease in postmenopausal control women (Pöllänen *et al.*, 2010).

Advancing age is strongly associated with decreasing muscle function, mass, and quality (Cruz-Jentoft *et al.*, 2010), which could be partially explained by the changes in hormonal milieu, but most probably also other aging-related factors are involved. Furthermore, the measured neuromuscular phenotypes result from complex interactions between several tissue types involving functions of central and peripheral nervous system, cardiovascular system as well as musculoskeletal system, which makes it difficult to dissect the exact mechanisms behind the observed deteriorations. Nevertheless, we found no associations between measured muscular hormone concentrations and specific muscle force, but the higher amounts of infiltrated fat within muscle tissue (lower attenuation values) were associated with the higher E<sub>2</sub> and testosterone concentration in muscle tissue. Therefore, it is possible that fat infiltration increases local steroid synthesis within muscle tissue. The result fits well with previous understanding that visceral and subcutaneous adipose tissue is able to take up and convert steroid hormones for intracrine as well as endocrine purposes (Belanger *et al.*, 2002). In contrast, the serum concentration of IGF-1, DHEAS, androstenedione, E<sub>1</sub>, and E<sub>2</sub> correlated positively with muscle quality, i.e. less fat within QF muscle and higher specific force, but not with muscle or fat CSA<sub>QF</sub>. Serum concentration of SHBG had negative associations with muscle quality. Taken together, these observations indicate direct or indirect involvement of steroid hormones in the regulation of muscle properties and support the idea that systemic and local steroids have their own distinct influences on the measured neuromuscular properties.

At the molecular level, systemic and local steroids may involve activation of distinct signaling routes in the target tissue. Systemic signaling may favor activation of membrane-bound receptors as proposed elsewhere (Rosner *et al.*, 1999) while intracrine effects of steroid hormones may involve other type of receptors. As already discussed that binding of SHBG to yet undetermined membrane receptor, following activation by interaction with androgens or estrogens, leads to accumulation of intracellular cAMP, which is considered to mark G-protein-mediated signal transduction (Nakhla *et al.*, 1999). Similarly, the interaction of E<sub>2</sub> with GPR30 has been shown to induce cAMP and mediate intracellular signaling changing the metabolic fate of the cell (Maggiolini & Picard, 2010). GPR30 belongs to the family of G-protein-coupled receptors spanning the membrane seven times and has been recently recognized as a membrane-bound ESR and signed with new official name: G-protein-coupled ESR1 (GPER). In addition to nuclear ESR1/2 and AR, skeletal muscle expresses also GPER (Baltgalvis *et al.*, 2010). In the current study, the gene expression of *GPER* tended to be lower in muscle of postmenopausal women than in premenopausal, which could indicate impairments in the

GPER-coupled signaling. However, the difference was not statistically significant. The opposite trend was observed for the muscle expression of *ESR1*, which tended to be higher in postmenopausal than in premenopausal women. Also, other studies have shown that lower systemic levels of estrogens tend to increase the expression of *ESR1* in muscle (Baltgalvis *et al.*, 2010) and adipose tissue (Lundholm *et al.*, 2008), which may indicate menopause-related withdrawal of negative regulation on the expression of *ESR1*.

Local intracrine target cell-specific production of active sex steroids at their final site of action may be very effective, but the low mRNA levels of steroidogenesis-related enzymes technically limited the amount of successful measurements, which may have affected the power and significance of statistical testing. Another limitation of the current study is that it was not possible to define the origin of the sex steroid hormones measured from skeletal muscle tissue because they might reflect systemic endocrine or local intracrine processes. Further, sex steroids could originate from paracrine secretion by surrounding tissues such as adipose tissue instead of direct intracrine production within muscle cells. Also, the amount of infiltrated fat within muscle tissue seems to have an impact on the amount of measured steroid hormones. The overall body composition did not differ between the study groups, and all eye visible fat was removed from the muscle samples before hormone measurements. However, as seen by QF attenuation, postmenopausal women had more fat infiltration within and between muscle cells. Some of such nonvisible fat can also be a source for the steroids, and therefore, it is impossible to distinct the role of muscle cells from infiltrated adipose cells in local production of steroid hormones. However, according to the immunofluorescence assays, the steroidogenic enzymes STS, aromatase, and SRD5A1 are expressed in muscle cells evenly staining the whole cross-sections of muscle samples. Concerning the ELISA analyses, there may be some inborn cross-reactivity in the tests used, even though the manufacturer ensures that it is not substantial. The possible cross-reactivity may falsely elevate the muscle concentration of the measured hormone. However, in that case, the error is similar in both study groups. Therefore, we are confident that, the above-mentioned possible limitations do not change our observations at tissue level or our interpretation of the results.

