INTRODUCTION AND BACKGROUND

Alkaline phosphatases (ALPs, EC3.1.3.1) are ubiquitous and membrane-bound glycoprotein enzymes that catalyse the hydrolysis of phosphate monoester at an alkaline environment. There are four genes in the human genome encoding the alkaline phosphatase isoenzymes. Three genes are tissue-specific: intestinal ALP (IALP), placental ALP (PALP) and germ cell ALP (GCALP). The fourth gene ALPL (liver, bone,

Abstract

Pregnane X receptor (PXR) is a xenobiotic-sensing nuclear receptor that regulates drug metabolism in the liver and intestine. In our clinical trials on healthy volunteers to discover novel metabolic functions of PXR activation, we observed that rifampicin, a well-established ligand for human PXR, 600 mg daily for a week, increased the plasma alkaline phosphatase (ALP) significantly compared with the placebo. Further analysis with lectin affinity electrophoresis revealed that especially the bone form of ALP was elevated. To investigate the mechanism(s) of bone ALP induction, we employed osteoblast lineage differentiated from human primary bone marrow–derived mesenchymal stromal cells. Rifampicin treatment increased ALP activity and mRNA level of bone biomarker genes (ALP, MGP, OPN and OPG). PXR expression was detected in the cells, but the expression was very low compared with the human liver. To further investigate the potential role of PXR in the ALP induction, we treated mice and rats with a rodent PXR ligand pregnenolone 16α-carbonitrile (PCN). However, PCN treatment did not increase plasma ALP activity or bone ALP mRNA expression. In conclusion, rifampicin treatment induces the bone form of ALP in the serum of healthy human volunteers. Further studies are required to establish the mechanism of this novel finding.

KEYWORDS

alkaline phosphatase, bone, pregnane X receptor, pregnenolone 16α-carbonitrile, rifampicin
kidney) encodes the tissue-non-specific ALP (TNALP).\(^1,3,4\) IALP is expressed throughout the gastrointestinal tract with the highest expression at the duodenum, and it plays a major role in the regulation of inflammation, infection and gut microbiota via dephosphorylation of lipopolysaccharide.\(^5,6\) PALP is highly expressed in the placenta, and trace amounts may be detected in normal sera of adults.\(^1\) ALPL is highly expressed in liver and bone tissues and represent about 95% of the total serum ALP with a ratio of 1:1 (liver to bone) in healthy, non-pregnant adults.\(^7,8\) The differential glycosylation of ALPL results in tissue-specific isoforms differing from each other only on the post-translational modifications,\(^9\) which are utilized in analytical methods to distinguish between the isoenzymes. Total ALP is commonly measured as a marker of liver function, and the isoenzyme-specific measurement is only employed when deemed clinically necessary. Deactivating mutations in the \(ALPL\) gene results in hypophosphatasia disorder, which is characterized by defective bone mineralization and deficiency in TNALP activity.\(^7\)

Bone ALP (BALP) is a homodimer anchored to the membrane of osteoblasts and matrix vesicles, and it plays a major role in bone formation and mineralization. BALP is cleaved by the phospholipase, and the circulating soluble BALP can be used as a marker of bone formation.\(^10\) BALP activity reflects the mineralization and formation of bone more sensitively and more specifically than total ALP.\(^11,12\) BALP promotes tissue mineralization and vascular calcification by hydrolysing the pyrophosphate (the potent inhibitor of tissue mineralization and vascular calcification).\(^13-15\) BALP is expressed mainly on the surface of the osteoblasts but it can also be expressed by other cells such as B-type lymphocytes and vascular smooth muscle cells.\(^16,17\) It has been reported that retinoic acid, 1,25-dihydroxyvitamin D\(_3\), thymoxine and p38 mitogen-activated protein kinases are involved in the transcriptional, post-transcriptional and/or post-translational regulation of the \(ALPL\) gene expression in various bone-derived cell lines.\(^18,23\)

Pregnane X receptor (PXR), a member of the nuclear hormone receptor superfamily, is activated by a wide variety of endobiotic substances, pharmaceutical agents, dietary compounds and environmental toxicants.\(^24\) While the PXR is expressed at high levels in the liver and intestine, its expression in other tissues like kidney, lung and bone is low.\(^25\) PXR is one of the key regulators of drug metabolism and induces the expression of the drug-metabolizing enzymes such as several cytochrome P450 (CYP) enzymes and drug transporters.\(^26\) The PXR-mediated induction of CYP3A4 activity can be assessed by measuring the serum/plasma 4β-hydroxycholesterol (4βHC), an oxysterol metabolite of cholesterol, formed by CYP3A4.\(^27\) PXR has been implicated in the bone formation. Vitamin \(K_2\) was able to induce osteoblast marker genes including ALP via the PXR in human osteosarcoma cell lines.\(^28\) Another study reported that PXR knockout in female mice resulted in osteopenia with reduced bone formation and enhanced bone resorption.\(^29\) Interestingly, a recent study demonstrated that PXR plays a role in warfarin (the vitamin \(K\) epoxide reductase–inhibiting anticoagulant)-induced human aortic valve calcification suggesting a role for PXR in calcium homeostasis or mineralization.\(^30\) Furthermore, it has been suggested that PXR activation could cause osteomalacia through increased catabolism of 1,25-dihydroxyvitamin D\(_3\). However, the mechanisms are somewhat controversial and induction of both CYP3A4 and CYP24A1 has been suggested to play a role.\(^31-34\)

