



Inhibition of peripheral serotonin synthesis by LP533401 and disturbances in calciotropic hormones attenuated excessive osteoblastogenesis with simultaneous improvement of bone mineral status in 5/6 nephrectomized rats

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ABSTRACT

Chronic kidney disease (CKD) is a pathological condition associated with renal osteodystrophy for which there are limited treatment options. Gut-derived serotonin (GDS) is one of the key signaling factors controlling the osteoblast proliferation. Previously, we shown that inhibition of GDS synthesis by LP533401 improved bone mineral status of rats with 5/6 nephrectomy-induced CKD model. Here, we investigated whether the use of LP533401 can modify GDS-dependent molecular pathway involved in osteoblast formation and bone mineralization in CKD rats. The 8-weeks of pharmacological manipulation after a complete CKD development reduced GDS and lead to the advantage of endogenous vitamin D [25(OH)D] over serotonin and parathyroid hormone (PTH) in rats treated with LP533401. The imbalance between GDS - 25(OH)D - PTH resulted in the intensified expression of cAMP- responsive element-binding protein (Creb), whereas the expression of myelocytomatosis oncogene (c-Myc) was simultaneously reduced. This lead to disruption of Foxo1- activating transcription factor 4 (Atf4) complex, and decrease in the expression of the major osteogenic markers. The weakening of excessive osteoblastogenesis was associated with better bone mineral status in all rats with CKD, and especially in LP533401-treated animals. In conclusion, the inhibition of GDS synthesis resulted in the mitigation of osteoblastogenesis observed in CKD, which translated into improvement of bone mineral status. This study provides key mechanistic insights into how modification of GDS-dependent molecular pathway affects bone mineral status in CKD and lays the groundwork for translating the role of functional serotonin signaling in the origin of impaired bone mineral status in patients with CKD.

1. Introduction

Mineral and Bone Disorder is a common component in the course of Chronic Kidney Disease (CKD-MBD), which causes a high incidence of skeletal fractures and contributes to the high mortality rates associated with kidney disease [1]. The kidney plays a critical role in the regulation of bone metabolism because it is the major organ controlling calcium and phosphate homeostasis, which are essential elements for bone mineralization and development [2]. As a consequence of CKD, the disturbance of bone turnover develops, being a result of a dysregulation between bone formation by osteoblasts and bone resorption by osteoclasts [3]. Many signaling factors, such as hormones, growth factors and cytokines modulate the activity of osteoblasts and osteoclasts,

and therefore affect the balance between bone formation and resorption [4].

One of the signaling factors that can affect bone metabolism is gut-derived serotonin (GDS) [5], although its role in bone metabolic processes is not entirely clear. Some experimental studies support that serotonin enhance bone formation [6,7], whereas others demonstrate the opposite [8]. Studies in patients treated with antidepressants which increase plasma serotonin levels, revealed increase of bone loss [9,10]. However, the elevated GDS was positively associated with markers of bone formation and resorption in hemodialyzed patients, indicating that possibly this substance increases both osteoblastic and osteoclastic activity in this population [11].

The molecular mechanisms of GDS action on osteoblasts were

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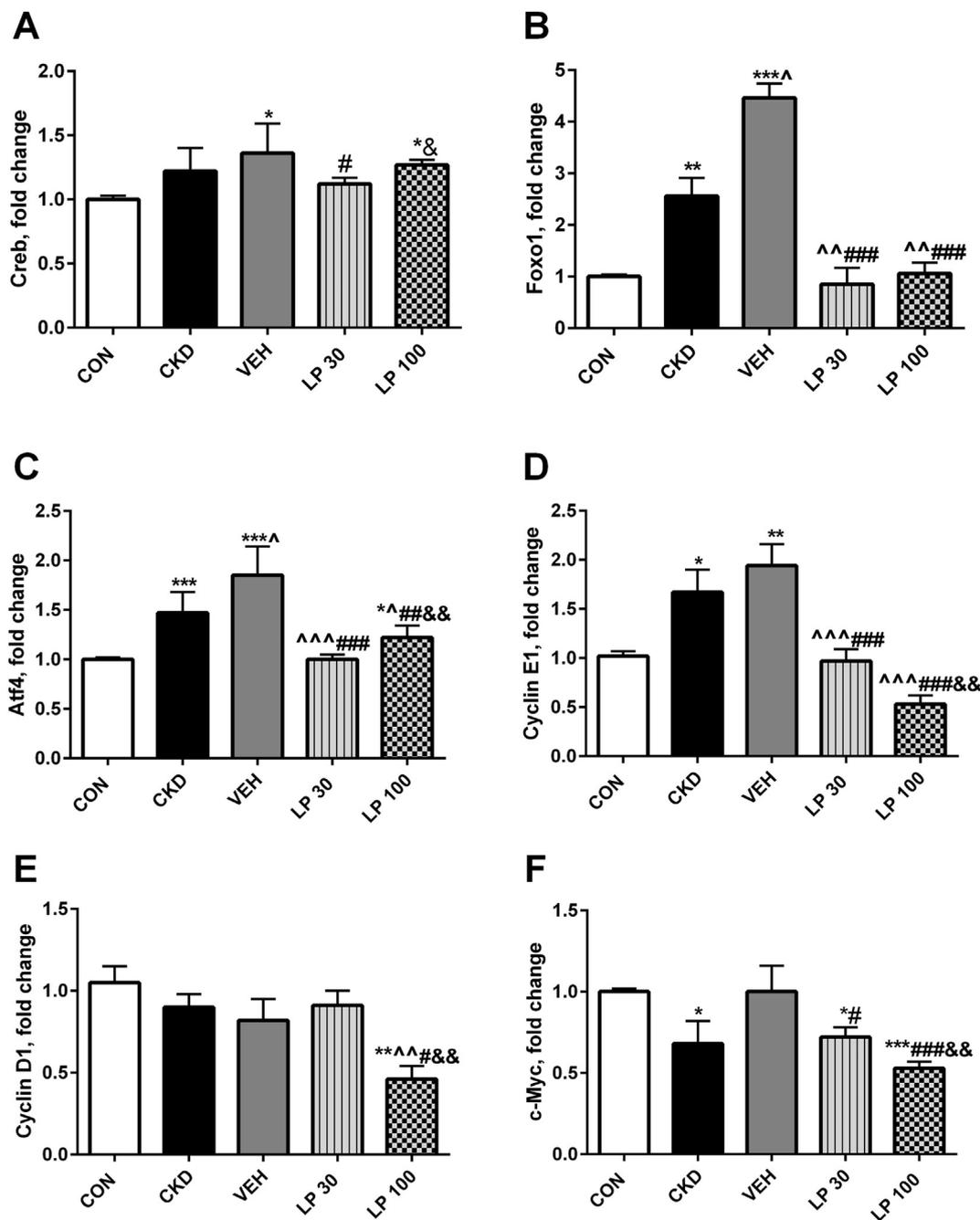


Fig. 1. The effect of LP533401 treatment on GDS-dependent genes and c-Myc mRNA levels in bone of rats with chronic kidney disease (CKD).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ CON versus others; $\hat{p} < 0.05$, $\hat{\sim}p < 0.01$, $\hat{\sim\sim}p < 0.001$ CKD versus others; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ VEH versus others; & $p < 0.05$, && $p < 0.01$ LP 30 versus LP 100; Data are mean \pm SEM.