The most important achievement of our study was to demonstrate that despite menopausal cessation of ovarian sex hormone secretion, the skeletal muscle tissue does not suffer from extensive lack of steroid hormones. The local steroid synthesis, however, was not sufficient to prevent aging-related deterioration of neuromuscular functions. Therefore, it is possible that intracrine steroidogenesis has its own distinct functions differing from those coming through systemic signals. This study opens a new branch of hormone research and inspires further research to clarify the differences in actions of intra- and endocrine hormonal signals to understand how skeletal muscle properties are regulated and maintained as we age.

## Experimental procedures

### Study design and participants

Thirteen premenopausal women ( $33 \pm 3$  years) not using any contraceptives and 13 postmenopausal women ( $64 \pm 2$  years) who did not use any estrogen-containing HRT were recruited into this study.

An invitation to the study was sent to two thousand premenopausal women (39.1% of the entire cohort) randomly selected from the entire 30- to 40-year-old age cohort (born in 1967–1977) and living in the City of Jyväskylä. Screening for the study participants fulfilling the inclusion criteria was performed using a short prequestionnaire sent along with the invitation. The women were asked for their past and current history of being treated with hormonal contraceptives (contraceptive pills and plasters, hormonal intravaginal and hormonal intrauterine devices) or progesterone preparations. Altogether, 163 women returned the prequestionnaire. Subsequently, 118 women fulfilling the inclusion criteria, i.e. not being treated with hormonal contraceptives or progesterone preparations within the past five years and willing to participate in the study were randomly selected for telephone interviews. Altogether, 96 women were interviewed over telephone about their gynecological status, medication, and potential contraindications for participation (chronic musculoskeletal diseases, type 1 or 2 diabetes, mental disorders, asthma with oral glucocorticosteroid treatment, cancer, drug or alcohol abuse, and Crohn's disease). Irregular menstrual cycles, breastfeeding, and planned pregnancy were also used as exclusion criteria. Based on the interview, 62 women were eligible and willing to participate to the study. Two participants discontinued after the second measurement day, and one was excluded by the physician because of a contraindication for muscle tissue sampling. For this study, a subgroup of 13 subjects was randomly selected for the full battery of biochemical analyses. The random sampling feature of the PASW Statistics software (SPSS Inc., IBM, Chicago, IL, USA) was used in all randomization steps.

The postmenopausal women of this study had earlier participated in an experimental study ran by our department [the Ex/HRT-trial (Sipilä *et al.*, 2001)]. Invitation to participate to a ten-year follow-up was sent to all 80 women who took part in the baseline measurements of the Ex/HRT-trial. From these, two were deceased, eight did not respond, and 23 refused or were unable to participate. Finally, 47 women took part in the 10-year follow-up. The past and current use of any estrogen-containing pharmaceuticals and the health were carefully evaluated by a medical doctor. Among these women, 19 had never used any oral estrogen-containing HRT, and two had used < 6 months at some point during the first 5 years following the initiation of the Ex/HRT trial and not at all during the past 5 years. Eight women did not consent to muscle biopsy or could not be studied because of insufficient tissue samples. Finally, 13 postmenopausal women were studied according to the current protocol.

This study was proved by Local Ethical Committee and performed following guidelines of the Helsinki declaration and good clinical and scientific practice. An informed consent form was signed by all study participants before the measurements, tissue sampling and analysis.