rifampicin is an antibiotic commonly used for the treatment of tuberculosis and a well-known efficient activator of human PXR.\(^35\) The long-term rifampicin treatment may lead to hepatotoxicity including ALP elevation, and high rifampicin concentrations have been shown to inhibit the osteogenic differentiation of human mesenchymal stem cells of the bone marrow in vitro.\(^36-39\) Several studies in humans have demonstrated that treatment with rifampicin and PXR-activating antiepileptic drugs (carbamazepine, phenytoin, phenobarbital) increase the serum total ALP levels.\(^40-44\) Interestingly, some, but not all, studies investigating patients treated with antiepileptic drugs have reported that the bone ALP is the main ALP form elevated.\(^45-47\) Only one previous study has presented data on the ALP isoforms induced by rifampicin treatment: 1-week dosing of rifampicin induced the mRNA expression of intestinal ALP in the duodenum of healthy volunteers.\(^48\) No data were presented on the other ALP isoforms, including BALP. Human bone marrow mesenchymal stem cells participate not only in the regulation of bone turnover but they may be involved in chronic tuberculosis dormancy.\(^49\) Hence, rifampicin effect on the bone ALP may be of clinical interest. In the current study, we investigated the isoenzyme pattern of rifampicin-elevated ALP and the role of PXR in the ALP induction in both cell and animal models.

## 2 | MATERIALS AND METHODS

### 2.1 | Subjects

Healthy volunteers were recruited to the four trials Rifa-1,\(^50\) Rifa-2,\(^51\) Rifa-BP\(^52\) and Rifa-Stea (unpublished). The studies were designed to explore the effects of PXR activation on glucose tolerance (Rifa-1), incretin secretion (Rifa-2), blood pressure regulation (Rifa-BP) and hepatic steatosis (Rifa-Stea). The inclusion criteria were healthy volunteers with age between 18 and 40 years (45 years in Rifa-2). The body mass index (BMI) criterion was between 19-28 kg/m\(^2\) in Rifa-2, 19-30 kg/m\(^2\) in Rifa-BP and 18.5-25 kg/m\(^2\) in Rifa-Stea, while Rifa-1 did not have a BMI limit. Inclusion
criterion for the systolic blood pressure was 95-140 mm Hg in Rifa-BP, and blood pressure over 150 mm Hg was an exclusion criterion in Rifa-Stea while the other studies had no blood pressure limits. The exclusion criteria included major medical or psychiatric conditions including any liver disease (as judged by the study physician on the basis of history, physical examination and basic laboratory values); any continuous medication, including oral contraceptives; hypersensitivity to rifampicin; pregnancy and breastfeeding; continuous use of soft contact lenses (rifampicin may colour); history of difficult venipuncture; drug or alcohol abuse; and participation in any other trial within 1 month. The studies were approved by the Ethics Committee of Northern Ostrobothnia Hospital District (Oulu, Finland) and the Finnish Medicines Agency. Written informed consent was obtained from each subject. The study procedures were in accordance with the ethical standards of the Declaration of Helsinki and guidelines on Good Clinical Practice. Moreover, the study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.53 The participants were financially compensated for participation. The original sample size calculations were targeted for fasting glucose, 24-hr blood pressure and hepatic steatosis, and thus, for the ALP analyses, this data set represents a sample of convenience. These trials were registered at ClinicalTrials.gov.50-52 Rifa-Stea is registered as NCT02329405.

### 2.2 Study design and experimental protocols

The Rifa-1 (n = 12), Rifa-Stea (n = 16) and Rifa-BP (n = 22) studies had the same randomized, open (in Rifa-BP, the study personnel were blinded), placebo-controlled, cross-over design, while Rifa-2 (n = 12) study had a one-arm, open design. In all the trials, volunteers were given 600-mg rifampicin (Rimapen; Orion Corporation) a day for 1 week. The participants were asked to abstain from the use of alcohol, over-the-counter medications, and dietary and herbal supplements for 5 days before and during the study arms. The studies were conducted on outpatient basis at the Research Unit of Internal Medicine of the Oulu University Hospital. The experimental protocols are described in detail in the previous publications.50-52 The manuscript on the main Rifa-Stea results is under preparation. The subjects had the last rifampicin or placebo dose on the evening of the 7th day in all four trials. In the one-arm Rifa-2 study, the blood samples were taken before (1st day) and after (8th day) 1-week rifampicin dosing. In the studies with the cross-over design (Rifa-1, Rifa-BP and Rifa-Stea), the blood samples were taken on the morning of 8th day except for Rifa-BP where blood samples were taken on the morning of 9th day (Figure 1).

