

Effects of endogenous hypercortisolism on bone mRNA and microRNA expression in humans

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Received: 4 July 2017 / Accepted: 21 September 2017 / Published online: 4 October 2017
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Abstract

Summary Hypercortisolism in humans suppresses osteoblastogenesis and osteoblast function through the upregulation of Wnt-signaling antagonists (sclerostin, Dkk1) and changes in microRNAs levels (miR-125b-5p, miR-218-5p, miR-34a-5p, miR-188-3p, miR-199a-5p) which are associated with mesenchymal stem-cell commitment to adipocytes or cartilage cells over the osteoblasts.

Introduction The purpose of this study was to evaluate the responses of bone to chronic glucocorticoid (GC) excess by measuring the levels of selected mRNA and microRNA (miR) in bone samples of patients with Cushing's disease (CD).

Methods Bone samples were obtained during transsphenoidal adenectomy from the sphenoid bone (sella turcica) from 16 patients with clinically and biochemically evident CD and 10 patients with clinically non-functioning pituitary adenomas (NFPA) matched by sex, age, and body mass index. Quantitative polymerase chain reactions (qPCR) were used to examine the expression of genes (mRNA and miRs) known to be involved in bone remodeling regulation based on studies in animals and cell culture.

Results Hypercortisolism was associated with the downregulation of genes involved in osteoblast function and maturation (ACP5, ALPL, BGLAP, COL1A1, COL1A2, BMP2, RUNX2, TWIST1). An excess of GC caused increased expression of Wnt-signaling antagonists (Dkk1, SOST) and changes in the levels of miRs that are known to suppress osteoblastogenesis (miR-125b-5p, miR-218-5p, miR-34a-5p, miR-188-3p, miR-199a-5p) $p < 0.05$, $q < 0.1$. Interestingly, compensatory mechanisms were found in long-term hypercortisolism: upregulation of Wnt10b, LRP5, and LRP6; downregulation of SFRP4; changes in miRs involved in osteoblastogenesis (miR-210-5p, miR-135a-5p, miR-211, miR-23a-3p, miR-204-5p); and downregulation of genes associated with osteoclastogenesis. None of these changes prevented the suppression of bone formation.

Conclusions An excess of endogenous GC in humans suppresses bone formation through the upregulation of Wnt-signaling antagonists and dysregulation of miRs involved in mesenchymal stem-cell commitment. Both Wnt-signaling antagonists and miRs seem to be promising targets for further research in therapeutic intervention in glucocorticoid-induced osteoporosis.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00198-017-4241-7>) contains supplementary material, which is available to authorized users.

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Keywords Cushing's disease · Glucocorticoid-induced osteoporosis · microRNA · Sclerostin · Wnt-signaling

Glucocorticoid-induced osteoporosis (GIO) is the most common and severe form of secondary osteoporosis and is associated with significant morbidity secondary to the development of low-traumatic fracture in up to 50% of patients [1, 2]. Despite new advances in our understanding of GIO pathophysiology and improvements in its clinical management over the past decades, the mechanisms that account for glucocorticoid (GC)-induced inhibition of bone formation remain a

subject of investigation, and modern diagnostic and treatment approaches remain less effective in GIO than in primary osteoporosis [3]. The pathophysiology of GIO is most frequently studied in a rodent model. However, and besides other differences, a major limitation of the rodent models of GIO is that loss of cancellous bone is not consistently observed [4], whereas it is preferentially lost in humans in the presence of an excess of GC [3].

The first description of multiple vertebral fractures caused by an excess of GC in humans was done in the famous historical description of endogenous hypercortisolism caused by Cushing's disease (CD). Clinical presentations of hypercortisolism in patients with CD develop because of excessive and continuous adrenal production of cortisol due to increased adrenocorticotropin (ACTH) secretion from a pituitary adenoma [5]. Excessive and continuous tissue exposure to endogenous GC results in a similar or an even more severe set of symptoms than is usually observed in patients subjected to chronic administration of supra-physiological doses of GC [3, 5]. However, the bone deterioration caused by an excessive dose of GC in humans may be affected by the disease for which the treatment was prescribed and almost all disorders for which GC are prescribed could themselves influence bone remodeling and be a cause of osteoporosis [6, 7].

Nevertheless, in large studies, it was shown that the doses of GC or the levels of 24-h urinary free cortisol (24hUFC) are the only and most significant predictors of fractures in exogenous or endogenous hypercortisolism [1, 8].

We suggest that CD, usually affecting young and otherwise healthy patients, is a good model to examine the effects of GC excess on the expression of mRNAs and microRNAs known to affect bone homeostasis in human bone samples, minimizing other confounding factors including the disease itself, the dose and type of GC, as well as the compliance of the patients.

Materials and methods

The Institutional Review Board of the National Research Centre for Endocrinology (NRCE) approved the study protocol.

Subjects

Sixteen patients with clinically evident and biochemically proven CD and ten patients with a benign clinically non-functioning pituitary adenoma (NFPA), who had signed a formal informed consent form, were enrolled into the study. The patients with CD were proven positive for endogenous hypercortisolism in at least three of the following tests: 24-h urinary free cortisol (24hUFC) (reference range 60–413 nmol/24 h), serum cortisol after low-dose dexamethasone suppression test (cutoff value for

suppression, 50 nmol/L) [5], late-night salivary cortisol (LNSC) (reference range 0.5–9.4 nmol/L) [9], and awake serum cortisol at 23:00 (reference range 46–270 nmol/L). CD was confirmed based on the normal or elevated morning ACTH levels and the presence of a benign pituitary adenoma on MRI or positive for CD inferior petrosal sinus sampling test. The histological material obtained was sufficient to confirm CD in all these patients. Among patients with CD, amenorrhea was registered in 11 cases, menstrual irregularity in three women and one woman reported normal menstrual function. Five patients had steroid-induced diabetes, but glycemia was controlled at the moment of surgery.