### Body anthropometry, muscle composition and strength and physical activity assessment

Body weight was measured with a beam scale and height with a stadiometer. Body fat mass and LBM were measured with a multifrequency bioelectrical impedance analyzer (InBody 720; Biospace, Seoul, Korea). Computed tomography (CT) scans (Siemens Somatom Emotion Scanner; Siemens, Erlangen, Germany) were obtained from the mid-part of *m. vastus lateralis*, and the scans were analyzed using Geanie software (version 2.1; Commit, Espoo, Finland) developed for cross-sectional CT image analysis and enabling separation of fat and muscle tissue based on radiological density. Total CSA of the QF, muscle CSA<sub>QF</sub>, and intramuscular fat area within QF (fat CSA<sub>QF</sub>) was measured. The relative muscle area within the muscle compartment was calculated by dividing muscle CSA<sub>QF</sub> by total CSA<sub>QF</sub>. Mean attenuation coefficient expressed in Hounsfield units (HU) was defined for the muscle CSA<sub>QF</sub>. Low HU values are associated with high amount of lipids infiltrating into muscle tissue (Goodpaster *et al.*, 2000). Therefore, low attenuation values indicate poor muscle quality. Maximal isometric knee extension force was measured in a sitting position using an adjustable dynamometer chair at a knee angle of 60° from full extension (Good Strength, Metitur, Palokka, Finland). After familiarization with the test, the participants were encouraged to produce maximal force as rapidly as possible. Three to six maximal efforts were conducted, and the highest recording was used as the test result. As another muscle quality indicator, the muscle force per CSA<sub>QF</sub> was calculated by dividing knee extension force by muscle CSA<sub>QF</sub> from which the fat CSA<sub>QF</sub> was excluded. Information concerning physical activity was collected using the six-point scale of Grimby (Grimby, 1986) with slight modifications. The interassay coefficient of variations (CV) between two consecutive measurements in our laboratory is 1–9% for CSA, 1% for attenuation, and 6% for knee extension strength (Sipilä *et al.*, 2001; Taaffe *et al.*, 2005).

### Serum hormone measurements

Blood samples were taken from the antecubital vein with the study participant in a supine position at the same morning as muscle sampling was performed. From the premenopausal women, the additional sample was collected during the early follicular phase of the menstrual cycle to determine the normal basal levels of E<sub>2</sub>. The aliquoted sera were stored in –70 °C until analyses. Serum concentrations of SHBG, FSH, LH, and IGF-1 were measured using solid-phase, chemiluminescent immunometric assays (Immulite 1000; Diagnostic Products, Los Angeles, CA, USA). Serum E<sub>2</sub> levels were determined in duplicates using