### 2.3 Analytic methods

The clinical laboratory analyses were performed by the Clinical Laboratory of Oulu University Hospital (NordLab) and were validated for clinical use. Photometric method was utilized for the measurement of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and total ALP. The measurements of serum ALP isoenzymes were analysed with lectin affinity electrophoresis method. Photometric vanadate oxidation method was employed in the assays of serum total and conjugated bilirubin. The serum calcium ion was measured with the ion selective electrode method and the plasma phosphate with photometric molybdate reaction.

In addition, the measurement of serum intact procollagen type I N-terminal propeptide (PINP) was performed with radioimmunoassay (UniQ PINP; Aidian Oy) in accordance with the manufacturer’s recommendations. The liquid chromatography-electrospray-high-resolution mass spectrometry was utilized for the measurement of the plasma 4β-hydroxycholesterol (4βHC) at the Admescope Ltd. as described.54

### 2.4 Experimental protocol for the differentiation of the human osteoblasts from the bone marrow–derived mesenchymal stromal cells

Bone marrow–derived human mesenchymal stromal cells (hMSCs) were obtained from three donors undergoing a hip replacement operation for osteoarthritis. Primary isolation and culture of hMSCs was performed as described in detail earlier.55 The protocol was approved by the Ethics Committee of Northern Ostrobothnia Hospital District. Briefly, after primary isolation, the hMSCs were plated in a culture flask (T175) and cultured in α-MEM (minimum essential medium; Gibco), supplemented with 10% heat-inactivated FBS (foetal bovine serum; Autogen Bioclear), 20 mmol/L HEPES (Gibco), 100 U/mL of penicillin,

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**FIGURE 1** A schematic figure representing the design of the clinical studies.
0.1 mg/mL of streptomycin (Gibco) and 2 mmol/L L-glutamine (denoted as basal medium “BM” hereafter) and incubated at 37°C in 5% CO₂ and 95% air. The medium was replaced at a rate of 50% every 3 days until the cells reached 90% confluence, after which the cells were passaged and replaced into a 96-well culture plate for osteogenic differentiation. The cells were then cultured for 3 and 5 weeks in two different media (OS and BM), and then, the cells were treated with different concentrations of (1 µmol/L, 10 µmol/L) rifampicin (human PXR ligand) and (0.1, 1 µmol/L) hyperforin (structurally different PXR ligand) or DMSO vehicle control for 48 hours. The OS medium promotes osteogenic differentiation, and it consists of BM medium supplemented with 100 nmol/L dexamethasone, 10 mmol/L sodium β-glycerophosphate and 0.05 mmol/L ascorbic acid-2-phosphate (all from Sigma-Aldrich). Alkaline phosphatase activity was measured from the cells using colorimetric assay kit (BioVision) according to the manufacturer's protocol.

### 2.5 In vivo animal experiments

The animal experiments have been approved by the Animal Experiment Board in Finland (licence numbers ESAVI/6357/04.10.07/2014 and ESAVI/8240/04.10.07/2017) according to the European Union directive 2010/63/EU. All the laboratory animals were obtained from the Laboratory Animal Center, University of Oulu. Animals were housed in individual cages under standard conditions with a 12-hour dark-light cycle. Eight-week-old C57BL/6N male mice (n = 6-7 mice/group, weighing 23-25 g) were given a daily i.p. injection of pregnenolone 16α-carbonitrile (PCN, a rodent PXR agonist) 50 mg/kg dissolved in DMSO/corn oil or vehicle (DMSO/corn oil) for 4 days. Two-month-old male Sprague Dawley rats (n = 5 rats/group, weighing 240-300 g) were given daily i.p. injection of PCN (40 mg/kg) or vehicle (DMSO/corn oil) for 6 days. Rodents dosed with PCN vs vehicle control are an established model for the study of the effects of PXR activation. The animals were killed by CO₂ inhalation, and blood samples and tissues were collected and frozen in liquid nitrogen for further analyses. Alkaline phosphatase activity was measured in the serum samples of mice and rats as described above.

### 2.6 RNA extraction and quantitative reverse transcriptase PCR

Total RNA was extracted from the human osteoblast cells and human liver cells and from the femur bone of the PCN-treated mice using RNaZol (Sigma-Aldrich) according to the manufacturer's protocol. To produce complementary DNA, 1 µg RNA was reverse transcribed using random hexaprimer (Roche Diagnostic) and reverse transcriptase enzyme (Thermo Scientific). The quantitative reverse transcriptase PCRs (40 cycles) were performed using FastStart Universal SYBR Green Master Mix (Roche Diagnostic) or TaqMan chemistry (Applied Biosystem) using Applied Biosystems 7300 Real-Time PCR instrument. The sequences of the primers of the genes of interest were obtained from Thermo Scientific and are presented in Table 1.