Abbreviations: *Creb* - cAMP responsive element-binding protein; *Foxo1* - Forkhead box transcription factor 1; *Atf4* - activating transcription factor 4; CON - controls; CKD - chronic kidney disease; LP 30 - LP533401 at the dose of 30 mg/kg; LP 100 - LP533401 at the dose of 100 mg/kg.

explained by Kode et al. [12], who showed that the transcription factor Foxo1 and its interaction with cAMP- responsive element-binding protein (Creb) or activating transcription factor 4 (Atf4) is a crucial determinant of the effects of GDS on bone formation. On the other hand, Yadav et al. [8] demonstrated that serotonin determines the bone formation by controlling osteoblast proliferation through the regulation of Cyclins expression by Creb.

Findings from our previous investigation have demonstrated that increased GDS can influence the integrity and metabolism of bone in experimental model of CKD in rat [13]. Next, we identified a previously unknown molecular pathway, by which elevated GDS can shift in Foxo1 target genes from Creb to Atf4 - dependent response, resulting in

enhanced expression of osteoblast differentiation genes in bone of rats with CKD [14]. Recently, we demonstrated that LP533401, a small molecule inhibitor of tryptophan hydroxylase-1 (Tph-1) - an enzyme responsible for the synthesis of GDS, restores bone health in 5/6 nephrectomized rats by a decrease of GDS and regulation of serum phosphate [15].

The aim of the present study was to determine the impact of LP533401 treatment on GDS-dependent molecular pathway involved in osteogenesis, and potential consequence of this regulation for bone mineral status of uremic rats. Unexpectedly, we discovered that not only GDS, but also disturbances in calciotropic hormones can affect Foxo1-Creb-Atf4 balance, which translated into the process of

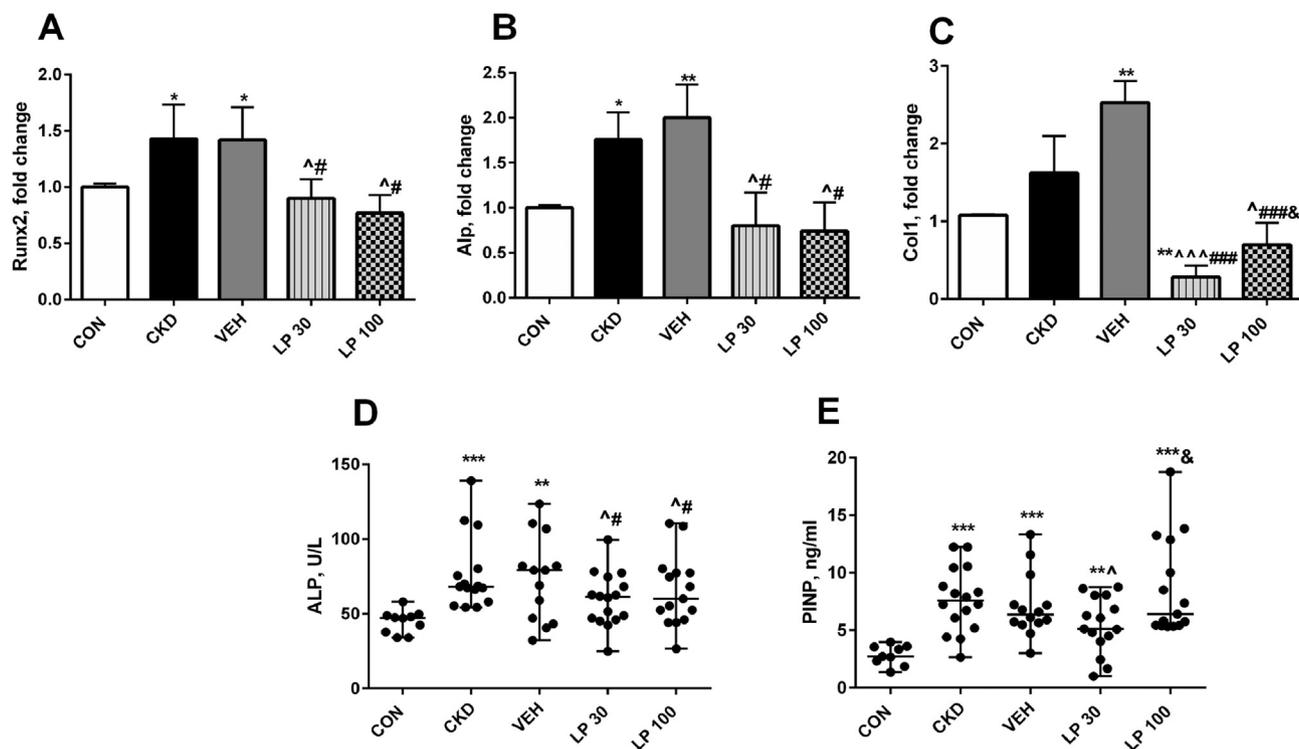


Fig. 2. The expression of early osteoblast differentiation genes and serum levels of their proteins in rats with CKD treated with LP533401.

Panels A–C – the expression of genes in bone. Data presented as mean \pm SEM; Panels D–E – serum levels of bone formation markers. Data presented as median with range.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ CON versus others; ^ $p < 0.05$, ^^ $p < 0.001$ CKD versus others; # $p < 0.05$, ### $p < 0.001$ VEH versus others; & $p < 0.05$ LP 30 versus LP 100.

Abbreviations: *Runx2* – Runt-related transcription factor 2; *Alp* – alkaline phosphatase; *col1* – type I collagen; ALP – alkaline phosphatase; PINP – procollagen type 1 N-terminal propeptide; CON – controls; CKD – chronic kidney disease; LP 30 – LP533401 at the dose of 30 mg/kg; LP 100 – LP533401 at the dose of 100 mg/kg.

osteoblast proliferation/differentiation and bone mineral status.

2. Materials and methods

2.1. Experimental design

Previously, we described in detail the experimental design, applied procedures, national and institutional guidelines for the care and use of animals and sample collection [15,16]. Briefly, 74 four-week old male Wistar rats were subjected to a two-step surgical 5/6 nephrectomy (CKD, $n = 64$) or sham-operation by renal decapsulation (CON, $n = 10$). After 16 weeks of CKD progression, the rats were randomly assigned to 4 subgroups that were administered for the next 8 weeks: only sterile water (CKD, $n = 16$); two doses of LP533401: 30 or 100 mg/kg/per day dissolved in polyethylene glycol and 5% dextrose in the ratio of 40:60 (LP 30, $n = 16$ and LP 100, $n = 15$); and polyethylene glycol with 5% dextrose (40:60 ratio) as the vehicle (VEH, $n = 14$). The reasons for the different animals number in the studied group were previously explained [15].