an extraction radioimmunoassay as previously described (Ankarberg-Lindgren & Norjavaara, 2008). E<sub>1</sub> was measured as a dansyl derivative using liquid chromatography–tandem mass spectrometry (LC–MS/MS) on API 4000 mass spectrometer as previously described (Nelson *et al.*, 2004). Serum testosterone (Turpeinen *et al.*, 2008), DHT and androstenedione were measured separately using LC–MS/MS method. Before DHT and androstenedione analysis, 30 µL of 0.1 µM deuterated DHT or androstenedione in 50% (vol/vol) methanol [internal standards (IS)] was added to 250 µL of serum before extraction with 5 mL of diethyl ether. After mixing for 3 min, the upper layers were collected and evaporated to dryness under nitrogen. The residues were dissolved in 250 µL of 50% methanol. Calibrators containing 0.2–25 nM of DHT or 0.5–50 nM of androstenedione were prepared in 50% methanol. Forty microlitre (DHT) or 25 µL (androstenedione) of sample extracts and calibrators were analyzed on an LC–MS/MS system equipped with an API 3000 triple quadrupole mass spectrometer (AB Sciex; Applied Biosystems, Foster City, CA, USA) with the electrospray ionization probe and an Agilent series 1200 HPLC system with a binary pump. Separation was performed on a SunFire C18 column (2.1 × 50 mm; Waters, Milford, MA, USA). The mobile phase was a linear gradient consisting of methanol and 100 mM ammonium acetate in water, at a flow rate of 250 µL min<sup>–1</sup>. DHT and androstenedione were detected as protonated ions in the positive mode with the following transitions: *m/z* 287 to *m/z* 97 (androstenedione), *m/z* 294 to *m/z* 100 (IS) and *m/z* 291 to *m/z* 255 (DHT), *m/z* 295 to *m/z* 259 (IS), respectively. Data were acquired and processed with the Analyst Software (Ver 1.4, AB Sciex; Applied Biosystems). E<sub>2</sub>, E<sub>1</sub>, testosterone, DHT, androstenedione, and SHBG concentrations were used to calculate the corresponding free hormone levels (FE<sub>2</sub>, FE<sub>1</sub>, FT, and FDHT) according to a recently presented spreadsheet method that takes into account the competitive binding of the different hormones present in sera (Mazer, 2009). The CV was 8.4% for SHBG at 32.4 nM, 5.5% for FSH at 38.5 IU L<sup>–1</sup>, 8.1% for LH at 30.0 IU L<sup>–1</sup>, 4.7% for DHEAS at 5.2 µM, and 6.9% for IGF-1 at 25.5 nM. Limit of quantification (LOQ) was 0.2 nM for SHBG, 0.1 IU L<sup>–1</sup> for FSH and LH, 0.08 µM for DHEAS, and 2.6 nM for IGF-1. For E<sub>2</sub>, LOQ was 4 pM, while the interassay CV was 19% at 6 pM and below 14% at 12 pM and above. For E<sub>1</sub> interassay, CV was 7.8% at 200 pM, while the LOQ was 10 pM. LOQ was 70 pM and interassay CV 5.2% for testosterone at 4.7 nM. For DHT, LOQ was 100 pM and interassay CV 9.1% at 3.5 nM, and for androstenedione, LOQ was 70 pM and CV 5.5% at 3.2 nM.

### Muscle biopsy sampling

Muscle biopsies were obtained from the mid-part of *m. vastus lateralis*. The biopsy protocol was standardized to avoid variation because of sampling. All muscle biopsies were taken always by the same experienced physician between 7:00 and 10:00 hours from the same site as CT scanning. Visible blood and fat were removed before two parts of the samples were snap frozen in liquid nitrogen and stored at –80 °C until used for mRNA, pro-

tein, or hormone analysis. The total protein and muscle hormone measurements were taken from the same part of the biopsy. Third part of the biopsy was mounted transversely on a cork with Tissue Tek Optimal Cutting Temperature compound (Sakura; Alphen aan den Rijn, Netherlands) and frozen rapidly in 2-methylbutane (Sigma-Aldrich Corporation, ST. Louis, MO, USA) precooled to  $-160^{\circ}\text{C}$  in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . This part of the biopsy was used for the immunofluorescence analysis.

### Muscle hormone measurements

Muscle samples were homogenized on ice in a buffer (Tissue extraction Reagent I; Invitrogen, Carlsbad, CA, USA; 15  $\mu\text{L}$  buffer per mg muscle) containing 80  $\mu\text{L mL}^{-1}$  PMSF, 40  $\mu\text{L mL}^{-1}$  aprotinin, 40  $\mu\text{L mL}^{-1}$  leupeptin and 1  $\mu\text{L}$  per 100  $\mu\text{L}$  Inhibitor Cocktail I (all from Sigma-Aldrich Corporation) using plastic tissue grinder. The homogenate was gently mixed in rotation for 15 min at  $+4^{\circ}\text{C}$  following centrifugation at 10 000  $g$  for 15 min at  $+4^{\circ}\text{C}$ . A sample of the supernatant (1:10 dilution) was used for the measurement of total soluble protein concentration, immediately following centrifugation, using Pierce BCA Protein Assay-kit (Thermo Scientific, Rockford, IL, USA). ELISA tests from IBL-International (Hamburg, Germany) were used to determine  $\text{E}_2$ , testosterone, DHT, and DHEA concentrations in 1:10-diluted muscle supernatants in duplicate. The concentrations of all hormones were standardized using total protein concentration and expressed as  $\text{nmol } \mu\text{g}^{-1}$  muscle protein. Interassay CVs for  $\text{E}_2$ , testosterone, and DHEA after correction for total protein concentrations as determined in our laboratory were 14%, 23%, and 11%, respectively. The used ELISA tests are highly specific for the tested hormones. According the manufacturer, the cross-reactivity of the  $\text{E}_2$  test is 0.2% for the  $\text{E}_1$  and  $\leq 0.05\%$  for number of other steroids, the cross-reactivity of the  $t$ -test is 3.3% for 11 $\beta$ -hydroxytestosterone and 19-nortestosterone, 0.9% for androstenedione, 0.8% for DHT, and  $\leq 0.1\%$  for other steroids, the cross-reactivity of the DHT test is 8.7% for testosterone, 2.0 for 5 $\beta$ -DHT, and 0.2% for androstenedione, and the cross-reactivity of the DHEA test is  $< 0.08\%$  for all the tested steroids including, e.g.,  $\text{E}_2$ , testosterone, and DHT.