| Gene | Probe | Forward primer (5’-3’) | Reverse (5’-3’) |
|------|-------|------------------------|----------------|
| ALPL | ALPL FAM Hs01029144-m1 | CATACAAGGCCATCCCCG | CTGTCCCTCCCAGGGCTG |
| OSP  | 6-FAM CCTGAAACGCCCTTCTGATTGG-TAMRA | GGGAGCGCTGTAGTACACAG | CGATTATAGGGATGTATCCA |
| MGP  | 6-FAM TTTGCAACGCTACGCATGGTT-TAMRA | TGTACAGCAAAGTGGAAGACCG | GGTGTGCCACGTGTCTGTGT |
| OPG  | 6-FAMTGGGCCCTTGGCTGGACC-TAMRA | TGTACAGCAGAAATGGAAGACCG | GGTGTGCCACGTGTCTGTGT |
| 18S  | -     | CTCAAACCGGGAAACCTCAC | CGTCCTCCAACTAAGAACG |
| PXR  | -     | TTGCCATCGAGGACGACTAT | GTCTCCGCTGTAACACTGT |
| Alpl | -     | CCAACTTTTTGTGAGCAGAGA | GGTATCTGGTGTTAGCTTTT |
| Osp  | -     | AGCAAGAAACTCTTTCAAGCAA | GTGAGATTCTCGATATCAGAG |
| Mgp  | -     | GGCAACCCTTGGCTACGAAT | CCGGAGCCTCTTCTTGGCGGTTA |
| Opg  | -     | CTCTCCACCTACAGGCTGATT | AGAACATCGTAAGGATGCCTTG |

### Table 1 Sequences of the primers used in quantitative RT-PCR
bone biomarker genes *ALPL*, matrix gla protein (*MGP*), osteopontin (*OSP*) and osteoprotegerin (*OPG*) were normalized against 18S rRNA control levels using the comparative Ct (ΔΔCt) method.

### 2.7 Statistical analyses

Prism software (GraphPad Software) was used for statistical analyses. The parameters were compared across treatments by two-tailed Student’s *t* test (paired test for the human studies; unpaired for rodent studies), Wilcoxon matched-pairs signed rank test, or one-sample Wilcoxon test of the ratio (rifampicin/placebo) of individual values. Multiple groups were compared by one-way ANOVA followed by Dunnett’s multiple comparisons test. Pearson correlation was used to analyse the correlation of the 4βHC with total and bone ALP *P* < .05 was considered statistically significant.

## 3 RESULTS

### 3.1 Rifampicin increases the plasma total alkaline phosphatase in healthy volunteers

To investigate novel functions of PXR activation, we performed three randomized, cross-over, placebo-controlled clinical trials on healthy volunteers: (a) Rifa-1 (*n* = 12), (b) Rifa-Stea (*n* = 16) and (c) Rifa-BP (*n* = 22). We also performed a trial with 1-week rifampicin dosing with no control arm (Rifa-2, *n* = 12). In all the trials, 600-mg rifampicin was given orally to the subjects once daily for a week. Rifampicin increased the 4βHC plasma levels significantly in all the study subjects as expected, indicating adherence to the rifampicin dosing (Tables 2 and 3).

Several safety biomarkers were measured in the Rifa-1, Rifa-Stea and Rifa-BP studies (Table 2) including liver function test ALP, AST, ALT, GGT and bilirubin. Rifampicin significantly increased the plasma level of total ALP compared to the placebo arm in all three trials as well as in the combined data set (Rifa-1, Rifa-BP and Rifa-Stea) (Table 2). Furthermore, rifampicin significantly increased the plasma AST level in the Rifa-BP and in the combined data set. Rifampicin had no effect on plasma ALT levels. Rifampicin increased significantly the plasma level of GGT in Rifa-Stea and combined Rifa-1 and Rifa-Stea (not measured in Rifa-BP). On the other hand, the serum levels of bilirubin were significantly decreased by rifampicin dosing in all three trials while serum conjugated bilirubin was decreased by rifampicin in Rifa-1 (not measured in the other trials).

### 3.2 Rifampicin increases the bone form of alkaline phosphatase in serum

Alkaline phosphatase has several different isoenzymes originating from various tissues. Therefore, we performed further analysis of the serum ALP with lectin affinity electrophoresis to determine the isoenzymes affected by the rifampicin treatment. We measured ALP isoenzymes from the serum samples of Rifa-Stea and Rifa-2 trials.

In Rifa-Stea, the bone-specific ALP was increased significantly by rifampicin compared to the placebo arm (Table 4) while in the smaller Rifa-2 study (*n* = 12), only the intraindividual ratio of rifampicin to placebo for bone ALP increased statistically significantly (Table 3). The combined results of the two trials (Rifa-Stea and Rifa-2) showed significant increase of BALP (Table 5). Rifampicin also increased significantly the liver+bone ALP compared to controls (Tables 4 and 5), but not the liver ALP.

The plasma 4βHC concentration, a marker of PXR-regulated CYP3A4 activity, was not correlated with the total ALP in the rifampicin or placebo arms (*r* = −.17, *P* = .99; *r* = −.23, *P* = .07, respectively). There was also no correlation between the 4βHC levels and the bone ALP in the rifampicin or placebo arms (*r* = .01, *P* = .99; *r* = −.197, *P* = .32, respectively).