Subjects with NFPA did not have any clinical symptoms of CD and were shown to be at the eucortisolism stage by at least two tests that are effective at verifying endogenous hypercortisolism or hypocortisolism (24hUFC, LNSC, morning and late-night serum cortisol). They were also tested for other pituitary hormone production and their levels were shown to be within the reference range. Patients with NFPA were referred to surgery because of optic chiasm compression in nine cases and in one case because of severe headache.

Exclusion criteria were pregnancy, glucocorticoid use, alcohol abuse, exacerbation of chronic disease, severe conditions (i.e., renal and liver insufficiency, heart attack, stroke), terminal conditions, mental insanity, prolonged immobilization (> 1 week), any other cause of secondary osteoporosis at present or in a 5-year medical history, or any prolonged treatment with drugs documented to influence bone metabolism in humans during the previous 12 months [10] including treatment with antiresorptive or anabolic compounds for osteoporosis or treatment to resolve hypercortisolism.

Sample collection and RNA isolation

Bone samples were taken during transsphenoidal adenomectomy from the sphenoid bone (sella-turcica) using Blakesley forceps before the removal of the benign pituitary adenoma. The specimens were washed with sterile 0.9% NaCl solution to remove visible blood. A scalpel was used to make the bone samples a similar size of 5×5 mm. Bone samples were immediately placed in lysis buffer (QIAzol). Bone tissue was harvested in 1 ml QIAzol Lysis Reagent (Qiagen, 79306) and homogenized by TissueLyser LT. Total RNA isolation from bone tissue with on-column digestion of the genomic DNA was carried out with a miR-Neasy Mini Kit (Qiagen, 217004) on the automatic station "QIAcube" as per the manufacturer's protocol. To prevent degradation, 1 unit of RiboLock RNase Inhibitor (Thermo Fisher Scientific, EO0382) was added per 1 µl of RNA solution. The concentration of total RNA in the aqueous solution was evaluated on a NanoVue Plus spectrophotometer (GE Healthcare).

Quantification of messenger RNA (mRNA) transcripts by qRT-PCR

A two-step quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was carried out using a High-Capacity RNA-to-cDNA Kit (Thermo Fisher, 4368814) and Custom TaqMan Array 48 Plus plates (Thermo Fisher Scientific, 4413258), run in 96-well format on the StepOnePlus instrument (Applied Biosystems), according to the manufacturer's protocol. Kits used for qRT-PCR are listed in Supplementary Table S1. Data analyses were done using SDS software (version 2.3, Applied Biosystems). All samples were normalized to GUSB and levels of GAPDH, HPRT served as secondary internal controls.

Quantification of microRNA (miRNA, miR) transcripts by qRT-PCR

A qRT-PCR was carried out using a TaqManTM Advanced miRNA cDNA Synthesis Kit (Thermo Fisher, A28007) and TaqMan[®] Advanced miRNA Assays (Thermo Fisher Scientific, A25576), run in 96-well format on the StepOnePlus instrument (Applied Biosystems), according to the manufacturer's protocol. Kits used for qRT-PCR are listed in Supplementary Table S2. Data analyses were done using the SDS software (version 2.3, Applied Biosystems). All samples were normalized to hsa-miR-191 levels and spike-in control cel-miR-39-3p.

Serum measurements

All subjects had fasting 8–9 AM serum samples taken to obtain routine biochemistry, hormonal status, and bone turnover markers (osteocalcin (OC), carboxyterminal cross-linked telopeptide of type I collagen (CTx)).

Serum samples on OC, CTx, ACTH, cortisol in serum, and saliva were assayed by electrochemiluminescence (ECLIA) Cobas 6000 Module e601 Roche. Vitamin D (25OHD₃) levels were measured by immunochemiluminescence assay (Liaison). Routine biochemistry was performed on Architect 8000 (Abbott, USA).

24hUFC was measured by an immunochemiluminescence assay (extraction with diethyl ether) on a Vitros ECI.

Bone mineral density and fracture assessment

At the time of enrollment, all participants were questioned regarding any recent low-traumatic fractures, back pain, and height changes. Height was measured by stadiometer and a body mass index (BMI) was calculated as kilograms per meter squared.

Patients underwent standard spinal radiographs in anterior-posterior and lateral positions of the vertebrae Th4-L4 (Axiom

Icons R200 “Siemens”). A deformity was considered a fracture if the visual inspection perceived a reduction in vertebral height (anterior, posterior, or middle) of 20% or more [11].

Bone mineral density (BMD) was measured by dual energy X-ray absorptiometry (iDXA, GE) at the anteroposterior lumbar spine (L1-L4) and femoral neck (the lower of two Z-scores was chosen) positions according to the standard protocol. Quality control procedures were carried out in accordance with the manufacturer's recommendations.

Statistical analysis

Data is presented using mean (M) values and 95% confidence intervals (95% CI). Comparisons between the descriptive parameters of patients with CD and NFPA were made using non-paired, two-sided *t* tests. Fisher's exact test was used to compare two independent groups for qualitative parameters.

All statistical analysis and graphical output were performed using R software version 3.4.0 (2017-04-21). The original scale and log₂-transformed data were plotted using “ggplot” package and descriptive statistics were calculated using basic “stats” package. Each parameter was tested using a paired *t* test to evaluate any differences between CD and NFPA. Spearman's rank test was used to correlate quantitative parameters within the group.

A *p* value less than 0.05 was considered statistically significant. In order to account for multiple comparisons, a correction for the false discovery rate (*q* values < 0.10) was calculated using the Benjamini-Hochberg adjustment [12, 13]. All analytical statistics were performed using basic “stats” package only.

No power calculations were made for this study in advance and there was not a statistical hypothesis as it was a pilot rather than confirmatory study. Yet, post-hoc power analysis was conducted on the samples, and it was confirmed that for the majority of comparisons made the power of the calculation exceeded 90% (ranging from 39.2 to 99.9% across mRNA and miR parameters tested).

