

## Sclerostin and bone metabolism markers in hyperthyroidism before treatment and interrelations between them

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Sclerostin, which is a glycoprotein produced by osteocytes, reduces the formation of bones by inhibiting the Wnt signal pathway. Thyroid hormones are related with Wnt signal pathway and it has been reported that increased thyroid hormones in hyperthyroidism fasten epiphysis maturation in childhood, and increase the risk of bone fractures by stimulating the bone loss in adults. The aim of this study was to examine the sclerostin serum levels, the relation between sclerostin and thyroid hormones as well as the biochemical markers of the bone metabolism in patients with hyperthyroidism (including multinodular goiter and Graves' disease), whose treatments have not started yet. No difference was found in the serum sclerostin levels between the hyperthyroidism group (n=24) and the control group (n=24) ( $p=0.452$ ). The serum osteocalcin levels and 24-hour urinary phosphorus excretion were found to be higher in the hyperthyroid group than in the control group ( $p<0.001$ ,  $p=0.009$ ). A positive correlation was determined between the sclerostin and bone alkaline phosphatase levels ( $p<0.001$ ); a negative correlation between the osteocalcin and thyroid stimulating hormone (TSH) ( $p<0.05$ ); a positive correlation between the osteocalcin and thyroid hormones (FT<sub>3</sub>, FT<sub>4</sub>) ( $p<0.001$ ); and a positive correlation between the deoxyypyridinoline and hydroxyproline ( $p<0.001$ ). No correlation was determined between sclerostin and TSH, FT<sub>3</sub>, FT<sub>4</sub> ( $p>0.05$ ). Therefore, we consider that a long-term study that covers the pre-post treatment stages of hyperthyroidism, including both the destruction and construction of the skeleton would be more enlightening. Moreover, the assessment of the synthesis of sclerostin in the bone tissue and in the serum level might show differences.

**Key words:** hyperthyroidism, sclerostin, bone metabolism markers

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**Abbreviations:** ALT, alanine transaminase; AST, aspartate transaminase; BALP, bone alkaline phosphatase; BMD, bone mineral density; BMP, bone morphogenetic protein; Ca, calcium; DPD, deoxyypyridinoline; ELISA, enzyme-linked immuno sorbent assay; FT<sub>3</sub>, free triiodothyronine (free thyroid hormone 3); FT<sub>4</sub>, free tetraiodo-L-tyronine (thyroxine); GGT,  $\gamma$  glutamyl transpeptidase; ISCD, international society for clinical densitometry; P, phosphorus; RANKL, nuclear factor  $\kappa$ B receptor activator ligand; TSH, thyroid stimulating hormone

### INTRODUCTION

Thyroid hormones have a regulatory effect on cellular metabolism in several tissues, which is important in nutrition and mineral metabolism, thermogenesis,

cardiovascular system, stimulation of growth and development of various tissues at critical periods, including the central nervous system and skeleton (Ogilvy-Stuart, 2002). Thyroid hormones play significant roles in bone formation in fetal life, bone modelling during childhood and remodelling in adults (Ben-Shlomo *et al.*, 2001). On the other hand, it has been reported that hyperthyroidism has a lifelong influence on bone tissues, in that it may accelerate epiphyseal maturation by increasing bone mineralization in childhood, and may induce bone loss in adults (Cardoso *et al.*, 2014). As a result, this increases the risk of bone fractures (Vestergaard *et al.*, 2000; Basset *et al.*, 2007). During the bone formation, T<sub>3</sub> stimulates osteoblast differentiation and activity by direct influence on thyroid receptors as well as it induces growth factors (IGF-1, its receptor), osteocalcin, type 1 collagen, alkaline phosphatase and metalloproteinases. T<sub>3</sub> also stimulates osteoclast differentiation and activity, but it still remains unclear whether the effects of T<sub>3</sub> on bone resorption result from direct actions in osteoclasts or indirectly through effects on osteoblasts. During the bone resorption, T<sub>3</sub> stimulates factors that are important in the differentiation of osteoclasts such as interleukin-6,8, prostaglandin-E<sub>2</sub> and RANKL. T<sub>3</sub> also has synergistic effects with osteoclastogenic hormones such as vitamin D and parathormone (Cardoso *et al.*, 2014). The effects of thyroid hormones on the bone formation and bone loss might occur *via* Wnt/ $\beta$ -catenin signaling pathway (O'shea *et al.*, 2012; Tsourdi *et al.*, 2015; Wang *et al.*, 2007). Wnt signaling pathway, which consists of several proteins, takes part in the regulation of several features of cells such as cell growing, differentiation, function and death (Krishnan *et al.*, 2006).

