High dickkopf-1 levels in sera and leukocytes from children with 21-hydroxylase deficiency on chronic glucocorticoid treatment

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Submitted 31 October 2012; accepted in final form 3 January 2013

Children with 21-hydroxylase deficiency (21-OHD), the most frequent and severe form of drug-induced osteoporosis. In this study, we enrolled 18 patients (9 females) and 18 sex- and age-matched controls. We found in 21-OHD patients high serum and leukocyte levels of dickkopf-1 (DKK1), a secreted antagonist of the Wnt/β-catenin signaling pathway known to be a key regulator of bone mass. In particular, we demonstrated by flow cytometry, confocal microscopy, and real-time PCR that monocytes, T lymphocytes, and neutrophils from patients expressed high levels of DKK1, which may be related to the cGC therapy. In fact, we showed that dexamethasone treatment markedly induced the expression of DKK1 in a dose- and time-dependent manner in leukocytes. The serum from patients containing elevated levels of DKK1 can directly inhibit in vitro osteoblast differentiation and receptor activator of NF-κB ligand (RANKL) expression. We also found a correlation between both DKK1 and RANKL or COOH-terminal telopeptides of type I collagen (CTX) serum levels in 21-OHD patients on cGC treatment. Our data indicated that DKK1, produced by leukocytes, may contribute to the alteration of bone remodeling in 21-OHD patients on cGC treatment.

dickkopf-1; 21-hydroxylase deficiency patients; glucocorticoid-induced osteoporosis; leukocytes; glucocorticoids

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Children with 21-hydroxylase deficiency (21-OHD), the most common cause of congenital adrenal hyperplasia (CAH) due to deletions or mutations of the P450 21-hydroxylase gene (CYP21), need chronic glucocorticoid (cGC) therapy as soon as the diagnosis has been made to replace congenital deficit of cortisol synthesis, and this therapy is the most frequent and severe form of drug-induced osteoporosis. In this study, we enrolled 18 patients (9 females) and 18 sex- and age-matched controls. We found in 21-OHD patients high serum and leukocyte levels of dickkopf-1 (DKK1), a secreted antagonist of the Wnt/β-catenin signaling pathway known to be a key regulator of bone mass. In particular, we demonstrated by flow cytometry, confocal microscopy, and real-time PCR that monocytes, T lymphocytes, and neutrophils from patients expressed high levels of DKK1, which may be related to the cGC therapy. In fact, we showed that dexamethasone treatment markedly induced the expression of DKK1 in a dose- and time-dependent manner in leukocytes. The serum from patients containing elevated levels of DKK1 can directly inhibit in vitro osteoblast differentiation and receptor activator of NF-κB ligand (RANKL) expression. We also found a correlation between both DKK1 and RANKL or COOH-terminal telopeptides of type I collagen (CTX) serum levels in 21-OHD patients on cGC treatment. Our data indicated that DKK1, produced by leukocytes, may contribute to the alteration of bone remodeling in 21-OHD patients on cGC treatment.

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Recently, DKK1 has been described as a central molecular player in bone loss-associated diseases such as multiple myeloma (50), rheumatoid (52), and psoriatic arthritis (7) as well as Paget’s disease (28).

Thus, in the present study, we evaluated the DKK1 serum levels and the intracellular expression on leucocytes obtained from patients affected with 21-OHD, as well as the effects of dexamethasone on the expression of DKK1 in human leucocytes from healthy donors in vitro. Moreover, we examined the effects of the conditioned media by the serum of the patients on OB differentiation and RANKL expression. In the same patients, we also evaluated the BMD and the biochemical markers of bone turnover.