Results

The general characteristics and biochemical parameters of patients are summarized in Table 1. Subjects with CD had elevated levels of 24hUFC—1243 (95% CI 633–1853) nmol/24 h, late-night serum cortisol—649 (95% CI 403–895) nmol/L, late-night salivary cortisol—13.6 (95% CI 7.3–20.0) nmol/L with the mean estimated duration of hypercortisolism being 2 (0.5–5) years. Subjects with NFPA had cortisol levels within the reference range. As expected, serum levels of OC were decreased in patients with CD versus patients with NFPA, but no difference in CTx levels were found. Notably, more than 50% of patients with CD had

Table 1 Clinical and biochemical characteristics of enrolled patients with Cushing's disease (CD) and clinically non-functioning pituitary adenoma

Variable	Clinically non-functioning pituitary adenoma mean (95% CI)	Cushing's disease mean (95% CI)	<i>p</i> value
<i>N</i>	10	16	
Age (years)	45 (33–57)	40 (33–48)	0.353
Body mass index (kg/m ²)	27 (23–31)	30 (26–35)	0.55
Sex female (%):male (%)	6 (60):4 (40)	14 (87.5):2 (12.5)	0.16
Calcium (mmol/L)	2.35 (2.33–2.43)	2.38 (2.33–2.43)	0.93
Creatinine (mkmol/L)	72 (64–79)	69 (63–75)	0.67
Osteocalcin (ng/ml)	23.5 (12.3–34.6)	9.1 (5.8–12.4)	0.015
CTx (ng/ml)	0.58 (0.28–0.89)	0.33 (0.24–0.41)	0.102
Vitamin D (ng/ml)	12.2 (5.9–18.4)	11.5 (8.0–13.1)	0.932
L1-L4 Z-score	-0.4 (-1.8 to 0.9)	-1.2 (-2.1 to -0.2)	0.297
Femur neck Z-score	-0.2 (-1.2 to 0.7)	-0.8 (-1.5 to -0.07)	0.325
Low traumatic fractures <i>N</i> (%), type	1 (10%), in 1 case vertebral fracture	9 (56%), in 6 cases vertebral fractures and in 6 cases non-vertebral fractures (4 cases of rib fractures and 2 cases of ankle fractures)	0.037

low-traumatic fractures mainly vertebral and rib fractures, and the rate of fractures was significantly higher in patients with CD as compared to NFPA (Table 1).

Effects of glucocorticoid excess on gene expression critical for osteoblast differentiation and maturation

As shown in Fig. 1, we have found downregulation of some of the genes responsible for osteoblast function and bone formation [14] (ACP5, ALPL, BGLAP), including more than two-fold downregulation of collagen synthesis (COL1A1, COL1A2). Several growth factors and receptors that affect osteoblastogenesis [14, 15] (BMP2, IGF1, FGR2, VEGFA) were downregulated. As might be expected, osteoblast transcription factors (TWIST1, RUNX2) were also downregulated by more than 50%. Extracellular antagonists of the Wnt-signaling pathway—Dkk1 (3.24 (1.98–4.50) $p = 0.002$, $q = 0.039$) and SOST (5.13 (1.86–8.41) $p = 0.017$, $q = 0.259$) were among the upregulated genes. Surprisingly, receptors for Wnt proteins LRP5 and LRP6 and Wnt10b which promote osteoblastogenesis and TGFB1, which stimulates matrix protein synthesis, were significantly upregulated, specifically Wnt10b was increased by up to 12-folds. A secreted antagonist of the Wnt-signaling pathway—SFRP4 [14, 16] was found to be among the downregulated genes in bone samples of patients with CD versus NFPA.

To support our findings, we performed correlation analysis between serum osteocalcin and bone mRNA levels and found the following significant association: ACP5 $\rho_o = 0.663$ $p = 0.002$, ALP $\rho_o = 0.469$ $p = 0.043$, BGLAP $\rho_o = 0.518$ $p = 0.023$, BMP2 $\rho_o = 0.534$ $p = 0.019$, CD40 $\rho_o = -0.523$ $p = 0.022$, COL1A1 $\rho_o = 0.497$ $p = 0.030$, Dkk1 $\rho_o = -0.500$ $p = 0.029$, FGFR2 $\rho_o = 0.507$ $p = 0.027$, IGF1 $\rho_o = 0.544$

$p = 0.016$, IL6 $\rho_o = 0.667$ $p = 0.003$, MMP2 $\rho_o = 0.595$ $p = 0.007$, RUNX2 $\rho_o = 0.691$ $p = 0.001$, SFRP1 $\rho_o = 0.471$ $p = 0.042$, SOST $\rho_o = -0.509$ $p = 0.026$, SPP1 $\rho_o = 0.483$ $p = 0.036$, STAT1 $\rho_o = -0.516$ $p = 0.024$, TGFB1 $\rho_o = -0.493$ $p = 0.032$, TIMP2 $\rho_o = 0.504$ $p = 0.028$, TNFSF11 $\rho_o = 0.518$ $p = 0.023$, VEGFA $\rho_o = 0.554$ $p = 0.014$. A few genes were close to demonstrate correlation with serum osteocalcin levels, but did not reach a significant value probably due to a low sample size: COL1A2 $\rho_o = 0.419$ $p = 0.074$, SFRP4 $\rho_o = 0.449$ $p = 0.054$, Wnt10b $\rho_o = -0.634$ $p = 0.06$.

Of the tested microRNAs (Table 3, Fig. 2), we reported up-regulation of miR-125b-5p, miR-188-3p, and miR-34a-5p, all of which are expected to have negative effects on osteoblastogenesis and positive on adipogenesis [17–20]. Downregulation of miR-218 found in bone of patients with CD most likely also exerts negative effects for osteoblastogenesis, because it was shown to act as a positive regulator of osteogenesis in vitro [21]. Downregulation of miR-199a-5p with the main target of SMAD1 [22] should be beneficial for chondrocyte differentiation over the osteoblasts.

The other dysregulated miRs target the negative regulators for osteoblastogenesis (miR-210-5p) [23] or attenuate negative effects on RUNX2 [24–26] (miR-135a-5p, miR-211, miR-23a-3p, miR-204-5p).