Sclerostin is a glycoprotein, which is mainly produced in osteocytes. It is a product of sclerostin gene and it reduces bone formation in osteoblasts by inhibiting Wnt signaling pathway (Van Bezooijen *et al.* 2004, 2007; Lin *et al.*, 2009). In gene encoding sclerostin knockout mice, a phenotype with high bone mass, similar to sclerostosis patients, was observed (Xiaodong *et al.*, 2008). On the contrary, rats overexpressing sclerostin gene exhibited low bone mass (Winkler *et al.*, 2003). Lin *et al.* (2009) in wildtype mice with avoided mechanical loading, demonstrated increased synthesis of sclerostin in opposition to decreased Wnt/ $\beta$ -catenin signaling. However, the bone loss detected after reduced mechanical loading was not identified in sclerostin negative mice.

Since thyroid hormones and sclerostin have an influence on bone tissues *via* Wnt signaling pathway, it seemed reasonable to search both of them in hyperthyroidism patients. In this study serum sclerostin levels were studied in hyperthyroidism patients. The relation between

en sclerostin and thyroid hormones and the relation of both, with bone metabolism markers such as serum BALP, intact osteocalcin as markers for bone formation and urine DPD, OH proline for bone resorption were investigated.

## MATERIALS AND METHODS

**Patients and setting.** Twenty four patients with hyperthyroidism, yet untreated, were admitted to the Clinics of Endocrine and Metabolism, and 24 voluntary normal persons were included in the study. The hyperthyroidism group consisted of multinodular goiter and Graves' disease patients. Subacute thyroiditis patients were eliminated. The study was approved by the BEU Clinical Studies Ethics Committee (11.02.2014.2013-138-31/12) and informed consents were obtained from the patients. Exclusion criteria were the presence of hormonal disorders other than hyperthyroidism, malabsorption, rheumatic diseases, malignancy, diabetes, hypertension, menopause, smoking and alcohol use, previously suffered drug therapy for thyroid diseases, radiotherapy and surgery of the thyroid gland. Control groups were created as non smoking, alcohol free, healthy men and non menopause women.

**Study procedures.** Blood and urine samples were obtained from the patients with the diagnosis of hyperthyroidism before the initiation of the therapy. Twentfour-hour urine samples were collected. After 12-hour fasting period, the blood samples were drawn. The samples in the control group were obtained in the same way. After the blood clotted, the samples were centrifuged and aliquots of serum were immediately stored at  $-80^{\circ}\text{C}$ .

Levels of serum sclerostin were determined using the human sclerostin (SOST) ELISA kit (Bioassay technology laboratory, cat no: E3068Hu, China), serum bone alkaline phosphatase (BALP) using the human BALB ELISA kit (Bioassay technology laboratory, cat no: E1493Hu, China), 24-hour urine deoxypyridinoline (DPD) using the human DPD crosslinks kit (Bioassay technology laboratory, cat. no: E1455 Hu, China) with the double antibody labeled with biotin solid phase sandwich method in an ELISA device (ELX 800 G, BIO-TEC Instruments Winooski, USA). All three tests have intra-assay CV of  $<8\%$ , interassay CV of  $<10\%$ . Levels

of serum osteocalcin were determined using the human Osteocalcin ELISA kit (Invitrogen, Life Technologies, USA, cat. no: KAQ1381) with the solid phase enzyme amplified sensitivity immunoassay method in an ELISA device (ELX 800 G, BIO-TEC Instruments Winooski, USA). Intra-assay CV of  $<4.7\%$ , interassay CV of  $<3.5\%$ .

The levels of urine hydroxyproline (OH proline) were measured by using the Bergman & Loxley (1963) method. The principle of the test was colorimetric measurement of the coloured complex created with p-dimethylaminobenzaldehyde of pyrrole after the oxidation of OH-pyrrole to pyrrole compound with chloramine T using a spectrophotometer (Shimadzu UV 1601, Tokyo, Japan) at 558 nm. As a standard, trans-4-Hydroxy-L-proline (Aldrich) was used. 24-hour urine OH proline/ 24-hour urine creatinine ratio was calculated (intra-assay CV of  $<5.4\%$ ).