2.2. Quantitative real-time PCR

Total RNA was isolated from femoral tissue using Thermo Scientific GeneJET RNA Purification Kit (Thermo Scientific, Lithuania). Quantification and quality control of RNA was determined using the Thermo Scientific NanoDrop 2000 spectrophotometer. Total RNA (1 μ g) was reverse transcribed using the RevertAid™ First Stand cDNA Synthesis Kit. Quantitative real-time PCR was performed using the SYBR Green Master Mix (EURx, Poland) as previously described [14]. The comparative CT method ($\Delta\Delta$ CT) was used for relative quantification of gene expression [17]. Sequences of primers used are presented

in Supplementary Table 1. For normalization of expression level, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

2.3. Serum markers of bone formation

Serum osteocalcin and procollagen type 1 N-terminal propeptide (PINP) levels were measured by Rat Osteocalcin ELISA Kit from Immutopics, Inc., San Clemente, CA and Rat/Mouse PINP EIA, purchased from Germany Immunodiagnostic Systems GmbH, Frankfurt, German; respectively. Serum sclerostin assay was performed using Rat (SOST) ELISA kit from Shanghai Sunred Biological Technology Co., Ltd., Shanghai, China. Alkaline phosphatase (ALP) activity was determined by colorimetric test from BioMaxima S.A., Lublin, Poland.

2.4. Bone mineral status measurements

Bone densitometry scans were performed on a Horizon QDR Series X-ray Bone Densitometer (Hologic Inc., Bedford, MA, USA), using the specific small animal software. The bone mineral area, bone mineral content (BMC) and bone mineral density (BMD) of the femur were measured for each animal, as has been previously described in detail [15]. In addition to the whole-femur measurements, the subregional BMC and BMD of small uniform areas were quantified at the distal metaphysis (R1 region), representing mixed trabecular and cortical bone, and midshaft (R2 region), constituted of cortical bone [15].

2.5. Statistical analysis

Shapiro-Wilk's test of normality was used for data distribution analysis. Multiple-group comparisons were performed with one-way

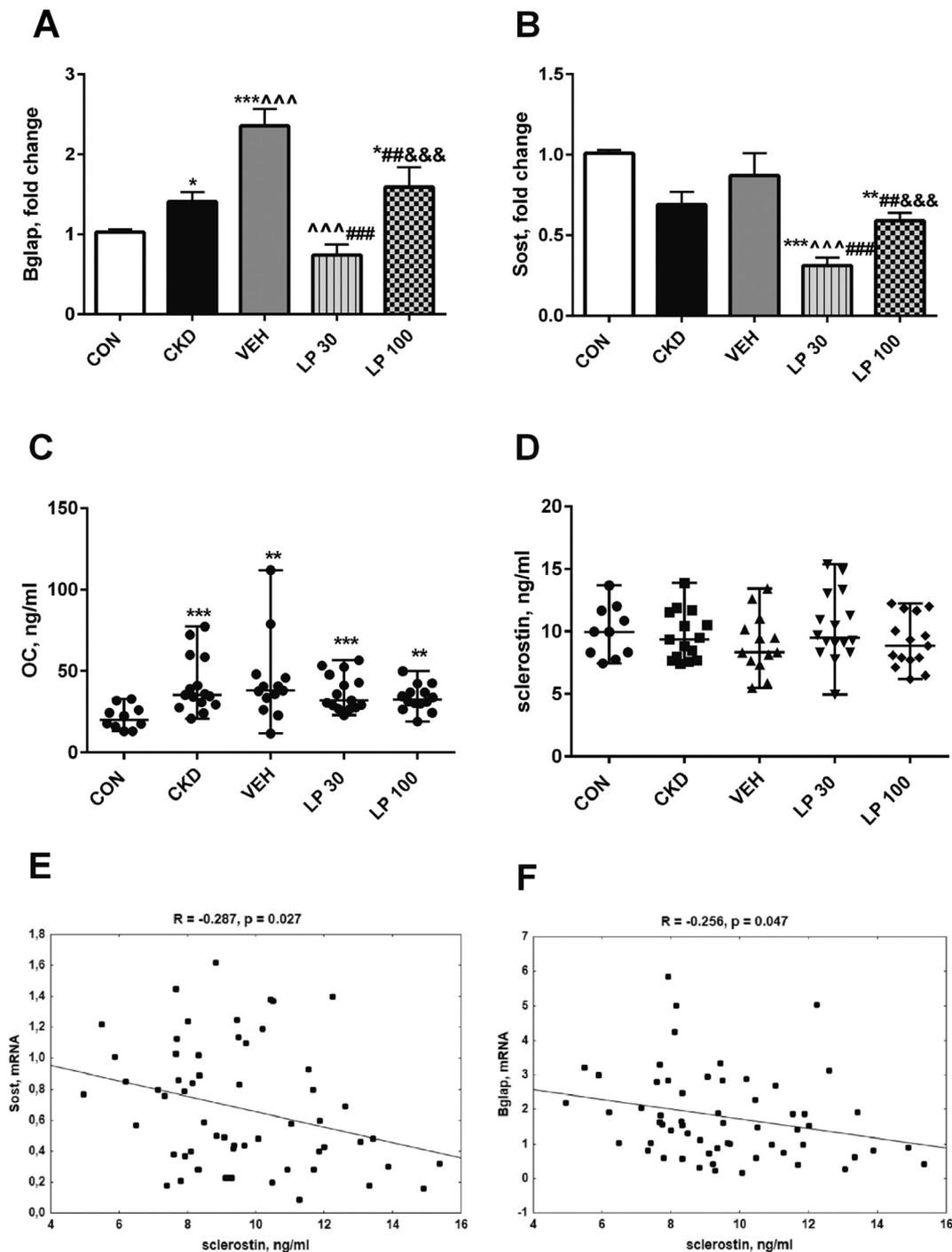


Fig. 3. The markers of the late stage of osteoblasts differentiation and osteocytes in bone and serum of rats with chronic kidney disease (CKD). Panels A–B – the expression of genes in bone. Data presented as mean ± SEM; Panels C–D – serum levels of bone formation markers. Data presented as median with range; Panels E–F – the relationship between serum sclerostin levels and bone expression of *Sost* and *Bglap*.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ CON versus others; ^^^ $p < 0.001$ CKD versus others; ### $p < 0.01$, #### $p < 0.001$ VEH versus others; &&& $p < 0.001$ LP 30 versus LP 100.

Abbreviations: *Bglap* – bone gamma-carboxyglutamate protein; *Sost* – sclerostin; OC – osteocalcin; CON – controls; CKD – chronic kidney disease; LP 30 – LP533401 at the dose of 30 mg/kg; LP 100 – LP533401 at the dose of 100 mg/kg.

analysis of variance (ANOVA), and significant differences between the groups were assessed using Duncan's post-hoc test at $p < 0.05$. Normally distributed data were expressed as mean ± SEM, and non-Gaussian data were presented as median with range. The correlations between study variables were calculated with Spearman's rank correlation analysis. A two-tailed p value of < 0.05 was considered statistically significant. Computations were performed using Statistica ver.10

computer software (StatSoft, Tulsa, OK, USA). Graphic design presentation of the results was performed using GraphPad Prism 6.0 software (USA) or R statistical software (version 3.3.2).