With regard to miR expression in bone samples, we also performed correlation analysis and found the following association between osteocalcin and miR-135a-5p $\rho_o = 0.618$ $p = 0.005$, miR-148a-3p $\rho_o = -0.502$ $p = 0.029$, miR-125b-5p $\rho_o = -0.477$ $p = 0.039$, miR-211 $\rho_o = 0.492$ $p = 0.032$, miR-10b-5p $\rho_o = -0.480$ $p = 0.037$, miR-21-3p $\rho_o = -0.473$ $p = 0.041$, miR-320a $\rho_o = 0.501$ $p = 0.029$, miR-26a-5p $\rho_o = -0.519$ $p = 0.023$, miR-96-5p $\rho_o = 0.748$ $p < 0.001$, miR-188-3p

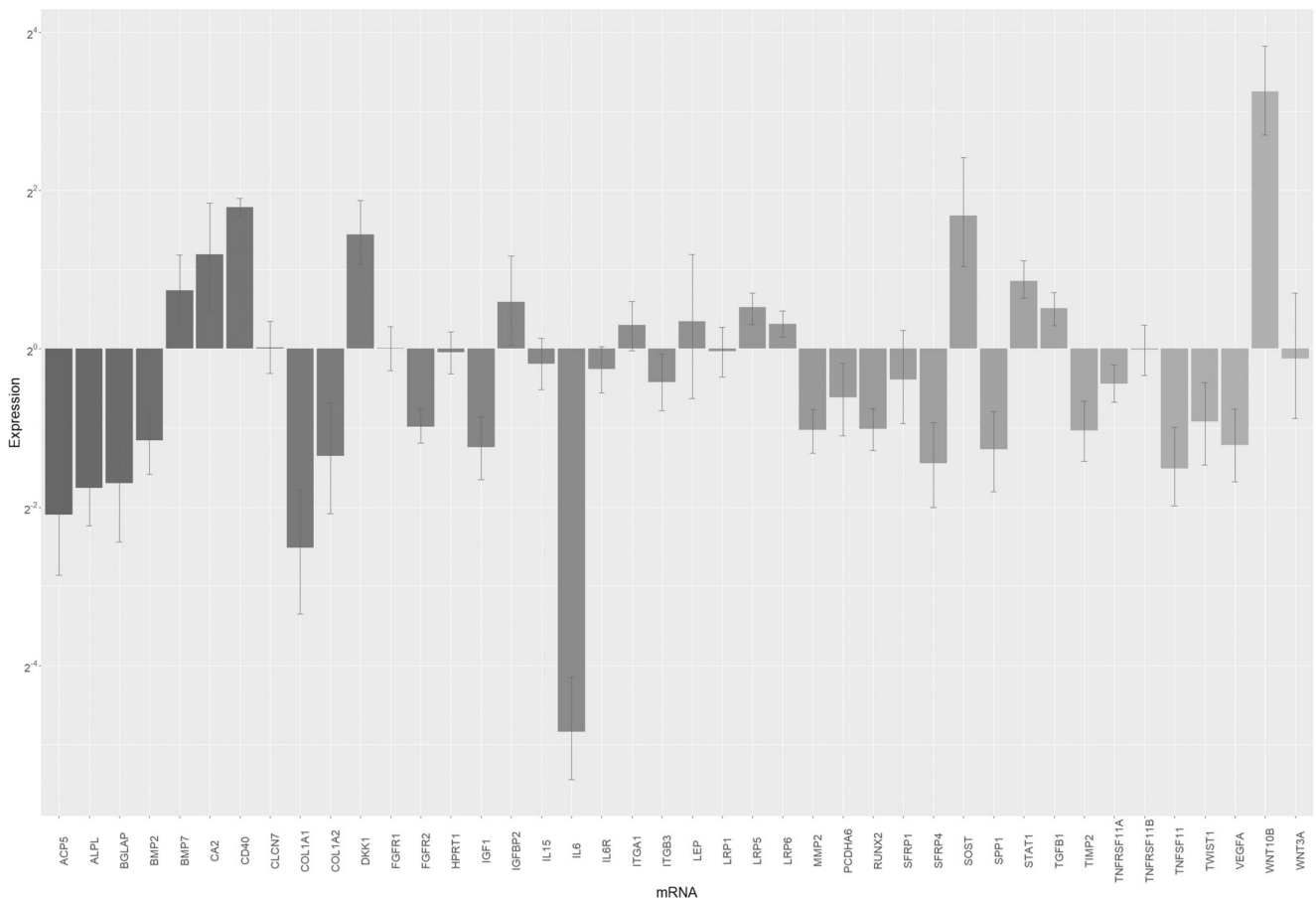


Fig. 1 The effects of GC excess on relative mRNA levels in bone samples of patients with endogenous hypercortisolism. The log-transformed data of upregulated and downregulated genes in bone

samples of patients with Cushing’s disease versus subjects with clinically non-functioning pituitary adenoma

$\rho_o = -0.657$ $p = 0.002$, miR-199a-5p $\rho_o = 0.476$ $p = 0.039$, miR-204-5p $\rho_o = 0.561$ $p = 0.012$, miR-218-5p $\rho_o = 0.736$ $p < 0.001$, miR-23a-3p $\rho_o = 0.722$ $p < 0.001$, miR-27a-3p $\rho_o = 0.493$ $p = 0.032$, miR-29a-3p $\rho_o = 0.481$ $p = 0.037$, miR-29c-3p $\rho_o = 0.695$ $p = 0.01$.

Effects of glucocorticoid excess on gene expression critical for osteoclast activation and function

MRNA levels of genes responsible for osteoclast function (MMP2, SPP1) [27] and osteoclastogenesis (TIMP2, IL6, TNFRSF11A, TNFSF11) [27] were decreased in bone samples from CD patients compared to NFPA. However, some genes that might affect osteoclastogenesis were upregulated (CD40, STAT1). We also found significant correlation between serum CTx and bone mRNA levels of FGFR2 $\rho_o = 0.536$ $p = 0.022$, SOST $\rho_o = -0.513$ $p = 0.029$, SPP1 $\rho_o = 0.465$ $p = 0.052$, TWIST1 $\rho_o = 0.510$ $p = 0.031$.