Beckman Coulter, Olympus AU 2700 (Japan) autoanalyzer device was used to determine serum and urine calcium, phosphorus, creatinine and serum urea, glucose levels, alanine transaminase (ALT), aspartate transaminase (AST), total alkaline phosphatase (total ALP),  $\gamma$  glutamyl transpeptidase (GGT) activity. Serum thyroid stimulating hormone (TSH), free thyroid hormone 3 (FT3), free thyroxine (FT4) levels were studied in the hormone autoanalyzer (Beckman Coulter Unicel DxI 600, USA).

Bone mineral density (BMD) was assessed by measuring lumbar spine and femoral neck bone mineral density in the Hologic QDR4500W (Hologic Inc., Bedford, Massachusetts, USA) device.

**Statistical analysis.** The Statistical Package for Social Sciences 19.0 (SPSS Inc, Chicago, IL, USA) was used for all data analyses. Descriptive statistics were expressed as mean, standard deviation (S.D.), median, and minimum-maximum for continuous variables and frequencies with percent for categorical variables. Shapiro Wilk test was used for test of normality. Student's *t*-test was used to test the significance of differences between hyperthyroidism and normal study groups for normal distributed variables and Mann Whitney U-test for non-normal distributed variables. Yates-corrected chi-square test was used for comparing categorical variables between groups. Spearman correlation analysis was performed to determine the relationship between continuous variables. For all statistical comparisons, a *p*-value below 0.05 was assumed as statistically significant.

**Table 1. Levels of serum TSH, FT3, FT4, ALT, AST, GGT, sclerostin, BALB, osteocalcin and 24-hour urine Ca, P, DPD, OH proline / creatinine ratios and BMI in the hyperthyroidism group and the control group**

	Hyperthyroidism group (n=24)	Control group (n=24)	<i>p</i> value
BMI	23.95 (17.10–32)	25.40 (20.80–35.80)	<i>p</i> =0.065
Serum TSH ( $\mu\text{IU/mL}$ )	0.02 (0.01–0.46)	1.50 (0.45–3.85)	***
Serum FT <sub>3</sub> (pg/mL)	8.57 (2.91–27.21)	2.77 (1.17–3.79)	***
Serum FT <sub>4</sub> (ng/dL)	2.71 (0.97–5.89)	0.91 (0.68–1.86)	***
Serum ALT (IU/L)	24.5 (9–37)	13.5 (5–53)	*
Serum AST (IU/L)	21.5 (12–31)	18.5 (12–46)	<i>p</i> =0.311
Serum GGT (IU/L)	27.5 (11–68)	22.0 (9–66)	<i>p</i> =0.577
Serum sclerostin (ng/mL)	5.97 (2.59–34.74)	7.00 (2–32)	<i>p</i> =0.452
Serum BALP (IU/L)	90.1 (34.4–240)	90.9 (34.6–240)	<i>p</i> =0.470
Serum osteocalcin (ng/mL)	10.08 (0.54–56.62)	3.40 (0.17–12.98)	***
Urine mg Ca/g creatinine/day	117 (21–409)	98 (17–218)	<i>p</i> =0.523
Urine mg P/g creatinine/day	569 (373–1198)	484 (211–885)	**
Urine nMol DPD/g creatinine/day	71.9 (29.1–158.3)	49.1 (20.2–123.2)	<i>p</i> =0.08
Urine mg OH proline/g creatinine/day	18.1 (8.0–33.7)	14.5 (6.0–89.0)	<i>p</i> =0.322
Median (min-max.)			