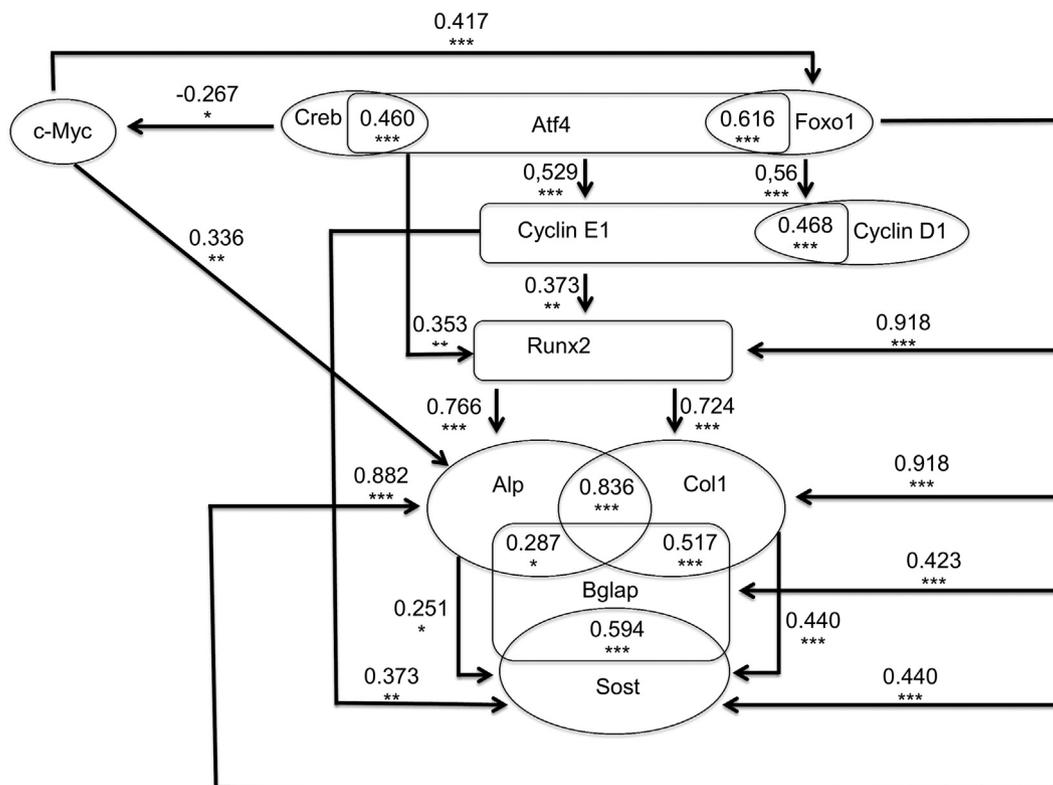


Fig. 4. Associations between the expression of GDS-dependent genes and the mRNA levels of genes participated in bone formation in femurs of all uremic rats. In the whole group of CKD rats, the strong association existed between *Atf4-Creb* and *Atf4-Foxo1*. Both *Atf4* and *Foxo1* gene expression was correlated with *Cyclin E1*, whereas *Atf4*, *Foxo1* and *Cyclin E1* were related to *Runx2* mRNA levels. *Alp* and *Col1* expression was directly regulated by *Runx2*, but also by *Atf4* and particularly by *Foxo1*. *Bglap* and *Sost* expression were under *Alp-Col1* and *Foxo1* control. Interestingly, *Creb* was inversely associated with *c-Myc*, which, in turn, was strongly and positively correlated with *Foxo1*. On the other hand, the positive association between *c-Myc* and *Alp* gene expression as well as between *Cyclin E1* and *Sost* mRNA levels were also observed.

Each value is Spearman's rank correlation coefficient (R) value between the genes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant values; negative value indicates inverse correlation between parameters.

Abbreviations: *Creb* – cAMP responsive element-binding protein; *c-Myc* – myelocytomatosis oncogene; *Foxo1* – Forkhead box transcription factor 1; *Atf4* – activating transcription factor 4; *Runx2* – Runt-related transcription factor 2; *Alp* – alkaline phosphatase; *Col1* – type I collagen; *Bglap* – bone gamma-carboxyglutamate protein; *Sost* – sclerostin.

3. Results

3.1. The effect of LP533401 treatment on the expression of GDS-dependent genes involved in osteoblast proliferation in rats with chronic kidney disease (CKD)

Previously, we showed the reduced turnover of serotonin in the whole group of rats treated with LP533401, however only LP533401 at the dose of 100 mg/kg (LP 100) was able to decrease intestinal and serum serotonin levels to the values observed in CON [15]. As has been shown in Fig. 1A, CKD rats treated with VEH and LP 100 had the highest *Creb* gene expression. The mRNA levels of *Foxo1*, *Atf4* and *Cyclin E1* were lower in animals treated with LP than those in CKD and VEH (Fig. 1B–D). Moreover, the expression of *Cyclin D1* gene was significantly reduced in LP100 group compared to others group with CKD and CON (Fig. 1E). Myelocytomatosis oncogene (*c-Myc*) is a multifunctional transcription factor that is involved in cell cycle, apoptosis and cellular differentiation [18], and previously we documented its effect on *Cyclins* expression in bone of animals with CKD [14]. In the present study, we observed gradually reduction in *c-Myc* mRNA levels in animals treated with LP, especially in group LP 100 (Fig. 1F) paralleled with diminished *Cyclin D1* expression in this group.

3.2. The impact of LP533401 treatment on bone formation markers in rats with chronic kidney disease (CKD)

The expression of key genes regulating the development of osteoblast from preosteoblasts stage to osteocytes and the serum level of bone formation markers were measured in studied groups. The expression of the early osteoblast differentiation genes: Runt-related transcription factor 2 (*Runx2*), alkaline phosphatase (*Alp*), type I collagen (*Col1*) were higher in uremic rats (CKD, VEH) than in CON, however their expression in rats treated with LP was significantly reduced compared to other uremic groups (Fig. 2A–C). These changes in bone formation markers expression corresponded with serum activity of alkaline phosphatase (ALP) and procollagen type 1 N-terminal propeptide (PINP) concentrations (Fig. 2D–E) – the recognized markers of osteoblast function in CKD [19]. The similar pattern of *Bglap* expression, the marker of the late stage of osteoblast differentiation, and serum osteocalcin (OC) concentrations was observed in the studied groups (Fig. 3A and C). Despite the intensified expression of proliferation/differentiation genes, the expression of sclerostin (*Sost*) – the marker of osteoblast transition to osteocyte [20] was slightly decreased in CKD and VEH group compared to CON; LP treatment significantly intensified this reduction, especially in the LP 30 group (Fig. 3B). Serum sclerostin levels were similar in all studied groups (Fig. 3D), whereas the inverse association was observed between this protein concentration and both *Sost* and *Bglap* mRNA levels (Fig. 3E–F). These results showed that osteoblast proliferation and differentiation was intensified