MATERIALS AND METHODS

Subjects

The samples included peripheral blood (PB) from 18 Caucasian patients (9 females) affected by 21-OHD patients aged 3–16 yr. Diagnosis was made on the basis of clinical evidence, basal serum concentrations, and peaks of 17α-hydroxyprogesterone after adrenocorticotropic test. Molecular analysis of the P450 21-hydroxylase gene (CYP21) gene was performed in all patients and in their parents. Subjects with risk factors for reduced bone mass (familial osteoporosis, prematurity, delayed puberty) were excluded from the study. The main characteristics of the patients and the dose of hydrocortisone at the time of the study consisted of hydrocortisone therapy beginning at early infancy, whereas the patients with the SV form were treated with GCs alone. Treatment at the time of the study consisted of hydrocortisone, expressed as dose per body surface per day (mg·m²·day⁻¹). The mean dose of GCs calculated over 4.3 yr because of advanced bone age or hirsutism. Four patients (1 female) had the classical salt-wasting (SW) form, and four subjects (4 females) had the simple virilizing (SV) form. Patients with the SW form received GCs and mineralcorticoid (9α-fludrocortisone: 0.1–0.2 mg/day) therapy beginning at the age of 7.6 ± 4.3 yr because of advanced bone age or hirsutism. Four patients (1 female) had the classical salt-wasting (SW) form, and four subjects (4 females) had the simple virilizing (SV) form. Patients with the SW form received GCs and mineralcorticoid (9α-fludrocortisone: 0.1–0.2 mg/day) therapy beginning at early infancy, whereas the patients with the SV form were treated with GCs alone. Treatment at the time of the study consisted of hydrocortisone, expressed as dose per body surface per day (mg·m²·day⁻¹), given twice or three times daily. The four patients with SW form received a total hydrocortisone dose of 25 mg·m²·day⁻¹. The patients with the SV and NC forms were treated with 10–15 mg·m²·day⁻¹ hydrocortisone. The mean dose of GCs calculated over the 5 yr preceding the investigation was 17.53 ± 4.49 mg·m²·day⁻¹. The hormonal control was established, with the serum concentrations of 17α-hydroxyprogesterone, Δ4-androstenedione, and testosterone evaluated from the patients’ records in the preceding 5 yr (43).

For control group, we studied 18 controls aged 3–16 yr (11 ± 5.9 yr) recruited from the same geographic area. All subjects were healthy, and none were involved in competitive sport activities. Candidates were excluded if they had a history of chronic illness, one or more fractures, or taken any medication, hormone, vitamin preparation, or calcium supplements regularly.

The study was approved by the Ethics Committee of the University of Bari Medical School, Bari, Italy, and the informed consent was obtained from all subjects or from their parents. The study was made in accordance with the principles of the Declaration of Helsinki.

Bone Mineral Measurements

BMD of 21-OHD patients and controls was measured at the proximal femur and lumbar spine (L2–L4), using dual-energy X-ray absorptiometry (ACN Unigamma X-Ray Plus; L’ACN Scientific Laboratories), and converted to SD scores (z-scores) in relation to age- and sex-matched normal population. Calcium and phosphate were also measured in the patients and controls.

Cells and Culture Condition

CD14⁺ and CD2⁺ cell and neutrophil isolation. PB samples obtained from controls or 21-OHD patients were subjected to Histopaque 1077 density gradient (Sigma-Aldrich, St. Louis, MO) centrifugation. The obtained buffy coat cell fraction was submitted to CD14⁺ monocyte and CD2⁺ T lymphocyte isolation using anti-CD14 and anti-CD2 antibody (Ab)-coated immunomagnetic Dynabeads (DynaL, Lake Success, NY), respectively. Only samples with a purity of >97%, checked by flow cytometry, were used. After Ficoll-Paque centrifugation, neutrophils were separated from erythrocytes by 3% dextran (GE Healthcare) density gradient sedimentation. Purity, determined by flow cytometry analysis on forward scatter/side scatter parameters, was routinely >98%. Freshly isolated CD12⁺ and CD14⁺ cells and neutrophils were plated for confocal immunostaining or subjected to RNA extraction.

Leukocyte stimulation. To stimulate DKK1 synthesis, 200 μl of heparinized PB from healthy donors was either nonstimulated or

Table 1. Main clinical and hormonal characteristics of patients with 21-OHD

<table>
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<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age, yr</th>
<th>Weight, kg</th>
<th>Height, cm</th>
<th>BMI, kg/m²</th>
<th>Clinical Form</th>
<th>17α-OHP, ng/ml*</th>
<th>Δ4-A, ng/ml*</th>
<th>Testosterone, ng/ml*</th>
<th>Dose of Hydrocortisone, mg/m²</th>
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21-OHD, 21-hydroxylase deficiency; M, male; F, female; SW, salt wasting; NC, nonclassical; SV, simple virilizing; *Mean value of the preceding 5 yr (except for patient no. 17).
stabilized with different concentrations of dexamethasone (ranging from $10^{-7}$ to $10^{-9}$ M) for 3 and 6 h. At the indicated times, the samples were processed for the DKK1 intracellular staining by flow cytometry analysis.