Decreased levels of miR-31-5p is expected to be negative for osteoclastogenesis as inhibition of miR-31 in cell culture by specific antagonomiRs suppressed the RANKL-induced formation of osteoclasts and bone resorption [28]. From the other

hand, upregulation of miR-148a-3p, miR-133a-3p, and miR-21-3p documented in our study was shown to be positive regulators for osteoclastogenesis [27, 29].

Some associations were found between serum CTx levels and miRs expressions in bone samples: miR-210-5p $\rho_o = -0.469$ $p = 0.05$, miR-148a-3p $\rho_o = -0.523$ $p = 0.026$, miR-211 $\rho_o = 0.595$ $p = 0.009$, miR-204-5p $\rho_o = 0.605$ $p = 0.008$, miR-23a-3p $\rho_o = 0.491$ $p = 0.039$, miR-29a-3p $\rho_o = 0.491$ $p = 0.038$.

All the tested mRNA (with abbreviation) and microRNA levels, p values, and corrections for multiple comparisons (q values) are summarized in Tables 2 and 3, respectively.

One woman among patients with CD reported normal menses. With regard to the gene expressions, she was not markedly different from the other patients. We also did not find any differences in mRNA or microRNA values in subjects with CD with and without diabetes. In order to analyze whether the duration of hypercortisolism exerts influence on gene expression, we divided patients on those whose approximate disease duration was 2 years and less and those who most likely suffered from hypercortisolism longer than 2 years. The only difference we found was in miR-210-5p expression which was 4.2 (95%

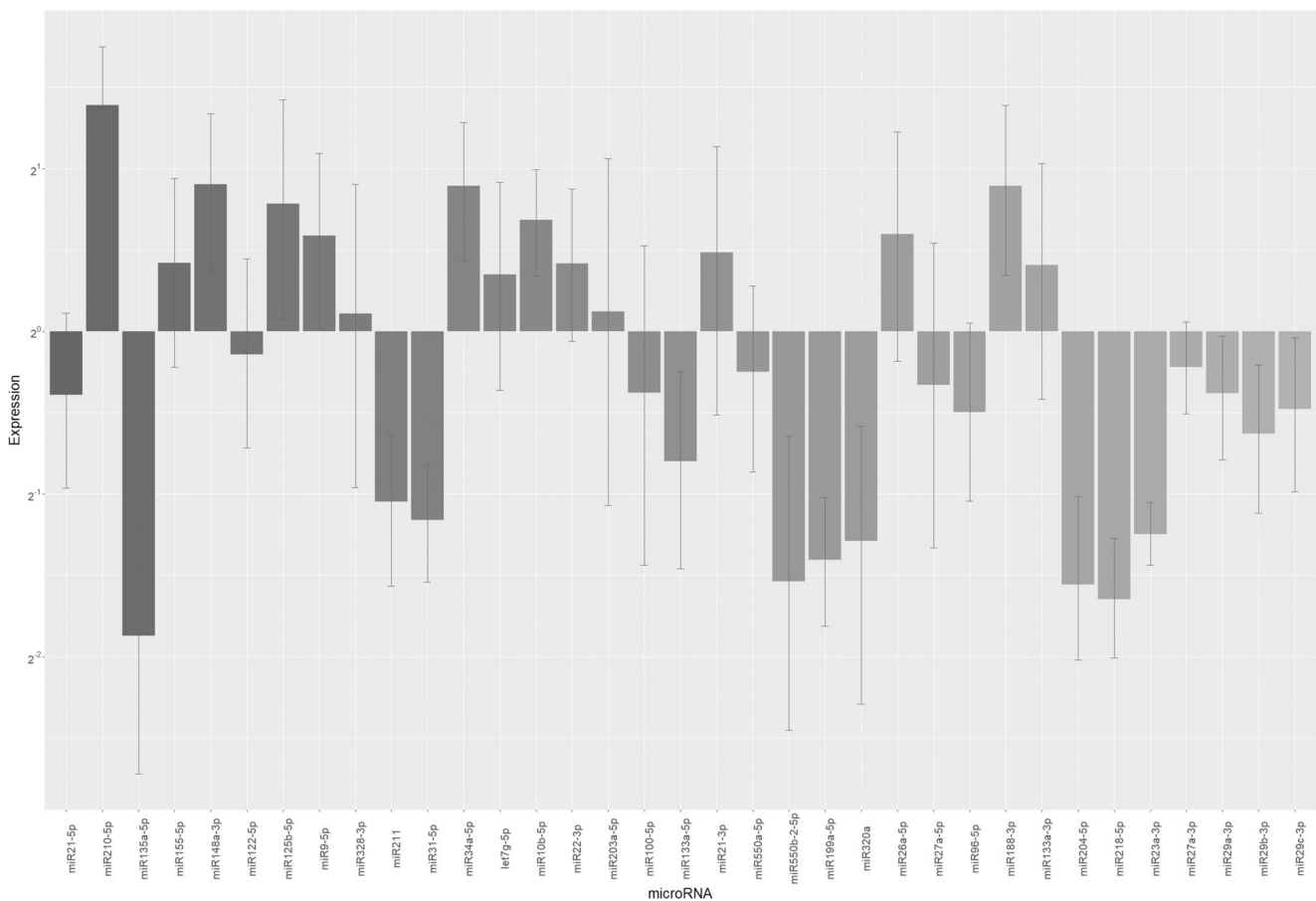


Fig. 2 The effects of GC excess on relative microRNA (miR) levels in bone samples of patients with endogenous hypercortisolism. The log-transformed data of upregulated and downregulated miRs in bone

samples of patients with Cushing's disease versus subjects with clinically non-functioning pituitary adenoma

CI 3.41–4.99) in patients with 2 years or less symptoms of hypercortisolism and 2.68 (95% CI 1.93–3.44) in those who most likely suffered from CD longer than 2 years $p = 0.012$.