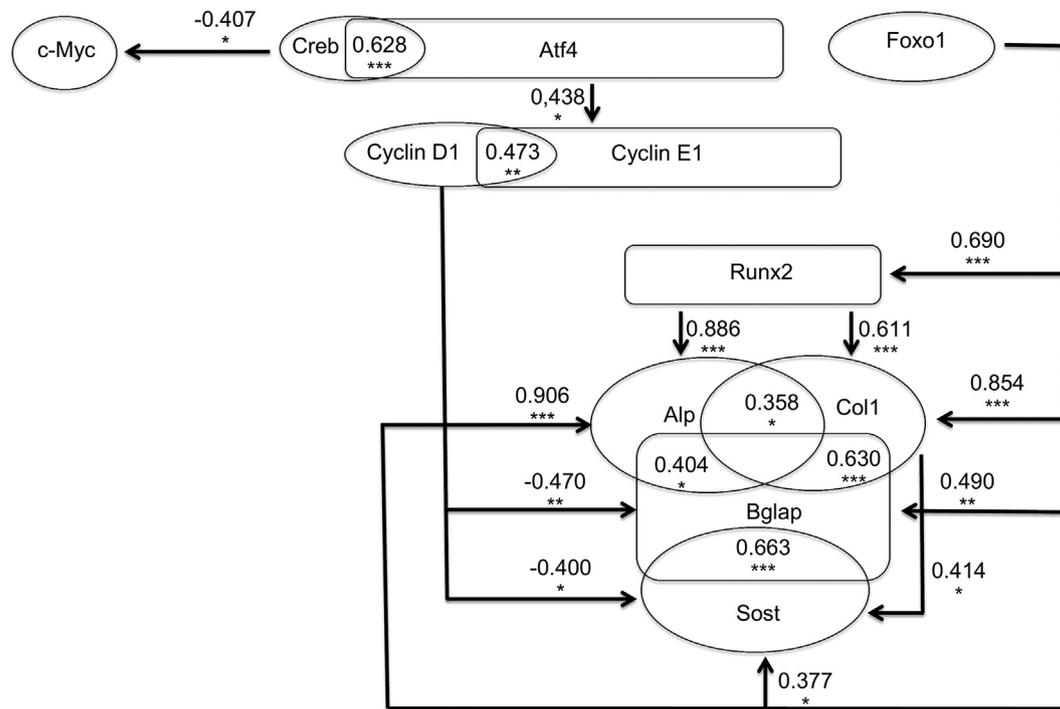


Fig. 5. Associations between the expression of GDS-dependent genes and the mRNA levels of genes participated in bone formation in femurs of uremic rats treated with LP533401.

Under conditions of reduced GDS and elevated 25(OH)D, the intensification in *Creb* expression, which was associated with reduction of *c-Myc* mRNA occurred in CKD rats treated with LP533401. In these animals, the relationship between *Atf4* and *Foxo1* was disrupted ($R = 0.339$, $p = 0.062$); while there was a weak dependence between *Atf4* and *Cyclin E1* expression. The attenuated *Foxo1* expression strongly affect *Runx2*, *col1*, *Alp*, *Bglap* and *Sost* mRNA levels. In contrast, a relationship between *c-Myc* and *Alp* gene expression was not observed in these conditions ($R = 0.321$, $p = 0.078$). Interestingly, *Cyclin D1* expression inversely and directly correlated both with *Bglap* as well as *Sost* mRNA levels in this group.

Each value is Spearman's rank correlation coefficient (R) value between the genes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant values; negative value indicates inverse correlation between parameters.

Abbreviations: *Creb* – cAMP responsive element-binding protein; *c-Myc* – myelocytomatosis oncogene; *Foxo1* – Forkhead box transcription factor 1; *Atf4* – activating transcription factor 4; *Runx2* – Runt-related transcription factor 2; *Alp* – alkaline phosphatase; *Col1* – type I collagen; *Bglap* – bone gamma-carboxyglutamate protein; *Sost* – sclerostin.

in CKD, and this is in line with our previous observation performed on young nephrectomized rats [14]. However, the inhibition of GDS synthesis by LP could diminished this process.

3.3. Mechanistic relationship between GDS-dependent genes and regulation of genes involved in bone formation

The relationships between the expression of GDS-dependent genes: *Creb*, *Foxo1*, *Atf4*, *Cyclins* and genes involved in bone formation process were analyzed both in the whole group of rats with CKD (Fig. 4) and separately in the group treated with LP533401 (Fig. 5). Collectively, data obtained from all uremic rats showed the existence of strong association between *Creb-Atf4-Foxo1* genes expression, whereas the expression of *Cyclin E1*, *Runx2* and other genes participated in osteoblasts differentiation and their transition into osteocytes were firmly dependent on the *Atf4-Foxo1* complex. Moreover, *c-Myc* expression was highly and positively related both to *Foxo1* and *Alp* mRNA levels (Fig. 4). Under conditions of intensified *Creb* and reduced *c-Myc* expression in rats treated with LP, the association between *Atf4* and *Foxo1* was disrupted. As a result, *Atf4* was associated only with *Cyclin E1* expression, whereas *Foxo1* strongly impact the expression of bone formation genes. Moreover, *Cyclin D1* expression was inversely related both to *Bglap* and *Sost* mRNA levels in LP treated animals (Fig. 5). These data suggest that the imbalance between *c-Myc* – *Creb* expression is originally responsible for reduced osteoblast proliferation/differentiation in rats with CKD treated with LP. However, diminished osteoblasts proliferation seems to be positively associated with terminally

differentiated osteoblast, which express osteocalcin and osteocytes – the most abundant cells in bone [21].

3.4. The effect of GDS and dysregulation of calciotropic hormones on *Creb* and *c-Myc* gene expression in rats with chronic kidney disease (CKD) treated with LP533401

Previously, we noticed that treatment with LP caused disturbances in calciotropic hormones and their interrelationships, which were further complicated by a reduction in serotonin levels [15]. In the current study, the decrease in GDS was inversely associated with *Creb* gene expression (Fig. 6A), whereas 5-HT had a tendency to positive correlation with *c-Myc* mRNA (Fig. 6B). Interestingly, 25-hydroxyvitamin D [25(OH)D] levels, and particularly 25(OH)D/5-HT ratio were positively associated with *Creb* (Fig. 6C and E), whereas they were strongly and inversely correlated with *c-Myc* expression (Fig. 6D and F). In contrast, a tendency to inverse association was noticed between serum parathyroid hormone (PTH) concentration and *Creb* expression ($R = -0.310$, $P = 0.088$), and a slight positive relation was observed between PTH and *c-Myc* mRNA levels ($R = 0.357$, $P = 0.048$). We also observed that the untreated animals with CKD had significantly lower 25(OH)D/PTH ratio than CON, and the administration of VEH and LP 30 further intensified this reduction. The treatment with LP100 was able to increase the values of this parameter to those observed in CKD, although they still remained lower than in healthy animals (Fig. 7). Moreover, the 25(OH)D/PTH ratio positively correlated with *Creb* and negatively with *c-Myc* (Fig. 6G–H). These data indicated that not only