**Human osteoblasts.** Clonetics Normal Human Osteoblasts (Lonza Walkersville, Walkersville, MD) were plated in six-well plates at a density of $10 \times 10^5$/well, using a medium composed of a-minimal essential medium supplemented with 20 ng/ml bone morphogenetic protein 2 (BMP2; R & D Systems, Minneapolis, MN) and 10% fetal calf serum (FCS; Gibco Life Technologies, Milan, Italy) or human serum obtained from controls or 21-OHD patients in the presence or absence of 5 µg/ml anti-DKK1 monoclonal Ab (mAb) (R & D Systems) or an anti-IgG Ab. After 48 h of culture, OBs were subjected to alkaline phosphatase activity evaluation or to protein extraction.

**Flow Cytometry Analysis**

For the DKK1 intracellular staining, 50 µl of heparinized PB from controls and 21-OHD patients and unconjugated anti-DKK1 mAbs were used. In particular, we utilized two different anti-DKK1 mAbs: clone 141119 (R & D Systems) and clone 2B12 (Abnova). Intracellular staining for unconjugated DKK1 was preceded by fixation and permeabilization with the Intraprep TM Kit (Instrumentation Laboratory) and then incubated for 25 min at 4°C. Cells were then washed and labeled with F(ab′) fragment secondary antibody Alexa Fluor 488 (Life Technologies, Milan, Italy) for an additional 25 min. Then, cells were washed twice and data acquired using a FC500 (Beckmann Coulter) flow cytometer and analyzed using Kaluza software. The area of positivity was determined using an isotype-matched mAb, and a total of $10^5$ events for each sample were acquired.

**Confocal Microscopy**

For each experiment, $1 \times 10^5$/cm² CD14+ cells, CD2+ cells, or neutrophils were plated on poly-l-lysine-coated coverslips (Sigma) and fixed in 3.7% paraformaldehyde.

Fixed cells were washed three times with PBS, permeabilized with 0.3% Triton X-100-PBS for 30 min, and blocked in 1% BSA and 1% fetal bovine serum in PBS for 1 h. CD14+ cells and neutrophils were incubated with mouse anti-DKK1 mAb, CD2+ cells were incubated with mouse anti-DKK1 mAb and rabbit anti-CD4 and anti-CD8 polyclonal Ab (10 µg/ml in blocking buffer; Santa Cruz Biotechnology). After washing, bound antibodies were detected using 10 µg/ml fluorescent-labeled goat anti-mouse or anti-rabbit F(ab′) fragment secondary antibody, Alexa Fluor 488, or Alexa Fluor 555 (Life Technologies). Nuclei were counterstained with TO-PRO-3 Iodide (Life Technologies). The cells were then visualized and photographed by laser confocal microscopy TCS SP5 (Leica Microsystems, Mannheim, Germany). Images (2,048 × 2,048 pixels) were acquired with an oil immersion objective (×63 1.4 HCX PL APO; Leica Microsystems). Overlay images were assembled.

**RNA Isolation and Real-Time PCR**

Total RNA was extracted from CD2+ cells, CD14+ cells, and neutrophils isolated from controls and 21-OHD patients using spin columns (RNaseasy; Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The extracted RNA was reverse-transcribed using the Super Script First-Strand Synthesis System kit for RT-PCR (Life Technologies, Carlsbad, CA); an RT mixture containing 1 µg of total RNA, deoxyribonucleoside triphosphates, oligo(dT), RT buffer, MgCl2, dithiothreitol, RNaseOUT, SuperScript II RT, and diethyl pyrocarbonate-treated water to final volume of 100 µl was prepared according to the manufacturer’s instructions. cDNA was amplified with the Takara SYBR Green supermix with ROX kit (Bio-Rad Laboratories, Hercules, CA), and the PCR amplification was performed using the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories). The following primer pairs were used for the real-time PCR amplification: sense DKK1, 5′-TTCAACGCTATACGAACCTG-3′; antisense DKK1, 5′-GGGAGGCTGCTCTCG-3′ (NM_012242.2); sense cyclophilin, 5′-CGAGGTTCGGCATCTTGGTCC-3′; antisense cyclophilin, 5′-TTGCTGTGTTGCTTCC-3′ (NM_021130.3). The running conditions were as follows: incubation at 95°C for 3 min and 40 cycles of incubation at 95°C for 15 s and 60°C for 30 s. After the last cycle, the melting curve analysis was performed into a 55°C to 95°C interval by incrementing a temperature of 0.5°C. The fold change values were calculated by Pfaffl’s method (37).