Discussion

This is the first study evaluating both mRNA and microRNA profiles in bone samples of patients with active endogenous hypercortisolism.

Consistent with previous reports [30–34], we found significant suppression of osteoblast activity, together with the downregulation of several growth factors (BMP2, FGFR2, IGF1, BMP7), and increased expression of Wnt-signaling antagonists (Dkk1, SOST). In addition to Dkk1 and SOST, an increase in SFRP1 was reported in animals and cell cultures subjected to GC excess [32, 35]. Contrary to the findings in mice and cell cultures, SFRP1 was not changed and SFRP4 was downregulated in patients with CD. These differences may be due to the fact that patients with CD have been exposed to GC for a much longer period than the usual duration of laboratory experiments. Moreover, rather unexpectedly,

Wnt-signaling agonists (Wnt10b, LRP5, and LRP6) were markedly upregulated, up to 12-folds, in CD bone samples versus a control group. Despite the increased mRNA levels of Wnt-signaling agonists, the downregulation of osteoblast specific transcription factors (TWIST1 and RUNX2) remained, indicating an overall suppression of osteoblastogenesis in subjects with CD. Consequently, these findings in humans might represent attempts to compensate for the negative effects of GC excess on osteoblastogenesis in long-term exposure.

The suppressed bone formation and osteoblastogenesis might be at least in part explained through our results in miRNA expression changes.

Consistent with previous findings in cell culture [20], we found miR-34a-5p to be upregulated, which, according to the literature, may reduce cell cycle-related proteins (CDK4, CDK6, Cyclin D1) leading to the inhibition of proliferation and inhibition of osteoblastogenesis [20]. MiR-125b-5p, which was upregulated in the present research, has been shown to inhibit osteogenic differentiation through the regulation of cell proliferation and mesenchymal stem-cell commitment [17, 18]. MiR-188-3p, upregulated in the present study, is a key regulator of the age-related switch between osteogenesis and adipogenesis

Table 2 The result of individual mRNA levels in Cushing's disease compared with clinically non-functioning pituitary adenoma patients with corrections for multiple comparisons (the gene name and function were taken from: <https://ghr.nlm.nih.gov/gene>)

Gene symbol	Gene name	Clinically non-functioning pituitary adenoma (control group)	Cushing's disease mean (95% CI)	<i>p</i>	<i>q</i>
ACP5	Acid phosphatase 5, tartrate resistant	1	0.32 (0.21–0.43)	< 0.001	< 0.001
ALPL	Alkaline phosphatase	1	0.35 (0.26–0.45)	< 0.001	< 0.001
BGLAP	Bone gamma-carboxyglutamate protein	1	0.42 (0.30–0.54)	< 0.001	< 0.001
BMP2	Bone morphogenetic protein 2	1	0.51 (0.38–0.64)	< 0.001	< 0.001
BMP7	Bone morphogenetic protein 7	1	2.04 (1.29–2.78)	0.01	0.165
CA2	Carbonic anhydrase 2	1	3.68 (0.87–6.50)	0.06	0.84
CD40	CD 40 molecule	1	3.5 (3.13–3.87)	< 0.001	< 0.001
CLCN7	Chloride voltage-gated channel 7	1	1.12 (0.84–1.41)	0.375	1
COL1A1	Collagen type I alpha 1 chain	1	0.27(0.16–0.38)	< 0.001	< 0.001
COL1A2	Collagen type I alpha 2 chain	1	0.53 (0.36–0.70)	< 0.001	0.001
Dkk1	Dickkopf 1	1	3.24 (1.98–4.50)	0.002	0.039
FGFR1	Fibroblast growth factor receptor 1	1	1.09 (0.87–1.30)	0.41	1
FGFR2	Fibroblast growth factor receptor 2	1	0.53 (0.44–0.62)	< 0.001	< 0.001
HPRT1	Hypoxanthine phosphoribosyltransferase 1	1	1.03 (0.83–1.24)	0.735	1
IGF1	Insulin-like growth factor 1	1	0.48 (0.37–0.60)	< 0.001	< 0.001
IGFBP2	Insulin-like growth factor binding protein 2	1	2.14 (0.84–3.44)	0.08	1
IL15	Interleukin-15	1	0.98 (0.72–1.23)	0.852	1
IL6	Interleukin-6	1	0.06 (0–0.13)	< 0.001	< 0.001
IL6R	Interleukin-6 receptor	1	0.9 (0.71–1.10)	0.304	1
ITGA1	Integrin subunit alpha 1	1	1.34 (1.04–1.63)	0.028	0.421
ITGB3	Integrin subunit beta 3	1	0.85 (0.62–1.08)	0.179	1
LEP	Leptin	1	2.53 (0.42–4.64)	0.142	1
LRP1	Low-density lipoprotein receptor protein 1	1	1.08 (0.82–1.33)	0.533	1
LRP5	Low-density lipoprotein receptor protein 5	1	1.5 (1.30–1.69)	< 0.001	0.002
LRP6	Low-density lipoprotein receptor protein 6	1	1.28 (1.12–1.45)	0.002	0.046
MMP2	Matrix metalloproteinase 2	1	0.52 (0.43–0.62)	< 0.001	< 0.001
PCDHA6	Protocadherin alpha 6	1	0.8 (0.54–1.05)	0.112	1
RUNX2	Runt-related transcription factor 2	1	0.53 (0.43–0.62)	< 0.001	< 0.001
SFRP1	Secreted frizzled-related protein 1	1	1.1 (0.51–1.69)	0.726	1
SFRP4	Secreted frizzled-related protein 4	1	0.46 (0.32–0.59)	< 0.001	< 0.001
SOST	Sclerostin	1	5.13 (1.86–8.41)	0.017	0.259
SPP1	Secreted phosphoprotein 1	1	0.51 (0.35–0.67)	< 0.001	< 0.001
STAT1	Signal transducer and activator of transcription 1	1	1.93 (1.54–2.32)	< 0.001	0.004
TGFB1	Transforming growth factor beta-1	1	1.49 (1.26–1.71)	< 0.001	0.008
TIMP2	TIMP metalloproteinase inhibitor	1	0.55 (0.41–0.69)	< 0.001	< 0.001
TNFRSF11A (RANK)	TNF receptor superfamily member 11a (receptor activator of nuclear factor κ B)	1	0.78 (0.64–0.93)	0.006	0.111
TNFRSF11B (OPG)	TNF receptor superfamily member 11b (osteoprotegerin)	1	1.09 (0.86–1.31)	0.422	1
TNFSF11 (RANKL)	TNF superfamily member 11 (receptor activator of nuclear factor kappa-B ligand)	1	0.48 (0.18–0.79)	0.002	0.047
TWIST1	Twist family bHLH transcription factor 1	1	0.66 (0.46–0.86)	0.002	0.046
VEGFA	Vascular endothelial growth factor A	1	0.53 (0.32–0.74)	< 0.001	0.007
WNT10b	Wnt family member 10B	1	12.22 (7.31–17.13)	< 0.001	0.008
WNT3a	Wnt family member 3A	1	1.59 (0.40–2.78)	0,303	1