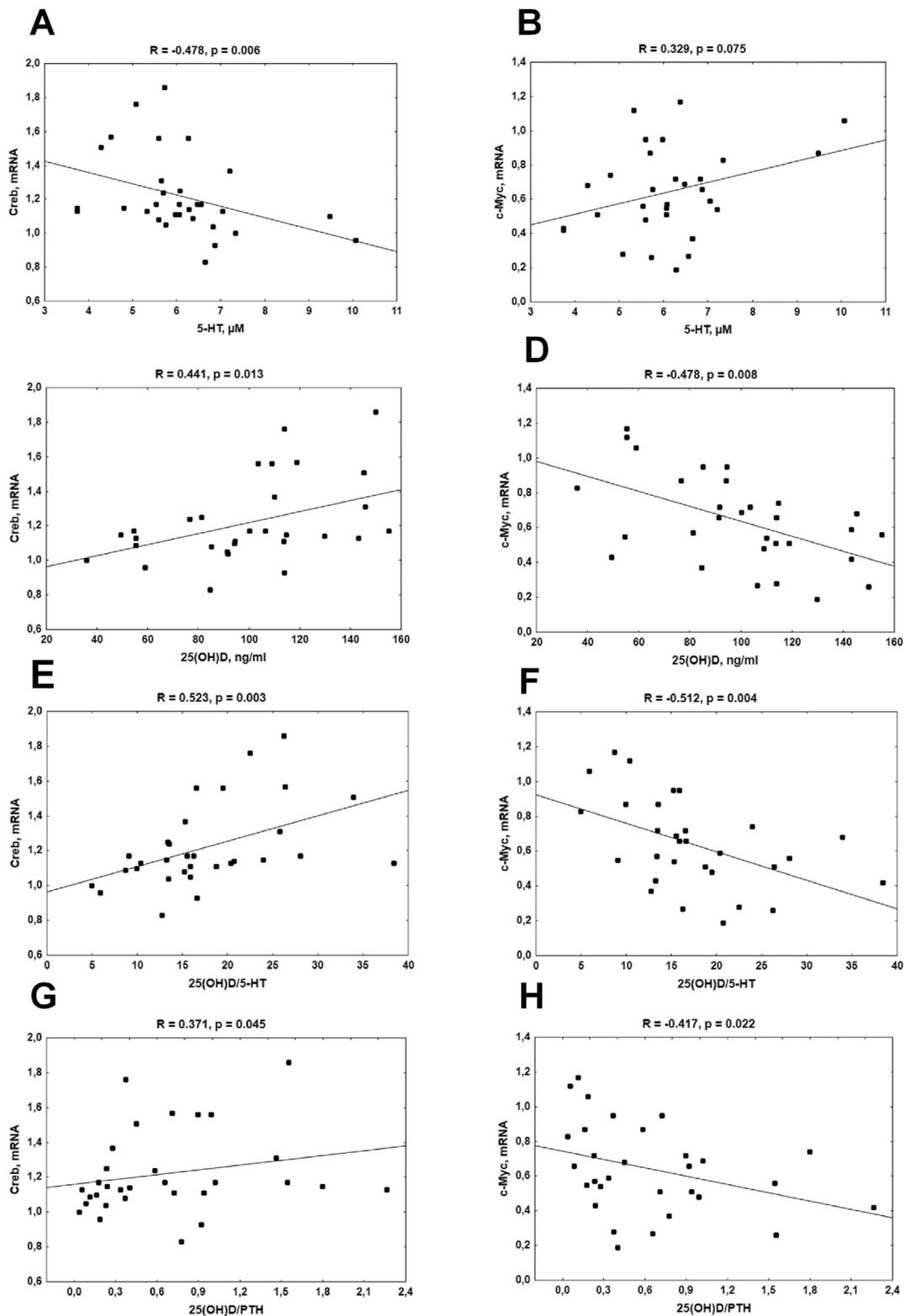


Fig. 6. The effect of gut-derived serotonin and disorders of calcitropic hormones on the regulation of *Creb* and *c-Myc* gene expression in rats with chronic kidney disease (CKD) treated with LP533401.

Abbreviations: *Creb* – cAMP responsive element-binding protein; *c-Myc* – myelocytomatosis oncogene; 5-HT – serotonin; 25(OH)D – 25-hydroxyvitamin D; PTH – parathyroid hormone.

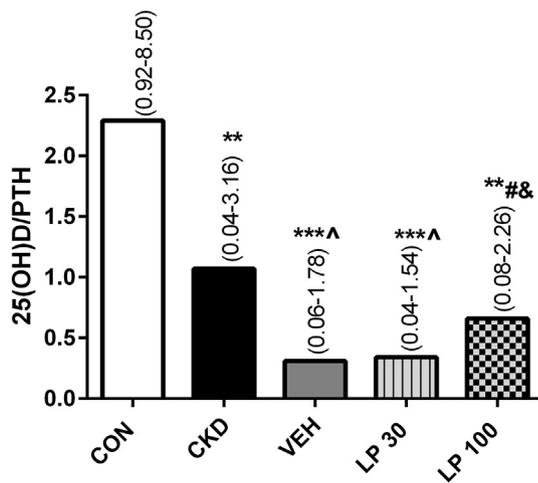


Fig. 7. The effect of LP533401 treatment on 25-hydroxyvitamin D to parathyroid hormone ratio [25(OH)D/PTH] in studied groups.

GDS, but also interrelationships between GDS and calciotropic hormones, especially 25(OH)D, can affect regulation of GDS-dependent genes in bone of rats treated with LP.

3.5. The improvement of bone mineral status in uremic rats treated with LP533401 was associated with reduced of Cyclin E1 and Sost gene expression

The treatment of uremic animals with LP533401 restores their femoral mineral status to the values observed in CON; this effect was particularly evident in the metaphyseal region R1, which contains more trabecular bone than diaphyseal region R2 [15]. To explain the potential impact of GDS-dependent genes as well as genes involving in bone formation on bone mineral status, we analyzed the correlations between the expression of these genes and the values of bone mineral area, bone mineral content (BMC) and bone mineral density (BMD) in the whole group of uremic rats and in rats treated with LP. Among the analyzed genes, the expression of *Cyclin E1* and *Sost* was inversely related to the studied parameters of bone mineral status in all uremic rats. The above relationships were especially visible in trabecular R1 region of the femur (Fig. 8). In animals treated with LP, *Cyclin E1* and *Sost* mRNA levels were also inversely related to bone mineral area (Fig. 9A). Moreover, the inverse relationships were noted between trabecular BMD and bone *Cyclins* expression (Fig. 9, B-C). This observation implies that mitigated osteoblastic cells proliferation/differentiation in uremic rats treated with LP can result in better bone mineral status.

4. Discussion

In the present study, we demonstrated that inhibition of GDS production by an inhibitor of Tph-1 – LP533401 (LP) was associated with the changes in GDS-dependent molecular pathway involved in osteoblast/osteocyte formation and bone mineral status. Moreover, we showed for the first time that the disturbance in the sophisticated balance between serotonin and calciotropic hormones, mainly 25-hydroxyvitamin D [25(OH)D] and parathyroid hormone (PTH), can impact GDS-dependent genes in bone of rats treated with LP, modifying osteoblast proliferation/differentiation process and bone mineral status.