The serum concentration of procollagen type I N-terminal propeptide (*PINP*), an osteoblast-derived marker of bone formation, or the serum levels of phosphate were not affected by rifampicin (Rifa-Stea) (Table 4). Furthermore, rifampicin had no effect on the serum levels of ionized calcium (1.22 ± 0.03 in the rifampicin or placebo arms, *P* = .48) measured in Rifa-1.

### 3.3 Rifampicin increases the alkaline phosphatase activity and induces the bone biomarker genes in human osteoblast cells in vitro

To investigate the mechanisms involved in the induction of ALP by rifampicin, we employed human osteoblast cells differentiated from bone marrow–derived mesenchymal stromal cells from donors. After 3 or 5 weeks of differentiation in 2 different mediums (BM and OS), the cells from two donors were treated with two concentrations (1 µmol/L and 10 µmol/L) of rifampicin for 48 hours. Then, the alkaline phosphatase activity was measured using colorimetric assay. Rifampicin increased the ALP activity in cells from donors 492 and 488 in BM medium at 3 and 5 weeks of differentiation (Figure 2A). In OS medium, rifampicin induced ALP activity only at 5 weeks (Figure 2B).
**Table 2**  Effect of 600 mg/day rifampicin vs placebo on liver function tests in healthy volunteers in three trials (Rifa-1, Rifa-Stea and Rifa-BP)

|                      | Rifa-1 |         |         | Rifa-Stea |         |         | Rifa-BP |         |         | Combination of Rifa-1, Rifa-Stea, and Rifa-BP |         |
|----------------------|--------|---------|---------|-----------|---------|---------|---------|---------|---------|-----------------------------------------------|---------|
|                      | N = 12 |         |         | N = 16    |         |         | N = 22  |         | N = 50  |                                               |         |
| Rifampicin           |        |         |         | Placebo   |         |         | Placebo |         |         |                                               |         |
| ALP (IU/L)           | 72.8 ± 15.5 | 65.3 ± 16.5 | .040    | 68.3 ± 22.5 | 62.2 ± 18.9 | .0051   | 67.4 ± 13.8 | 62.3 ± 13.1 | .0002   | 69 ± 17.20 | 63 ± 15.67 | <.0001 | 1.10 (3.83, 8.16) |
| ALT (IU/L)           | 20.4 ± 7.2 | 23.1 ± 9.4 | .45     | 19.7 ± 5.8 | 20.9 ± 5.4 | .45     | 21.5 ± 8.1 | 19.2 ± 6.1 | .21     | 20.7 ± 7.1 | 20.7 ± 6.3 | 1.0     | 1.00 (−2.55, 2.55) |
| AST (IU/L)           | 26.38 ± 4.9 | 23.8 ± 7.3 | .074    | 22.9 ± 4.6 | 22.8 ± 5.4 | .89     | 21.8 ± 7.7 | 18.2 ± 5.6 | .04     | 23.2 ± 6.4 | 21 ± 6.3  | .017    | 1.10 (0.40, 4.00) |
| GGT (IU/L)           | 20.2 ± 12.1 | 18.3 ± 16.2 | .22     | 15.9 ± 4.2 | 13.4 ± 3.4 | .0014   | -        | -        | -       | 17.71 ± 8.6 | 15.5 ± 11 | .004    | 1.14 (0.79, 3.70) |
| S. Bil (µmol/L)      | 8.8 ± 2.8 | 13.3 ± 4.3 | .0031   | 10.8 ± 3.9 | 16.3 ± 6.3 | .0014   | 8.0 ± 2.6 | 13.6 ± 5.8 | <.0001  | 9.2 ± 3.5 | 14.7 ± 6.1 | <.0001  | 0.63 (−7.33, −3.7) |
| S.conjugated Bil (µmol/L) | 3.2 ± 1.1 | 4.6 ± 1.8 | .0033   | -         | -        | -       | -       | -       | -       | -        | -        | -       | -       |
| 4βHC (ng/mL)         | 62.1 ± 20.7 | 17.5 ± 6.4 | <.0001  | 54.9 ± 18.4 | 17.1 ± 6.1 | <.0001  | 54.1 ± 18.1 | 16.1 ± 5.8 | <.0001  | 56.3 ± 18.7 | 16.8 ± 5.9 | <.0001  | 3.4 (35.24, 43.8) |

**Note:** The trials had a similar cross-over design with at least a 4-wk washout period.

Data are presented as mean ± SD, the ratio of arithmetic means and the 95% confidence interval CI of means of differences between arms. Student's paired t test was used as the statistical test.