Table 3 The result of individual microRNA levels in Cushing's disease compared with clinically non-functioning pituitary adenoma patients with corrections for multiple comparisons

MicroRNA (miR) symbol	Clinically non-functioning pituitary adenoma (control group)	Cushing's disease expression mean (95% CI)	<i>p</i>	<i>q</i>
miR-21-5p	1	0.94 (0.67–1.21)	0.663	1
miR-210-5p	1	2.97 (2.29–3.65)	< 0.001	0.001
miR-135a-5p	1	0.39 (0.27–0.51)	< 0.001	< 0.001
miR-155-5p	1	1.64 (1.22–2.07)	0.005	0.103
miR-148a-3p	1	2.22 (1.66–2.77)	< 0.001	0.007
miR-122-5p	1	1.22 (0.78–1.66)	0.311	1
miR-125b-5p	1	2.35 (1.56–3.13)	0.002	0.05
miR-9-5p	1	1.93 (1.30–2.57)	0.007	0.116
miR-328-3p	1	1.62 (1.13–2.11)	0.016	0.246
miR-211	1	0.57 (0.42–0.72)	< 0.001	0.001
miR-31-5p	1	0.51 (0.37–0.64)	< 0.001	< 0.001
miR-34a-5p	1	2.17 (1.61–2.74)	< 0.001	< 0.001
let7g-5p	1	1.64 (1.22–2.06)	0.005	0.103
miR-10b-5p	1	1.75 (1.38–2.12)	0.001	0.015
miR-22-3p	1	1.60 (1.14–2.06)	0.014	0.217
miR-203a-5p	1	2.07 (1.21–2.93)	0.019	0.280
miR-100-5p	1	1.43 (0.85–2.01)	0.134	1
miR-133a-5p	1	0.78 (0.49–1.07)	0.128	1
miR-21-3p	1	1.91 (1.50–2.32)	< 0.001	0.007
miR-550a-5p	1	1.09 (0.74–1.44)	0.576	1
miR-550b-2-5p	1	0.63 (0.33–0.93)	0.02	0.262
miR-199a-5p	1	0.44 (0.32–0.56)	< 0.001	0.001
miR-320a	1	0.59 (0.41–0.76)	< 0.001	0.004
miR-26a-5p	1	2.17 (1.41–2.93)	0.005	0.103
miR-27a-5p	1	1.33 (0.78–1.88)	0.219	1
miR-96-5p	1	0.92 (0.58–1.26)	0.627	1
miR-188-3p	1	2.28 (1.65–2.90)	0.001	0.013
miR-133a-3p	1	1.86 (1.10–2.62)	0.029	0.345
miR-204-5p	1	0.45 (0.23–0.66)	< 0.001	0.002
miR-218-5p	1	0.37 (0.25–0.49)	< 0.001	< 0.001
miR-23a-3p	1	0.44 (0.37–0.51)	< 0.001	< 0.001
miR-27a-3p	1	0.93 (0.72–1.14)	0.476	1
miR-29a-3p	1	0.86 (0.67–1.06)	0.15	1
miR-29b-3p	1	0.77 (0.55–0.98)	0.038	0.414
miR-29c-3p	1	0.87 (0.60–1.13)	0.302	1

of bone marrow mesenchymal stem cells (BMSCs) [19]. BMSC-specific overexpression of miR-188 in mice reduced bone formation and increased bone marrow fat accumulation [19]. In contrast, Real-time PCR showed that miR-218 is upregulated during osteogenic differentiation [21]. Overexpression of exogenous miR-218 enhanced osteogenic differentiation in vitro, whereas inhibition of miR-218 would suppress osteogenic differentiation [21]. Consequently, the downregulation of miR-218-5p and upregulation of miR-125b-5p, miR-188-3p, and miR-34a-5p in bone samples of patients with CD are likely to contribute to the suppression of osteogenesis.