The interaction between Foxo1 and Creb or Atf4 gene expression in bone was recognized as a crucial determinant of GDS-dependent effect on bone formation [12]. Foxo1 plays a pivotal role in the regulation of osteoblast function - it affects several key functions such as the transcriptional control of the major osteogenic markers: Runx2, Alp and type I collagen [12,22]. In the condition of high GDS in CKD and VEH groups [15], the expression of Foxo1, Atf4, Cyclin E1 and the osteoblast

differentiation markers were intensified (Figs. 1–3) and inter-dependent (Fig. 4). This is in agreement with the observation of Kode et al. [12] that serotonin can inhibit the interaction of Foxo1 with Creb, whereas it may increase the interaction of Foxo1 with Atf4. The similar associations between Atf4 - Foxo1- Cyclins expression in bone were noticed previously by us in young rats with CKD [14]. In contrast, animals treated with LP, which had lower GDS concentration than other studied groups with CKD [15], showed significantly lower expression of Foxo1, Atf4, Cyclins and osteoblast differentiation markers, whereas Creb mRNA levels in their bones was elevated. Interestingly, in this group the positive association between Creb-Atf4 maintained, but relationship between Foxo1 and Atf4 as well as Cyclin E1 disappeared (Fig. 5). The cyclins are important cell cycle regulatory factors in proliferating/differentiating osteoblasts [23,24], and their expression can be regulated by myelocytomatosis oncogene (c-Myc) [25]. In proliferating cells, c-Myc mRNA is expressed in all phases of the cell cycle [26], while a decrease in c-Myc mRNA synthesis is observed when cells are growth-arrested [27]. Previously, we showed that elevated Cyclins expression in bone of rats with CKD was related to the osteoblast differentiation markers and remained under Atf4 and c-Myc gene control [14]. As has been showed in Fig. 1F, c-Myc mRNA gradually decreased in animals treated with LP. Simultaneously, we noticed that c-Myc was positively related to Foxo1 and Alp expression in the whole group of rats with CKD, whereas it was inversely related to Creb mRNA levels (Fig. 4). In LP-treated group, this last relation was only visible (Fig. 5). These results indicated that LP treatment, through the reduction of GDS and intensification of Creb expression, could negatively impact of c-Myc, resulting in disruption of c-Myc – Foxo1 and c-Myc – Alp inter-relationship. Because the Atf4 – Foxo1 complex strongly participated in up-regulation of osteoblast differentiation markers in all rats with CKD (Fig. 4), the physiological consequence of Atf4 – Foxo1 disruption in animals treated with LP was decrease in the mRNA levels of the major osteogenic markers, paralleled with the reduction of their serum activity or protein levels (Figs. 2 and 3). This is in the line with recently published results [22], showing the inhibited expression of osteogenic phenotypic markers and reduced osteoblast differentiation in osteoblast-specific Foxo1 knockout mice model. In this context, our results provided the mechanistic explanation of the previous clinical study performed on patients with CKD, in which the number of proliferating cells and the expression of osteoblast markers are increased in primary human osteoblast-like cells obtained from dialysis patients when compared to healthy subjects [28].

The development of osteoblasts is regulated by different genes and characterized by stages from preosteoblasts to osteocytes [29]. Sclerostin is almost exclusively produced by osteocytes in the bone [29], and with the progression of CKD its levels in serum increase, in spite of an increased renal elimination [30]. Despite the accelerated expression of osteoblast proliferation/differentiation markers and the strong associations between them and Bglap (Figs. 3–5), *Sost* gene expression was not intensified in uremic animals compared to CON, and was especially low in LP-treated group compared to other studied groups. These data are complementary to the observation of Pereira et al. [28], who also showed the increase of osteoblastic markers occurred simultaneously with low osteocytic gene expression in cells obtained from dialyzed patients. These authors thought that the osteocytes do not migrate/or survive in the conditions of CKD. Interestingly, the inverse association has been observed by us between serum sclerostin levels and *Sost* as well as Bglap expression in uremic rats (Fig. 3E-F). This is in agreement with observation of Atkins et al. [31] that sclerostin was targeting late osteoblasts/preosteocytes and arresting them at the preosteocyte stage, blocking or retarding their differentiation into mature osteocyte-like cells. However, it is worth noting that in the whole uremic group, the proliferation/differentiation marker – Cyclin E1 was positively associated with *Sost* expression (Fig. 4), whereas in LP-treated rats Cyclin D1 was inversely related to both Bglap and *Sost* mRNA (Fig. 5). Because down-regulation of proliferation is necessary for transcription of genes

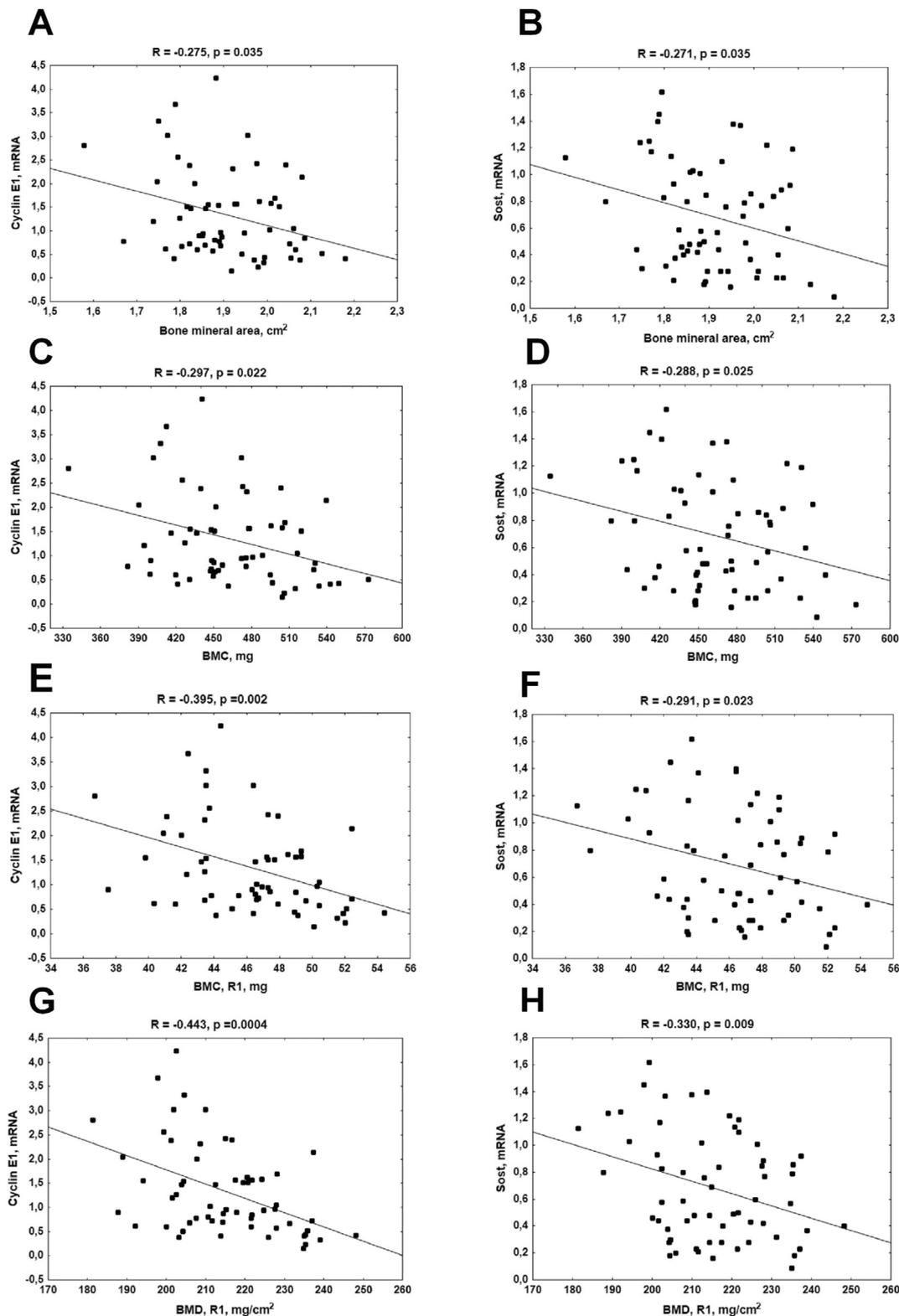


Fig. 8. Associations between *Cyclin E1*, *Sost* gene expression and bone mineral status of all rats with chronic kidney disease. Abbreviations: *Sost* – sclerostin; BMC – bone mineral content; BMD – bone mineral density; R1 – the region of trabecular bone.

functionally related to expression of the mature osteoblast phenotype [32], these results suggested that treatment of uremic rats with LP resulted in the sequential and mutually exclusive expression of genes involved in osteoblast growth, differentiation and maturation. In contrast, the coexpression of genes involved in the above mentioned processes has been observed in the whole uremic group, indicated that the

normal temporal sequence of osteoblast/osteocyte phenotype genes expression was not preserved in CKD, and particularly in VEH.