**Abbreviations:** 4βHC, 4β-hydroxycholesterol; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; S. Bil, serum bilirubin.
We also measured the mRNA expression levels of bone biomarker genes osteoprotegerin (OPG), osteopontin (OSP), matrix gla protein (MGP) and alkaline phosphatase (ALPL) using qPCR assay in cells from one donor. Rifampicin increased the mRNA expression levels of ALPL significantly in the BM medium at 1 and 10 µmol/L concentrations, while in the OS medium only the 1 µmol/L concentration had an effect. In both media, the 5-week differentiation was required for the effect (Figure 3A). The OSP expression was significantly induced by 1 and 10 µmol/L rifampicin after 3 weeks and by 10 µmol/L after 5 weeks in BM medium. In OS medium, only 10 µmol/L rifampicin, and only in the cells differentiated for 3 weeks, induced OSP statistically significantly (Figure 3B). The expression of OPG was increased by 10 µmol/L rifampicin in BM for 5 weeks and in OS for 3 and 5 weeks. MGP expression was induced by 10 µmol/L rifampicin both after 3 and 5 weeks in OS medium and by 1 µmol/L rifampicin after 3 weeks only (Figure 3D).

### TABLE 3 The effect of rifampicin on serum alkaline phosphatase isoenzymes (Rifa-2 study, n = 12)

|                          | After rifampicin | Before rifampicin | P-value paired Wilcoxon | Ratio and 95% CI of the median of differences | P-value, one-sample Wilcoxon |
|--------------------------|------------------|-------------------|-------------------------|-----------------------------------------------|-------------------------------|
| Total ALP (IU/L)         | 74.1 ± 32.3      | 70.9 ± 35.9       | .09                     | 1.04 (−3.00, 8.00)                             | .034                          |
| ALP (bone) (IU/L)        | 38.6 ± 24.5      | 36.2 ± 26.9       | .21                     | 1.06 (−5.00, 6.00)                             | .042                          |
| ALP (liver + bone) (IU/L)| 68.9 ± 33.2      | 66.6 ± 36.9       | .15                     | 1.03 (−2.00, 7.00)                             | .042                          |
| ALP (liver) (IU/L)       | 30.3 ± 10.9      | 30.3 ± 11.5       | .98                     | 1.0 (−3.00, 4.00)                              | .81                           |
| ALP (macromolecular) (IU/L)| 2.1 ± 1.4        | 1.8 ± 1.3         | .53                     | 1.13 (0.001, 1.00)                             | .28                           |
| ALP (intestinal<sup>a</sup>) (IU/L) | 6.3 ± 4.8      | 6.0 ± 6.4         | .37                     | 1.05 (−2.00, 4.00)                             | 1.0                           |
| ALP (bone tail form<sup>b</sup>) (IU/L) | 2.0            | 2.0               | -                       | -                                             | -                             |
| Plasma 4βHC (ng/mL)      | 70.2 ± 13.7      | 21.6 ± 6.3        | .0005                   | 3.3 (39.5,53.4)                                | .0005                         |

Note: Data are presented as mean ± SD, the ratio of arithmetic means and the 95% confidence interval CI of median of differences between arms. Wilcoxon matched-pairs signed rank test and one-sample Wilcoxon test were used as the statistical tests.

Abbreviations: ALP, alkaline phosphatase; 4βHC, 4β-hydroxycholesterol.

<sup>a</sup>Detect in six subjects.

<sup>b</sup>Only detected in one subject.

### TABLE 4 The effect of rifampicin on serum alkaline phosphatase isoenzymes and PINP (Rifa-Stea study, n = 16)

|                          | Rifampicin | Placebo | P-value, paired Wilcoxon | Ratio and 95% CI of the median of differences | P-value, one-sample Wilcoxon |
|--------------------------|------------|---------|-------------------------|-----------------------------------------------|-------------------------------|
| Total ALP (IU/L)         | 52.4 ± 18  | 46.6 ± 14| .006                    | 1.12 (4.00, 9.00)                             | .003                          |
| ALP (bone) (IU/L)        | 27.9 ± 12  | 24.9 ± 11| .046                    | 1.12 (0.001, 7.00)                             | .029                          |
| ALP (liver + bone) (IU/L)| 50.1 ± 17  | 45.1 ± 14| .007                    | 1.11 (0.001, 11.3)                             | .004                          |
| ALP (liver) (IU/L)       | 22.4 ± 14  | 20.3 ± 13| .38                     | 1.10 (−4.5, 6.3)                              | .119                          |
| ALP (macromolecular) (IU/L)| 0.65 ± 0.44| 0.58 ± 0.35| .26                    | 1.12 (−0.100, 0.300)                          | .304                          |
| ALP (intestinal<sup>a</sup>) (IU/L) | 3.8 ± 3.6      | 2.0 ± 1.9    | .38                     | 1.9 (−3.7, 8.6)                               | .156                          |
| ALP (bone tail form<sup>b</sup>) (IU/L) | 0.35 ± 0.07  | 0.30 ± 0.07  | -                       | 1.17 (−)                                     | -                             |
| Serum PINP (ng/mL)       | 90.0 ± 35   | 81.9 ± 37   | .274                    | 1.10 (−7.1, 17.4)                             | .10                           |
| Plasma phosphate (mmol/L)| 1.15 ± 0.13 | 1.18 ± 0.14| .79                     | 0.98 (−0.11, 0.11)                             | 92                            |

Note: Data are presented as mean ± SD, the ratio of arithmetic means and the 95% confidence interval CI of median of differences between arms. Wilcoxon matched-pairs signed rank test and one-sample Wilcoxon test of the ratio (rifampicin/placebo) of individual values were used as the statistical tests.