Statistically significant \**p*<0.05; \*\**p*<0.01; \*\*\**p*<0.001

Table 2. Levels of serum Ca, P, urea, creatinine, total ALP, glucose of the hyperthyroidism and control groups

	Hyperthyroidism group n=24	Control group n=24	p value
Age	37.5±10	37.1±10	p=0.909
♀/♂	15/9	11/13	p=0.384
Serum Ca (mg/dL)	9.9±0.4	9.7±0.4	p=0.078
Serum P (mg/dL)	3.6±0.53	3.5±0.42	p=0.745
Serum urea (mg/dL)	29.1±9.8	26.8±6.9	p=0.349
Serum creatinine (mg/dL)	0.72±0.17	0.82±0.17	p=0.057
Serum total ALP (IU/L)	88.7±37.6	66.4±19.0	*
Serum glucose (mg/dL)	96.1±7.8	100±14.5	p=0.247

Mean ± S.D.

Statistically significant \*p<0.05

## RESULTS

Sclerostin levels did not differ significantly in the hyperthyroidism group than in the control group ( $p=0.452$ ) (Table 1). Table 1 summarizes the median, minimum-maximum of variables that were not statistically normally distributed and body mass index (BMI) of the hyperthyroidism and healthy groups. The TSH levels in hyperthyroidism patients were lower than the ones in the healthy group ( $p<0.001$ ). On the other hand, serum  $FT_3$  ( $p<0.001$ ),  $FT_4$  ( $p<0.001$ ), ALT ( $p=0.033$ ), osteocalcin ( $p<0.001$ ), and urine mg P/g creatinine/day excretion ( $p=0.009$ ) were higher in the hyperthyroidism group than in the healthy group. Table 2 summarizes the mean ± S.D. scores of the variables which show normal distribution in both hyperthyroidism and healthy groups. Serum total ALP levels of the hyperthyroidism group were higher than those of healthy group ( $p=0.014$ ) (Table 2). According to ISCD-official-positions-adult; 2013, BMD z scores were measured both at lumbar spine and femoral neck in 11 patients. For 10 patients, the results were normal and for 1 patient the values were below the expected range for age ( $<-2$ ).

There was a significant positive correlation between sclerostin and BALP ( $r=0.805$ ,  $p<0.001$ ) but there was no significant correlation between sclerostin and osteocalcin, TSH,  $FT_3$ ,  $FT_4$ , urine DPD, urine OH proline. Similarly, there was a significant positive correlation between urine nMol DPD/g creatinine/day and urine mg OH proline/g creatinine/day ( $r=0.911$ ,  $p<0.001$ ) and there was a significant positive correlation between osteocalcin and  $FT_3$ ,  $FT_4$  respectively ( $r=0.580$ ,  $p<0.001$ ), ( $r=0.603$ ,  $p<0.001$ ) in opposition to negative correlation between osteocalcin and TSH ( $r=-0.474$ ,  $p<0.05$ )

(Table 3). Furthermore, there was no significant correlation between sclerostin and lumbar spine z scores ( $p=0.346$ ;  $r=-0.314$ ), femoral neck z scores ( $p=0.811$ ;  $r=-0.082$ ).

Sclerostin levels of men and women in the hyperthyroidism group [5.91 (2.59–23.12)], [6.57 (2.63–34.74)] did not differ significantly compared to those of men and women in the normal group [5.15 (2.00–32.00)], [7.32 (3.07–32.00)] ( $p=0.973$ ,  $p=0.237$ ).

## DISCUSSION

Thyroid hormones are important regulators of the skeleton that stimulate both the formation and resorption of bones and cause thinning in the mineralized bones (Mosekilde *et al.*, 1977; Ben-Shlomo *et al.*, 2001). To this end, it has been reported that the BMD decreases in patients with hyperthyroidism, and the risk of bone fractures increases (Vestergaard *et al.*, 2000; Basset *et al.*, 2007). It has been stated in the literature that the thyroid hormones (O'shea *et al.*, 2012; Tsourdi *et al.*, 2015; Wang *et al.*, 2007) and sclerostin (Van Bezooijen *et al.*, 2004, 2007; Lin *et al.*, 2009; Winkler *et al.*, 2003; Xiaofeng *et al.*, 2005; Poole *et al.*, 2005) were influential on bone tissue by Wnt/ $\beta$ -catenin signal pathway. There were several clinical (Skowronska-Jozwiak *et al.*, 2012, 2015) and experimental (Tsourdi *et al.*, 2015) studies that examine the sclerostin in hyperthyroidism.