### Quantitative PCR

Trizol-reagent (Invitrogen) was used to isolate total RNA from muscle biopsy samples homogenized on FastPrep FP120 apparatus (MP Biomedicals, Illkrich, France). Total RNA concentration was measured photometrically at 260 nm, and its purity was assessed based on 260/280 absorption, which was  $\sim 2.0$ . Two-microgram RNA aliquot from each muscle sample was reverse transcribed according to manufacturer's instructions using High Capacity cDNA Reverse Transcription-Kit (Applied Biosystems). Before cDNA synthesis, the total RNA was subjected to DNase treatment using Ambion Turbo DNA-free-kit (Applied Biosystems). The muscle gene expression of *STS* (forward primer [FP]: GGAAGGCCTTTTCTTCACC, reverse primer [RP]: AGGGTCTG

GGTGTGTCTGTC), *HSD3B1* (FP: TTGTCAAATAGCGTATTCACC TTC, RP: AGCTTGTGCCCTTGTCACTTT) *HSD3B2* (FP: TACT-TTGATTGGCCACGAT, RP: CATCAATGATACAG GCGGTG), *HSD17B1* (FP: AGCTGGACGTAAGGGACTCA, RP: GTGGGCG A GGTATTGGTAGA), *HSD17B3* (FP: TCGCTGAGATTCTCCAGAT G, RP: AATGGCTTGGGAGAAGGTTT), *HSD17B5* (FP: CCAGTT-GACTGCA GAGGACA, RP: TCGCTAAACAGGACGGATT), *aromatase* (FP: GTGGACGTGTTGACCCTTCT, RP: GCCATGCATC AAAATAACCTTGGA), *SRD5A1* (FP: ATGTTCTCGTCCACTAC GG, RP: GCCTCCCCTTGGTA TTTTGT), *SRD5A2* (FP: CTCAG-GAAGCCTGGAGAAAT, RP: AAATGCCAAATGCAAGTGCTG), *GAPDH* (FP: CCACCCATGGCAAATTCC, RP: TGGGATTCCATT-GATGACAA), and  $\beta$ -actin (FP: GACAGGATGCAGAA GGAGAT CACT, RP: TGATCCACATCTG CTGGAAGGT) was determined with CFX96 Real-Time PCR Detection Thermo cycler (BioRad, Hercules, CA, USA) using iQ SYBR Green supermix-kit (BioRad) for detection. The optimal annealing temperature was determined using temperature gradients before proceeding to qPCR. We were unable to determine the optimal annealing temperature for *HSD3B2*, *HSD17B1*, and *HSD17B3* because their expression levels in muscle tissue samples were too low for accurate determination by qPCR and they were therefore omitted from further analyses. The qPCR was performed in duplicate using the following program: 1 cycle of  $95^{\circ}\text{C}$  for 10 min and 40 cycles of  $95^{\circ}\text{C}$  for 10 s, the predetermined optimum annealing temperature for each gene (*STS*:  $61^{\circ}\text{C}$ , *HSD3B1*:  $62^{\circ}\text{C}$ , *HSD17B5*:  $61^{\circ}\text{C}$ , *aromatase*:  $62^{\circ}\text{C}$ , *SRD5A1*:  $62^{\circ}\text{C}$ , *SRD5A2*:  $62^{\circ}\text{C}$ , *GAPDH*:  $60^{\circ}\text{C}$ ,  $\beta$ -actin:  $60^{\circ}\text{C}$ ) for 30 s,  $72^{\circ}\text{C}$  for 30 s following the determination of the dissociation curves: 95 for 10 s, 65 for  $95^{\circ}\text{C}$ , with  $0.5^{\circ}\text{C}$  increment for 5 s. In addition, the expression of *ESR1* (Hs01046812\_m1), *AR* (Hs00171172\_m1), *GPER* (Hs01922715\_s1), *LRP2* (Hs00189742\_m1), and *GAPDH* (Hs99999905\_m1) was determined with ABI 7300 U (Applied Biosystems) using standard PCR conditions recommended for the TaqMan gene expression assays by the manufacturer (Applied Biosystems). Each gene was run in a separate plate and with the same reference sample (a mixture of several muscle samples) to control run-to-run variation. Dilution series of the reference sample were used to determine the amplification efficiency (E) for each gene. Both *GAPDH* and  $\beta$ -actin were measured as reference genes to normalize variation because of differences in the initial cDNA amounts. The gene stability measure M was determined according to (Vandesompele *et al.*, 2002) and found to be 0.6 for *GAPDH* and 0.5 for  $\beta$ -actin. Furthermore, the relative quantity (RQ) values of *GAPDH* and  $\beta$ -actin correlated significantly ( $r = 0.93$ ,  $P < 0.001$ ). Because both genes acted very similarly, but *GAPDH* is more widely used in muscle tissue studies, *GAPDH* was selected as the reference gene. Across the whole experiment, if standard deviation between quantification cycles ( $C_q$ ) of replicate samples was  $> 0.4$  or  $C_q$  was  $> 38$ , the result was considered unreliable or undetermined and the sample was excluded from the analysis. Relative quantity of each gene was determined as follows:  $\text{RQ} = E^{(C_q(\text{reference sample}) - C_q(\text{gene of interest}))}$  and normalized with RQ of *GAPDH*.