**Alkaline Phosphatase Activity**

Alkaline phosphatase activity was determined in cell lysates using the colorimetric Alkaline Phosphatase Assay Kit (Abcam, Cambridge Science Park). The kit uses p-nitrophenyl phosphate as a phosphatase substrate, which turns yellow when dephosphorylated by alkaline phosphatase. The absorbance at 405 nm was measured using a multiwell plate reader (550 Microplate Reader; Bio-Rad Laboratories). Cell lysates were analyzed for protein content using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories), and alkaline phosphatase activity was normalized for total protein concentration.

**Western Blot Analysis**

Cells were lysed by incubation on ice for 30 min in lysis buffer containing 50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 5 mmol/l ethylenediaminetetraacetic acid, 1% NP-40, and a commercial protease inhibitor mixture (Sigma-Aldrich). Western blot analysis was performed as described previously (10). The following primary Abs were used: anti-collagen 1 (Santa Cruz Biotechnology), anti-RANKL (Abcam), anti-OPG (Abcam), and anti-β-actin (Santa Cruz Biotechnology). After incubation with the appropriate fluorescent dye-conjugated secondary Ab (LI-COR Biosciences, Bad Homburg, Germany), specific reactions were revealed with the LI-COR’s Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE).

**ELISA**

DKK1, RANKL, OPG (Biomedica, Vienna, Austria), bone-specific alkaline phosphatase (Dade Behring, Newark, DE), and COOH-terminal telopeptides of type I collagen (CTX) (Serum CrossLaps; Immunodiagnostics Systems, Fountain Hills, AZ) were measured in the sera obtained from the 21-OHD patients as well as in the sera of age-matched healthy donors, using a commercially available ELISA kit according to the manufacturer’s instructions. The absorption was determined with an ELISA reader (550 Microplate Reader; Bio-Rad), and the results were expressed as means ± SE.

**Statistical Analyses**

Statistical analyses were performed by Mann-Whitney and Wilcoxon tests with the Statistical Package for the Social Sciences (spssx/win) software (SPSS, Chicago, IL). Correlations were analyzed with the Spearman test. The effect of the type of 21-OHD and sex was determined with a two-sample t-test. The results were considered statistically significant for $P < 0.05$.

**RESULTS**

**DKK1 Serum Levels in 21-OHD Patients**

DKK1 serum concentrations were significantly elevated ($P < 0.008$) in patients with 21-OHD (2,413 ± 218 pg/ml; range: 1,255–3,776 pg/ml) compared with control subjects (1,660 ± 88 pg/ml; range: 1,019–2,318 pg/ml) (Fig. 1). In particular, in the majority of 21-OHD patients, serum levels of DKK1 were above the mean serum amounts of controls. No significant correlation was found between DKK1 serum levels and GC dose (Rho = 0.02, $P = 0.86$), duration of treatment (Rho = 0.23, $P = 0.72$),
androgen levels (Rho = 0.14, P = 0.61), and age (Rho = -0.02, P = 0.94). Type of 21-OHD and sex of patients had no effect on serum DKK1 levels (P = 0.73).

**DKK1 Expression in Circulating Leukocytes From 21-OHD Patients**

To investigate which cells are responsible for DKK1 production, we analyzed the intracellular expression of DKK1 on leukocytes from 21-OHD patients on cGC therapy and from controls through flow cytometry and confocal microscopy. Flow cytometry analysis revealed a significant increase in DKK1 expression in all the three blood subpopulations (lymphocytes, monocytes, and neutrophils) in patients (Fig. 2B) compared with controls (Fig. 2A), as assessed by intracellular labeling of DKK1 vs the side scatter of cells (P < 0.025). Furthermore, in patients we observed that DKK1 was highly expressed in a portion of lymphomonocyte (5.4%) compared with controls, where it was barely detectable (0.2%) (P < 0.001).