Our study involves the hyperthyroidism patient group (including multinodular goiter and Graves' disease) whose treatments have not started yet. No differences were detected between the serum sclerostin levels of the group with hyperthyroidism and the control group

Table 3. The correlation analysis between the sclerostin, thyroid hormones, bone metabolism markers (n = 48)

	Correlation coefficient (r)	p value
Serum sclerostin-serum BALP	0.805	***
Serum sclerostin-serum osteocalcin	-0.143	>0.05
Serum sclerostin- urine nMol DPD/g creatinine/day	0.270	>0.05
Serum sclerostin- urine mg OH proline/g creatinine/day	0.208	>0.05
Serum TSH-serum sclerostin	0.124	>0.05
Serum $FT_3$ -serum sclerostin	-0.003	>0.05
Serum $FT_4$ -serum sclerostin	0.047	>0.05
Serum TSH-serum osteocalcin	-0.474	*
Serum $FT_3$ -serum osteocalcin	0.580	***
Serum $FT_4$ -serum osteocalcin	0.603	***
Urine nMol DPD/g creatinine/day-urine mg OH proline/g creatinine/day	0.911	***

Statistically significant \*p<0.05; \*\*\*p<0.001

in our study. This result differs from those reported by Skowronska-Jozwiak and coworkers (2012; 2015) and Tsourdi and coworkers (2015). Skowronska-Jozwiak and coworkers (2012; 2015) found high serum sclerostin levels in patients with hyperthyroidism when compared to the reference values. In their study, after the treatment, the sclerostin levels reduced significantly as opposed to the pretreatment levels. Tsourdi and coworkers (2015) reported that in 12 week-old male mice with hyperthyroidism, sclerostin mRNA expression, the number of sclerostin-positive cells and serum sclerostin levels increased, trabecular and cortical bone density and cortical thickness were found to have decreased. Thus the result of the present study suggests that the sclerostin levels may change depending on the duration of exposure of the bone tissue to increased thyroid hormone levels. More detailed data may be obtained in sclerostin by following-up the patients with hyperthyroidism for longer durations; however, it is another important fact that the treatment of the patients who are diagnosed with hyperthyroidism may not be delayed.

The studies of Wang (2007) and O'Shea (2012) revealed that the thyroid hormones stimulate the Wnt signal pathway and increase bone formation in the growing bone tissues. Wang and coworkers (2007) revealed that the applied T3 on cell cultures of chondrocyte growth plaques increased the Wnt4 mRNA, protein expression and  $\beta$ -catenin. When the Wnt antagonists were applied, the  $\beta$ -catenin induced by T3 and growth plaque cell maturation was inhibited. O'Shea and coworkers (2012) reported that in 2-week old homozygote *Thrb<sup>PV/PV</sup>* mutant mice with disturbed hypophysis-thyroid axes and increased thyroid hormone levels in circulation, they found increased bone mineral storage and increased canonic Wnt/ $\beta$ -catenin activation in the osteoblasts of rats during their growth. On the other hand, Tsourdi and coworkers (2015), in their study, examined the bones of 12-week-old male mice in histomorphometric terms, and determined that there was decreasing trabecular bone density in hyperthyroidism, and increased bone density in hypothyroidism. The interesting point here was, the thyroid hormones induced bone loss in bone tissues that have already completed their development and mineralization.

Poole and coworkers (2005) reported that newly-formed osteocytes lack sclerostin. However, after the primary mineralization is completed, widespread sclerostin staining occurs in adult osteocytes and in the dendrites of osteocyte channels. Taking the existing data in the literature in to account (Poole *et al.*, 2005; Van Bezooijen *et al.*, 2004; Van Bezooijen *et al.*, 2007; Lin *et al.*, 2009; Winkler *et al.*, 2003) we consider that sclerostin may be effective on bone tissue that has completed its mineralization in the form of bone loss.

No correlation was determined between sclerostin and thyroid hormones in our study ( $n=48$ ). With regard to the data in the literature, we consider that the influence of thyroid hormones and sclerostin on Wnt signal pathway, taking a great deal of cofactors and antagonists in consideration, are independent from each other.