## Immunofluorescence analysis

Frozen muscle tissue samples from pre- and postmenopausal women were cut by cryostat into 8- $\mu$ m sections, fixed with acetone (10 min at  $-20^{\circ}\text{C}$ ), and air-dried. To prevent nonspecific staining, the sections were blocked with 10% donkey normal serum for 30 min at room temperature. Sections were incubated with primary antibodies against STS, aromatase, and SRD5A1 (sc-33499, sc-14245, and sc-20396, respectively, all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Sections were washed with phosphate buffer solution and exposed to the fluorescence secondary antibody AlexaFluor donkey anti-goat 568 (Invitrogen). Sections were again washed with phosphate buffer and stained with DAPI to visualize nuclei. The specificity of the staining was controlled by omitting primary antibodies from the staining protocol.

## Statistics

All descriptive statistical results are presented using mean  $\pm$  standard deviation (SD). Normal distribution of the data was checked using Shapiro–Wilk test. For variables with a normal distribution, the independent samples *t*-test was used to test the eventual significance of the difference between study groups. If the variables had a skewed distribution, the nonparametric Mann–Whitney *U* test was used. The level of significance was set at  $P \leq 0.05$ . Pearson's correlation coefficient (*r*) was used to measure the strength of the associations between the variables. Data analyses were carried out using PASW Statistics software version 18 (SPSS Inc.).

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## Conflict of interest

The authors declare no conflict of interests.

## Author contributions

VK, SS, YK, and EP designed the study. EP, SS, MA, PR, JP, and HS contributed to the acquisition of the data, while VK super-

vised the process. MA acted as medical doctor during data acquisition and took muscle samples. CA-L, EH, and UT were responsible for the serum steroid hormone methods, YK for the immunofluorescence, and EP for the other methods as well as main data analysis. All authors contributed to the interpretation of the data, critical revision of the manuscript, and approving the final version of the manuscript, while EP drafted the article.

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