Coimmunostaining revealed DKK1 protein expression in CD8+ and CD4+ T lymphocytes (Fig. 2, C and D) from patients. Interestingly, we also observed strong DKK1 staining in monocytes and neutrophils (Fig. 2, E and F). A weak staining was found when cells from controls were evaluated (data not shown). To confirm DKK1 expression in whole blood leukocytes in 21-OHD patients, we next amplified RNA from purified monocytes, T lymphocytes, and neutrophils. As can be seen in Fig. 2, G–I, the lowest mRNA levels of DKK1 messenger RNA were detected in samples from controls. In striking contrast, DKK1 expression was higher in all investigated cell types (monocytes, T cells, neutrophils) isolated from patients. In detail, we found that, compared with controls, in 21-OHD patients DKK1 mRNA levels were 2.3 ± 0.4- (P < 0.001), 3.7 ± 0.4- (P < 0.001), and 4.8 ± 0.7-fold (P <

![Graph showing DKK1 serum levels in 21-OHD patients](image)

**Fig. 1.** Dickkopf-1 DKK1 serum levels (•) in patients with 21-hydroxylase-deficient (21-OHD). DKK1 serum concentrations were significantly elevated in patients with 21-OHD (2,413 ± 218 pg/ml; range: 1,255–3,776 pg/ml) compared with control subjects (1,660 ± 88 pg/ml; range: 1,019–2,318 pg/ml), P < 0.004.

![Flow cytometry analysis of DKK1 expression in permeabilized leukocytes](image)

**Fig. 2.** DKK1 expression in circulating leukocytes from 21-OHD patients. Flow cytometry analysis of DKK1 expression in permeabilized leukocytes revealed that DKK1 was significantly higher in all cell blood populations from patients (B) compared with the age-matched normal controls (A), especially in lymphomonocytes. Data shown are gated on all blood cell populations, and quadrants were established on the basis of DKK1 intracellular staining vs. cell side scatter (SS). C and D: confocal micrographic images showing coimmunostaining of DKK1 (green) protein and CD8+ (red) or CD4+ (red) T lymphocytes from patients. E and F: strong DKK1 staining in monocytes and neutrophils was also observed. Nuclei were counterstained with TO-PRO-3 iodide (blue). G–I: the expression of DKK1 was detected in T lymphocytes, monocytes, and neutrophils by real-time PCR. Results are depicted for 1 patient and 1 control but are representative of 8 different experiments.
increased in T lymphocytes, monocytes, and neutrophils, respectively (Fig. 2, G–I). Taken together, these data strongly suggest that a variety of different cell types contribute to the total pool of secreted DKK1 in the peripheral blood.

Effect of Dexamethasone on DKK1 Expression in Human Leukocytes

The enhanced expression of DKK1 by leukocytes from 21-OHD patients prompted us to investigate the effect of dexamethasone on human leukocytes from healthy donors. To this end, heparinized blood from controls was incubated with dexamethasone (10^-9 to 10^-7 M) for different times (0, 3, and 6 h). By flow cytometry, we found that dexamethasone induced the expression of DKK1 significantly compared with that in the unstimulated condition in a time- and dose-dependent manner. In particular, after 3 h of dexamethasone treatment, no differences in DKK1 expression were detected between the unstimulated and stimulated condition (data not shown). DKK1 expression increase effect was observed at 6 h of treatment, reaching the maximum amounts at the highest dose of dexamethasone used (10^-7 M, P < 0.001), maintaining elevated levels at 10^-8 M, and thus returning to control levels at 10^-9 M (Fig. 3). These results suggest that GCs induce the expression of DKK1 specifically in human leukocytes.

Effect of Serum From 21-OHD Patients on Osteoblast Differentiation In Vitro

To assess whether the high serum levels of DKK1 in the 21-OHD patients would be able to inhibit OB differentiation, we cultured human OBs in the basal condition (medium + 10% FCS), in conditioned media with sera from patients or controls, and in the presence or absence of anti-DKK1-neutralizing antibody or an anti-IgG. To promote OB differentiation, these cells were cultured in the presence of BMP2, which can stimulate OB differentiation through a mechanism that involves Wnt/β-catenin signaling (17).