BALP is an enzyme that ensures the phosphate hydrolyze from phosphate esters during the mineralization process of the bone, and the transportation of the phosphate to the OH apatite crystals (Zaitseva *et al.*, 2015). No difference was found at BALP activity in our study between the hyperthyroidism patients and the control group. A positive correlation was determined between BALB and sclerostin. Our study is in agreement with the study of Schouten and coworkers (2012) who showed

that BALP existed within reference values in hyperthyroidism patients in the pre-treatment period. It was reported in the study of Van Bezooijen and coworkers (2007) that sclerostin inhibits the BALP activity stimulated with BMP and sclerostin inhibits BMP-stimulated bone formation by antagonizing the Wnt signal pathway.

We found the ALT levels and the serum total ALP activity as being significantly higher in the hyperthyroidism group than in the control group. Considering these results, we may assume that the increase in total ALP activity stems from tissues other than bone tissue.

Osteocalcin is a Gla-protein of the bone matrix, synthesized from mature osteoblasts and hypertrophic chondrocytes (Zaitseva *et al.*, 2015). Unfortunately, osteocalcin is not only localized in cells at mineralized surfaces since it was reported that both osteocalcin mRNA and protein are synthesized at bone marrow megakaryocytes, peripheral blood platelets (Thiede *et al.*, 1994) and at bone marrow adipocytes (Benayahu *et al.*, 1997). The blood levels of intact osteocalcin show the bone formation theoretically. The release of immunoreactive fragments of osteocalcin to the blood is related with bone resorption process (Zaitseva *et al.*, 2015). Lee and coworkers (2000) reported that the majority of the osteocalcin is stored in extracellular bone matrix, and the osteocalcin in serum is the non-absorbed part of total osteocalcin with hydroxyapatite. The results of our study are consistent with the results reported by Skowronska-Jozwiak and coworkers (2015); Nagazaka and coworkers (1997); Pantazi & Papapetrou (2000); Barsal and coworkers (2004), who found the osteocalcin as being high in patients with hyperthyroidism. In addition, a positive correlation was determined between osteocalcin and FT<sub>3</sub>, FT<sub>4</sub>, and a negative correlation between the osteocalcin and TSH in our study. Schouten and coworkers (2012) revealed that the osteocalcin was within the reference limits before the treatment in hyperthyroidism. However, differing from our study Schouten measured the N-MID fragment, but we measured the intact form of osteocalcin.

Osteocalcin and BALB are both bone formation markers. In our study, in the hyperthyroidism group, osteocalcin was found to have increased compared to normals, whereas BALB levels did not differ from normals. A correlation between osteocalcin and thyroid hormones was found. The study of K. Ohishi and coworkers (1994) may help to identify these findings. In a cell culture study, they investigated the differentiation of rat calvaria osteoprogenitor cells to osteoblasts. T3 treatment on those cells resulted in the inhibition of the differentiation: T3 treatment also caused an increased functional activity of the mature osteoblasts. There was a decreased ALP activity at day 12. T3 treatment increased synthesis of osteocalcin in a dose dependent manner (Ohishi K *et al.*, 1994).

In our study, urine DPD (Vesper *et al.*, 2003) and OH proline/creatinine/day levels (Calvo *et al.*, 1996), which are the biological markers of bone destruction, did not show significant difference in the group with hyperthyroidism when compared with the control group. This result differs from previous studies (Nagazaka *et al.*, 1997; Akalin *et al.*, 2002; Sabuncu *et al.*, 2001), which reported that the urine DPD/creatinine levels were high in hyperthyroidism. However, in the present study, a positive correlation was detected between the urine DPD and urine OH proline levels, which is consistent with the study conducted by Ohishi and coworkers (1994). The urine parameters were

evaluated in 24-hour urine, which is different from the previous studies.

In our study, it emerged that the urine phosphorous excretion increased in the group with hyperthyroidism. Likewise, Mosekilde and coworkers (1977) reported that the Ca and P levels were high in 24-hour urine samples in hyperthyroidism patients.

In our study which included hyperthyroidism patient group whose treatments had not started yet, no differences were determined in sclerostin levels when compared with the control group. This result makes us think that sclerostin levels might change depending on the exposure duration of the bone tissue to high levels of thyroid hormones. A long-term study, which is to examine the skeleton mineralization destruction and construction, including pre-treatment and post-treatment periods of the hyperthyroidism patients, may provide us insight on sclerostin. We also consider that the assessment of the synthesis of the sclerostin in bone tissues and the serum levels may show differences.

### Conflict of interest

The authors declare no conflict of interest

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