Abbreviations: PINP, procollagen type I N propeptide. ALP, alkaline phosphatase.

<sup>a</sup>Detected in seven subjects.

<sup>b</sup>Only detected in two subjects.
TABLE 5  The effect of rifampicin on serum alkaline phosphatase isoenzymes (combined Rifa-Stea and Rifa-2 data set, n = 28)

|                          | Rifampicin | Placebo | P-value  | Ratio and 95% CI of the median of differences | P-value, one-sample Wilcoxon |
|--------------------------|------------|---------|----------|-----------------------------------------------|-----------------------------|
| Total ALP (IU/L)         | 61.7 ± 26.8| 56.8 ± 28.3| .0001    | 1.08 (3.2, 8.00)                              | .0001                       |
| ALP (bone) (IU/L)        | 32.5 ± 18.8| 29.8 ± 19.9| .019     | 1.09 (2.00, 5.00)                             | .0025                       |
| ALP (liver + bone) (IU/L)| 58.2 ± 26.4| 54.0 ± 28.1| .002     | 1.08 (2.00, 7.00)                             | .0003                       |
| ALP (liver) (IU/L)       | 25.8 ± 13  | 24.3 ± 13.4| .316     | 1.06 (−1.52, 4.52)                            | .21                         |
| ALP (macromolecular) (IU/L) | 1.4 ± 1.2 | 1.2 ± 1.1 | .181     | 1.17 (0.001, 0.300)                           | .14                         |
| ALP (intestinal\(^a\)) (IU/L) | 5.0 ± 4.2 | 3.5 ± 4.5 | .921     | 1.43 (−4.00, 4.30)                            | .19                         |
| ALP (bone tail form\(^b\)) (IU/L) | 0.9 ± 1.0 | 0.9 ± 1.0 | 1.0      | 1.0 (0.001, 0.10)                             | 1.0                         |
| Plasma 4βHC (ng/mL)      | 61.4 ± 17.9| 19.0 ± 6.5 | <.0001   | 3.23 (35.2, 45.8)                             | <.0001                      |

Note: Data are presented as mean ± SD, the ratio of arithmetic means and the 95% confidence interval CI of median of differences between arms. Wilcoxon matched-pairs signed rank test and one-sample Wilcoxon test were used as the statistical tests.

Abbreviations: 4βHC, 4β-hydroxycholesterol; ALP, alkaline phosphatase.
\(^a\)Detected in 13 subjects.
\(^b\)Only detected in three subjects.
**FIGURE 3**  Effect of rifampicin on mRNA expression of selected bone biomarker genes in human osteoblast cells from donor 492 in BM and OS medium for 48 h after 3 and 5 wk of differentiation. One-way ANOVA (Dunnett's multiple comparisons test) was used as the statistical test. 

*ALPL*, alkaline phosphatase (liver/bone/kidney); *MGP*, matrix gla protein; *OPG*, osteoprotogerin; *OSP*, osteopontin
We further treated the cells of an additional donor (694 in OS medium) with 0.1 and 1 µmol/L of hyperforin, a potent PXR agonist, for 48 hr after 3 and 5 weeks of differentiation. Hyperforin increased the mRNA expression levels of \textit{ALPL} after 5 weeks, and it was statistically significant with 0.1 µmol/L concentration (Figure 4A). The expression levels of \textit{OSP} and \textit{OPG} were not significantly affected by hyperforin (Figure 4B,C). 1 µmol/L hyperforin down-regulated the mRNA level of \textit{MGP}, but only after 3 weeks of differentiation in OS medium (Figure 4D).

Additionally, we measured the mRNA expression levels of PXR in the human osteoblasts with and without rifampicin treatment. The expression was detectable; however, the expression level was very low in comparison with the human liver and not affected by rifampicin treatment (Figure 5).

3.4 | Rodent PXR agonist does not increase the ALP plasma activity or bone expression in mice and rats

To further investigate the potential role of PXR on the alkaline phosphatase induction, we performed in vivo animal experiments in mice and rats. The mice and rats were given a daily i.p. injection of PCN (a rodent PXR ligand) 50 mg/kg for 4 days and 40 mg/kg for 6 days, respectively, or vehicle, and the ALP activity was measured in plasma. Also, the mRNA was extracted from the bones of the mice. PCN did not increase the plasma ALP activity either in the mice or the rats (Figure 6A,B). Furthermore, PCN had a tendency to down-regulate the mRNA expression levels of the bone biomarker genes (\textit{Alpl}, \textit{Mgp}, \textit{Opg} and \textit{Osp}) in the mouse bone; however, the difference was not statistically significant (Figure 6C).