In the cultures treated with BMP2 and FCS or serum from controls, the presence or absence of the anti-DKK1-neutralizing mAb did not exert effects on the activity of alkaline phosphatase, a specific marker of OB differentiation. On the contrary, OBs cultured with BMP2 and conditioned medium containing the serum of patients showed a significant reduction in alkaline phosphatase activity (21 ± 2%, P < 0.003; Fig. 4A) with respect to the OBs cultured with the serum from controls (or FCS). Moreover, we found that anti-DKK1-neutralizing mAb increased the alkaline phosphatase activity in the...
OBs significantly after 48 h of culture (20% ± 3%, P < 0.006) compared with conditioned medium containing the patient serum alone. No effect was found using anti-IgG antibody (not shown).

In the same culture system, we also evaluated the expression of collagen I as an additional marker of OB differentiation (Fig. 4B). In OBs cultured with FCS or serum from controls, the anti-DKK1-neutralizing mAb did not exert effects on collagen I expression. On the contrary, we found that conditioned medium containing the serum from 21-OHD patients and anti-DKK1-neutralizing mAb increased the collagen I expression in the OBs significantly after 48 h of culture (P < 0.007). No effect was found using anti-IgG antibody (not shown).

**Effect of Serum From 21-OHD Patients on Osteoblast Expression of RANKL and OPG**

On the basis of the knowledge that RANKL/OPG ratio can be unbalanced by Wnt inhibitors (38), we investigated whether in our culture system the serum from 21-OHD patients could affect, through DKK1 production, the expression of RANKL and OPG with pro- and antosteoclastogenic activity, respectively. In OBs cultured with FCS or serum from controls, the anti-DKK1-neutralizing mAb did not exert effects on RANKL or OPG expression (Fig. 5, A and B). On the contrary, the anti-DKK1 mAb strongly downregulated the RANKL expression (P < 0.003) and did not affect the OPG expression by OBs (Fig. 5, A and B). No effect was exerted by anti-IgG antibody (not shown). These data are supported further by the direct correlation between DKK1 and RANKL serum levels (Rho = 0.62, P < 0.003) in sera from 21-OHD patients (Fig. 5C). No significant correlation was found between DKK1 and OPG serum levels (Rho = 0.31, P < 0.12).

**Bone Mineral Measurements and Bone Biochemical Markers in 21-OHD Patients**

Proximal femur and lumbar spine BMD z-scores of 21-OHD patients were in the normal range (z-scores greater than −0.22) according to World Health Organization criteria for osteopenia and osteoporosis (19). However, a slight but significant reduction in bone mass was detected in 21-OHD patients (P < 0.05) with respect to the controls. The quantification of degradation products of CTX, an indicator of bone resorption, showed that patients had significantly higher serum levels with respect to controls (0.824 ± 0.17 ng/ml, range 0.34–1.70 ng/ml; 0.41 ± 0.09 ng/ml, range 0.15–0.87 ng/ml, P < 0.006). Interestingly, the higher levels of CTX were found in patients with lower BMD (P < 0.01). Moreover, we found that in sera from patients, DKK1 levels correlate with CTX concentrations (Rho = 0.65, P < 0.02). Serum levels of bone-specific alkaline phosphatase, total calcium, and phosphate of 21-OHD patients were within the normal range.