As we tested miRs that regulate the commitment of mesenchymal stem cells, we analyzed the expression of miRs involved not only in osteoblastogenesis, but also adipogenesis and cartilogenesis. Contrary to our study, miR-199a-5p levels were significantly increased in osteoblasts treated with dexamethasone [36]. Shi C et al. reported that the overexpression of miR-199a-5p increases the inhibition effect of Dex on osteoblast proliferation, but depleting miR-199a-5p significantly attenuated Dex-inhibited osteoblast proliferation through the Wnt-signaling activation [36]. However, other research suggests that miR-199a is a BMP-2-responsive microRNA,

which inhibits early chondrogenesis through its direct effect on SMAD1 levels, as revealed by the reduced expression of early marker genes for chondrogenesis such as cartilage oligomeric matrix protein (COMP), type II collagen, and Sox9 [22]. Indeed, in our research, both BMP2 and miR-199a-5p were downregulated; RUNX2 and all biomarkers of osteoblast activity remained suppressed. Consequently, we suggest that the downregulation of miR-199a-5p is likely to cause an increase in SMAD1 and an improvement in cartilage developments rather than activation of Wnt signaling and osteoblastogenesis. An additional positive factor in favor of cartilage might be the downregulation of miR-320a. MiR-320a overexpression enhanced the IL-1 β -induced downregulation of Col2 α 1 and ACAN and sGAG and increased the IL-1 β -induced overexpression of MMP-13 ($p < 0.01$) [37]. Consequently, the downregulation of miR-320a is expected to have preventative effects on osteoarthritis in GC excess.

Other changes in microRNA profiles seem to be protective of bone formation. MiR-204/211 act as endogenous negative regulators of RUNX2, which inhibit osteogenesis and promote adipogenesis of mesenchymal progenitor cells and BMSCs [25], and both were downregulated in our study. The other downregulated microRNA—miR-23a represses RUNX2 in the terminally differentiated osteocyte, representing a feedback mechanism to attenuate osteoblast maturation [26]. MiR-135 targets Smad5, a key transducer of the BMP2 osteogenic signal [24]. MiR-210, which was upregulated in this study, acts as a positive regulator of osteoblastic differentiation by inhibiting the TGF- β /activin-signaling pathway through inhibition of the activin A receptor type 1B (AcvR1b) gene expression and was shown to promote BMP-4-induced osteoblastic differentiation of bone marrow-derived ST2 stromal cells [23].

Excess GC is known to reduce the expression of miR-29a in rats [38]. Treatment with miR-29a in rats attenuated the adverse effects of GCs on bone by inducing Wnt3a and the mRNA levels of RUNX, IGF1 and by reducing Dkk1 and RANKL [38]. In our study, we did not find any statistically significant difference in miR-29a or Wnt3a, although miR-29b-3p was 30% downregulated $p = 0.038$, $q = 0.412$.

In a prospective evaluation of mice treated with GC, gene expression of RANK and RANKL was not significantly different during 56 days of observation [32]. In the present study, RANK and RANKL were downregulated along with some other genes responsible for osteoclastogenesis and osteoclast function (SPP1, MMP2). It is possible that osteoclastogenesis decreases following the inhibition of osteoblastogenesis or could be maintained but through another different mechanism. Downregulation of miR-31-5p was observed in the present study most likely add to attenuation of the RANKL-induced osteoclastogenesis and bone resorption through the upregulation of RhoA [28]. However, overexpression of miR-148a, documented in our study, is expected to promote osteoclastogenesis.

MiR-148a directly targeted V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) which is a transcription factor negatively regulating RANKL-induced osteoclastogenesis [29]. Downregulation of interleukin-6 (IL6) should also be positive for osteoclastogenesis because it was shown to directly inhibit osteoclast differentiation by suppressing receptor activator of NF- κ B-signaling pathway [39].

Interestingly, we have found that miR-21-3p, but not miR-21-5p; miR-133-3p, but not miR-133-5p were upregulated in bone samples from CD. According to current studies, both MiR-21 and miR-133 contribute to osteoclast activity and osteoclast survival [27, 40]. However, another research revealed that miR-21-3p inhibits Spry-1 and therefore promotes osteoblast differentiation [41]. Similarly, to our study, MiR-21-3p was significantly upregulated in bone marrow mesenchymal stem cells from mice with steroid-induced osteonecrosis of the femoral head [42].

This is the first study documenting gene expression in bone samples of humans suffering from endogenous hypercortisolism, and being a pilot study, it has certain limitations. We used whole bone samples including bone marrow to extract RNA samples; we could not distinguish whether the GC-induced transcriptional changes reflected changes in cellular composition or the response of a particular cell type. We also obtained bone samples from sphenoid bone (sella turcica) during neurosurgery, whereas usually, a bone biopsy is taken from the iliac crest. Nevertheless, the effect of hypercortisolism is systemic and the structures of bone are similar. We evaluated only the expression of genes which were previously reported to be associated with bone remodeling and mesenchymal stem-cell commitment [15, 27, 43–45], and we reported our findings in conjunction with currently known microRNA functions. However, we cannot be confident that we assessed all the relevant genes. In addition to this, in CD, elevated cortisol secretion (similarly to supra-physiological doses of exogenous GC) antagonizes other hormones, such as sex steroids, growth hormone, and vitamin D, and can cause steroid-induced hyperglycemia. These potential developments might themselves exert an influence on gene expression. As the effects of hypercortisolism in humans are multifactorial, the casual relationship between high levels of cortisol and their effects on bone is difficult to establish. Thus, the results of our study relates to hypercortisolism as a complex condition in humans. In addition to this, we cannot provide a precise estimate for the duration of hypercortisolism. Because the condition is rare and frequently misdiagnosed in primary care, there might be a long time between the first signs and diagnosis establishment. All the limitations related to the case-control cross-sectional design of our study should also be considered.

In summary, excess production of endogenous cortisol in humans suppresses osteoblastogenesis and osteoblast function through the upregulation of Wnt-signaling antagonists (Dkk1,

sclerostin) and changes in microRNAs levels which are associated with mesenchymal stem-cell commitment to adipocytes or cartilage cells over the osteoblasts. In patients with CD, some compensatory mechanisms are evident: upregulation of Wnt-signaling agonists (Wnt 10b, LRP5, LRP6), protective changes in certain microRNAs, and downregulation of osteoclast activity and osteoclastogenesis. The consistent suppression of RUNX2 indicates that the documented mechanisms of compensation are not sufficient in long-term hypercortisolism and new therapeutic intervention should be developed.

Acknowledgments We thank Geoffrey Clayson for his English editing work.

Financial support Russian Scientific Foundation 15-15-30032

Compliance with ethical standards

Conflicts of interest None.

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