\textbf{FIGURE 4} Effect of hyperforin on mRNA expression of selected bone biomarker genes in human osteoblast cells from donor 694 in OS medium for 48 h after 3 and 5 wk of differentiation. One-way ANOVA (Dunnett’s multiple comparisons test) was used as the statistical test. \textit{ALPL}, alkaline phosphatase (liver/bone/kidney); \textit{MGP}, matrix gla protein; \textit{OPG}, Osteoprotogerin; \textit{OSP}, osteopontin
4 | DISCUSSION

In this study, we show that the administration of the efficient human PXR agonist rifampicin for 1 week in healthy young adults increases the serum total ALP levels and especially the bone form of ALP. The liver and intestinal forms of ALP are not affected by rifampicin dosing. Rifampicin also induces the ALP activity in cultured cells differentiated from bone marrow–derived mesenchymal stromal cells of human donors. The cell model is resembling mature osteocytes (5 weeks of cell culture). Thus, we demonstrate that rifampicin induces the bone ALP activity in the serum of human healthy volunteers, and this effect is most likely explained by the direct rifampicin effect in osteocytes. While previous studies have reported that the rifampicin-induced hepatitis leads to elevated ALP levels, no prior study has examined the rifampicin effect on the isoenzymes of ALP.

We explored whether the PXR plays a role in the rifampicin-induced BALP activity. Rifampicin increased the ALP activity and mRNA expression levels of bone biomarker genes (\textit{ALPL}, \textit{MGP}, \textit{OPG}, \textit{OPN}) in the human osteoblast cells treated with rifampicin. This is in agreement with a study demonstrating that treatment of the osteosarcoma cell lines (HOS, MG-63 and Saos-2) with the PXR activators rifampicin, hyperforin and vitamin K$_2$ up-regulated the

![Figure 5](image_url)

**Figure 5** The mRNA expression level of PXR in control osteoblasts, rifampicin-treated osteoblasts and human liver. One-way ANOVA (Dunnett’s multiple comparisons test) was used as the statistical test. PXR, pregnane X receptor

![Figure 6](image_url)

**Figure 6** Effect of 4 d of i.p 50 mg/kg PCN in mice and 6 d of i.p 40 mg/kg PCN in rats in vivo on plasma ALP activity (A mice; B rats) and mRNA expression of selected bone markers in the bones of mice (C). Two-tailed Student’s $t$ test was used as the statistical test.
osteoblast marker genes. Also in murine primary osteocytes, PXR activators were able to induce OPG and MGP and this effect was dependent on the expression of PXR.

However, in our study, the PXR expression was very low in human primary cells differentiated from the human bone marrow–derived mesenchymal stromal cells compared to the human liver cells, and a potent PXR agonist hyperforin was not able to consistently induce ALP activity in vitro. In addition, the plasma concentration of 4β-hydroxycholesterol, an oxysterol metabolite of cholesterol formed by the PXR target enzyme CYP3A4, was not correlated with total ALP or bone ALP. Together, these findings suggest that the rifampicin induction of BALP in humans is perhaps not mediated by PXR. Furthermore, plasma ALP activity and the expression of bone marker genes including Alpl were not increased in mice and rats treated with the rodent PXR agonist PCN. Thus, if the rifampicin-elicted induction of bone ALP in humans is PXR-dependent, the effect is not conserved across species.

Previous studies have reported that the elevated BALP serum levels are positively associated with hyperinsulinemia, insulin resistance and vascular calcification. BALP levels have also been positively associated with mortality in patients with dialysis. In vitro experiments, insulin directly stimulates the osteoblasts and leads to an increase in BALP activity. In a cross-sectional study with 1,325 children and adolescents, BALP was positively correlated with the insulin and glucose levels during oral glucose tolerance test (OGTT). In our earlier study, we showed that rifampicin treatment for 1 week elevated insulin and glucose concentrations during OGTT in healthy volunteers. The hyperglycemic effect of PXR activation was also present in WT mice but not in PXR knockout mice. Thus, there may theoretically be a link between PXR activation-elicted post-prandial hyperinsulinemia and bone ALP levels. In the National Health and Nutrition Examination Survey (NHANES) 1999–2004 cohort in the United States, serum bone ALP concentration was positively associated with the blood pressure, and a structural equation model suggested that hypertension has direct effects on serum BALP levels. Also, the insulin resistance and low HDL cholesterol were associated with the bone ALP. As hypertension, insulin resistance and low HDL are all components of the metabolic syndrome, the authors suggested that their findings provide a possible mechanism for the increased vascular calcification in subjects with metabolic syndrome. In our recent study, rifampicin dosing for 1 week elevated systolic and diastolic 24-hr blood pressure in healthy volunteers. Thus, PXR activation has links with the blood pressure regulation as well as hyperinsulinemia which are associated with the elevated bone ALP levels. However, as rifampicin had a direct effect on ALP activity in osteocytes in vitro in our study, it is unlikely that rifampicin-elicted hypertension or hyperinsulinemia would mediate the effect of rifampicin on BALP.

In conclusion, we show that rifampicin induces total ALP and especially the bone-specific ALP in healthy volunteers. This effect is most likely explained by the direct rifampicin effect in osteocytes. However, our experiments cannot provide definitive evidence about the role of PXR in this phenomenon. Thus, further studies are required in the future to investigate the molecular mechanisms more precisely.

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CONFLICT OF INTEREST
None.

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