**DISCUSSION**

The current study results in three important observations. First, elevated serum levels of DKK1 have been found in 21-OHD patients on cGC treatment with respect to controls. The study included only 18 patients, with subjects with both the classical (SW or SV) and NC forms of 21-OHD, with the consideration that the incidence of the most severe forms (SW or SV) is about 1 in 10,000–15,000 people, whereas the incidence of milder forms (NC) is probably 10 times higher, with a prevalence ranges from 1 in 30 to 1 in 1,000 (33). Furthermore, whereas in the subjects with classical forms the GC treatment is started at the time of the diagnosis, generally in the newborn period, in those affected by the NC form the GC treatment needs to be directed toward the symptoms and should not be initiated merely to decrease abnormally elevated
have been selected from Ohnaka et al. (34), who demonstrated we have in our experiments. In particular, our used amounts in GIO, and most of them have used dexamethasone as GC, as and in vivo models have been used to explore the role of DKK1 treatment of 21-OHD patients (24). Moreover, several in vitro methasone can be used as glucocorticoid in the therapy of therapy. In fact, we found that in vitro dexamethasone treat-leukocytes in 21-OHD patients may be related to the cGC leukocytes, and neutrophils also express DKK1 further supports the tinction by T cells (10). Thus, the fact that monocytes, lympho-cytess from Paget’s lesions compared with control cultures from unaffected bone from the same patients or from other control subjects (32). During acute intestinal inflamma-tion, DKK1 expression is strongly induced in intestinal tissue in T lymphocytes, macrophages, neutrophils, and platelets (21). Here, we reported for the first time that circulating T lymphocytes, monocytes, and neutrophils from 21-OHD pa-tients express higher levels of DKK1 than controls. In the last decade, numerous scientists have highlighted the interactions between bone and immune cells, specifically in pathological conditions in which activation of both systems occurs (48). However, the biology regulating osteoimmune cross-talk is incompletely understood, and much research focuses on the identification of common molecules that can modulate the activation of immune cells as well as bone cells, and DKK1 could be another link between the two tissues. Moreover, in our previous work we demonstrated changes in gene expression following GC treatment in cultured mouse OBs (27). These data indicate that GCs inhibit the Wnt pathway and that this effect may be at least partially mediated by DKK1. Wang et al. (51) explored the role of DKK1 further in GIO. These authors treated MC3T3-E1 preosteoblasts with dexamethasone and reported the suppression of OB activity. Knockdown of DKK1 expression by DKK1-AS alleviated dexamethasone-induced suppression of OB activity and decreased OB apoptosis. Investigators also studied the changes in gene expression following GC treatment in a mouse model, using microarrays, and found that GC treatment led to a significant upregulation of DKK1 (53). Consistent with this finding, in the present study we demon-strated clearly that dexamethasone can induce the expression of DKK1 in human leukocytes.

Third, the serum from patients can directly inhibit OB differen-tiation in vitro as well as RANKL expression in OBs, and these effects were neutralized by the addition of an anti-DKK1 antibody in the cultures. We also found the correlation between DKK1 and RANKL or CTX serum levels in 21-OHD patients on cGC treatment. These positive correla-tions also suggest an effect of DKK1 on the bone resorption activity of osteoclasts that together with the DKK1 inhibitory effect on OB differentiation sustain the bone impairment that may be associated with cGC therapy. Thus, in these patients, the effects of DKK1 on bone seem to point to an important cross-talk between the bone-anabolic Wnt signal and the bone-catabolic RANKL pathway. Other studies have demonstrated a coregulation between DKK1 and RANKL expression in a range of diseases, including osteosarcoma (25) and prostate cancer (41), and in vitro studies using vascular progenitor cells (1) and murine mesenchymal stem cells (11). Additionally, it has been demonstrated that in sera from multiple myeloma patients, high DKK1 levels correlate with elevated CTX amount, a bone resorption marker (49).

In conclusion, the present study showed for the first time that dexamethasone treatment induces DKK1 expression in leukocytes, and in 21-OHD patients on cGC therapy the same cells represent an important source of this cytokine. Therefore, our findings suggest that the involvement of DKK1 secreted by leukocytes could be a general mechanism occurring not only in 21-OHD patients but in other forms of GIO. Thus, the period-ical monitoring of DKK1 levels could be useful for controlling the bone metabolism in these patients, and in those with a serious osteoporotic degree, DKK1 could represent a promising therapeutic target.

DISCLOSURES

The authors state that they have no conflicts of interest, financial or otherwise.
AUTHOR CONTRIBUTIONS

G.B. drafted the manuscript; M.F.F. and M. Grano contributed to the conception and design of the research; L.P., A.V., A.O., C.C., A.D.B., G.C., and M. Gigante performed the experiments; G.C. and L.G. interpreted the results of the experiments; G.M. analyzed the data; G.M., prepared the figures; S.C. edited and revised the manuscript; L.C. and M. Grano approved the final version of the manuscript.

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