In the previous studies [15,16], we noticed that the administration of VEH or LP caused the disturbance in the calciotropic hormones and their interrelationships, which can impact bone mineral status. Generally, PTH levels were the highest in VEH compared to other groups

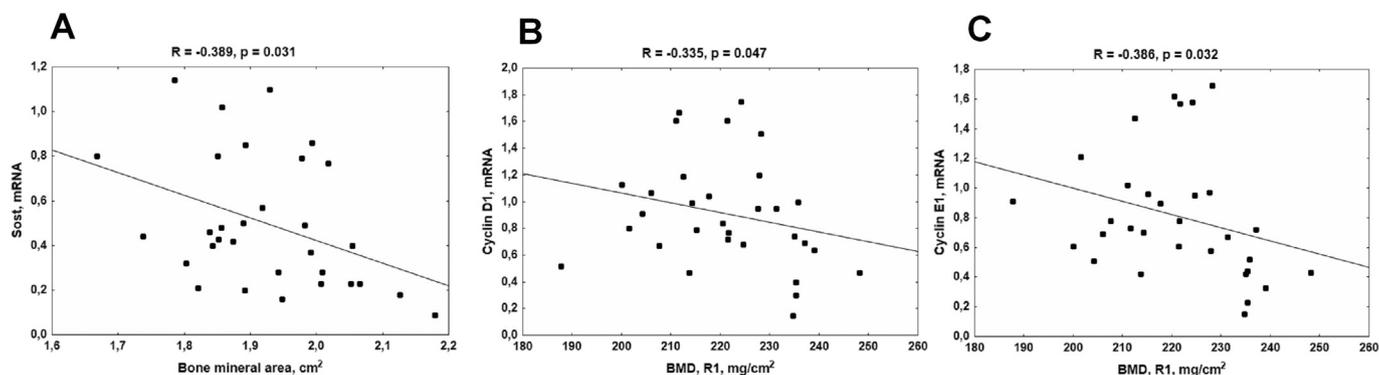


Fig. 9. Associations between bone *Sost*, Cyclins mRNA levels and bone mineral status of rats with chronic kidney disease (CKD) treated with LP533401. Abbreviations: *Sost* – sclerostin; BMD – bone mineral density; R1 – the region of trabecular bone.

[15], whereas 25(OH)D concentration was the most elevated in animals treated with LP 100 [16]. Both peripheral serotonin and mutual interactions between calcitropic hormones and 5-HT significantly affected the expression of *Creb* and *c-Myc* in LP-treated group (Fig. 6). These results indicated for the first time that not only GDS, but also the disturbed balance between GDS and calcitropic hormones, particularly 25(OH)D, can translate into expression of genes involved in osteoblastogenesis in this model. This new finding is in agreement with the study of Eelen et al. [33], who showed that vitamin D may be a potent inhibitor of osteoblastic cell cycle progression via downregulation of *c-Myc*. The clinical observation of Milinković et al. [34] also confirmed the inverse association between serum 25(OH)D and ALP activity in hemodialyzed patients.

Recently, we showed that LP treatment was able to reverse the CKD-induced decline in bone mineral status [15]. Thus, the second aim of the present study was to establish the potential consequence of GDS-dependent genes regulation for bone mineral status of uremic rats. As evidenced from our results, *Cyclin E1* and *Sost* turned out to be the only of the studied genes, the expression of which was inversely related to bone mineral status of uremic rats, particularly in trabecular rich bone region R1 (Fig. 8). To the best of our knowledge, there are only 2 studies currently available in the literature linking *Sost* gene deletion with renal osteodystrophy. Both Cejka et al. [35] and Kaesler et al. [36] found that renal osteodystrophy was more pronounced in wild type mice than in *Sost* – knockout mice, and our results are compatible with these observations. Moreover, our data demonstrated that the disturbance in the interrelationships between GDS and calcitropic hormones may ultimately translate into bone mineral status. This was especially seen in LP-treated group, where both *Sost* and *Cyclins* mRNA levels impacted bone mineral area and trabecular BMD values (Fig. 9). Consistent with cyclins role in cell cycle progression [23–25], the inverse relationship between their expression and bone mineral status could explain the phenomenon described previously by Pereira et al. [28], in which osteoblastic cells from patients with renal osteodystrophy proliferated more rapidly but mineralized more slowly than cells from healthy controls.

A limitation of the present study, besides its cross-sectional design, is the translation of rodent data to CKD patients. Renal osteodystrophy includes a wide spectrum of disturbances, which can affect bone structure. The study mainly focuses on GDS-dependent gene pathway involving in bone formation and mineral status, but obtained results indicated that the relative importance of an individual mechanism in CKD-MBD correction cannot be easily separated, due to interactions occurring between multiple mechanisms. Another limitation is that although osteoblastic and osteoclastic function are inter-dependent and the experiments were performed in vivo condition, the current study focuses only on the impact of GDS on osteoblastogenesis, but does not take into account the effect of this amine on osteoclastogenesis.

In conclusion, the inhibition of GDS synthesis, associated with the

disturbance of calcitropic hormones during LP533401 treatment, had a functional effect on serotonin-dependent signaling in bone of rats with CKD. The imbalance in *c-Myc* – *Creb* gene expression observed in animals treated with LP533401 induced *Atf4* – *Foxo1* complex disruption, resulting in the temporal sequence of osteoblast/osteocyte phenotype genes expression, and the improvement of bone mineral status in these rats. Our results are in line with clinical observation of Pereira et al. [28], and seem to explain previously undiscovered mechanism responsible for the intensified proliferation, but impaired mineralization of osteoblastic cells from CKD patients. Because both circulating factors and those intrinsic to osteoblasts themselves contribute to differences in osteoblast characteristics in patients with CKD [28,37], this study provided an additional molecular link, which may greatly improve understanding of the origins of impaired bone health in patients with renal osteodystrophy.

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Transparency document

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Authors' roles

Study design: DP and KP. Study conduct: TD, BS. Data collection: MJ. Data analysis: KP, TD and BS. Data interpretation: DP, KP, TD, BS, and MJ. Drafting manuscript: DP and KP. Revising manuscript content: TD, BS and MJ. Approving final version of manuscript: DP, KP, TD, BS and MJ. KP takes responsibility for the integrity of the data analysis.

Declaration of competing interest

All authors state that they have no conflicts of interest.

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