

TECHNISCHE UNIVERSITÄT MÜNCHEN

Experimentelle Unfallchirurgie

Klinik und Poliklinik für Unfallchirurgie

des Klinikums rechts der Isar

***In vitro* effects of pantoprazole on
human osteoblasts and osteoclasts**

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Abbreviations

AP	alkaline phosphatase
BMU(s)	basic multicellular unit(s)
BMD	bone mineral density
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dNTP(s)	deoxynucleoside triphosphate(s)
DPBS	Dulbecco's phosphate buffered saline
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
FCS	foetal calf serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
JNK	c-Jun N-terminal kinases
LSM	lymphocyte separation medium
M-CSF	macrophage colony-stimulating factor
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
M-BMM	murine bone marrow macrophages
NFATC1	nuclear factor of activated T-cells, cytoplasmic 1
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID(s)	non-steroidal anti-inflammatory drug(s)
PBMC(s)	peripheral blood mononuclear cell(s)
PBS	phosphate buffered saline
PCR	reverse transcription polymerase chain reaction
PPI(s)	proton pump inhibitor(s)
RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor κ B ligand
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction

SEM	standard error of the mean
SRB	sulforhodamine B
TNF	tumour necrosis factor
TRAP	tartrate resistant acid phosphatase
VATP	vacuolar adenosine triphosphatase
Wnt	wingless type

1 Introduction

1.1 PPIs

Pantoprazole, or 6-(difluoromethoxy)-2-[(3,4-dimethoxyphenyl)methylsulfinyl]-1H-benzimidazole, or $C_{16}H_{15}F_2N_3O_4S$, is a drug of the proton pump inhibitor (PPI) family. In the 1980s, this group of drugs was introduced into clinical practice, its first representative being omeprazole [Savarino 2009]. Since then, PPIs have been a major factor in treating peptic ulcers, gastroesophageal reflux disease, and preventing gastrointestinal complications under non-steroidal anti-inflammatory drug (NSAID) therapy, their efficacy and tolerance surpassing previously established therapies [Chey 2007, Katz 2013, Remes-Troche 2014].

Molecular alterations to the structural base, timoprazole, subsequently led to the introduction of pantoprazole (*Figure 1*) [Database 2005a, b], which has a lower potential for drug interaction [Devault 2007]. The high solubility and solution stability led to its additional admission for intravenous application [Kromer 1990].

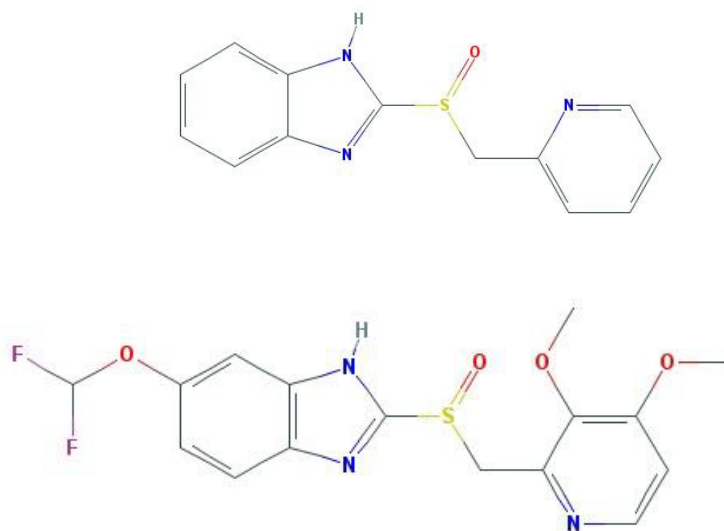


Figure 1: Chemical structure of the PPIs' structural backbone timoprazole (above) and pantoprazole (below) [Database 2005a, b]

PPIs are weak bases that convert from their inactive prodrug state into active metabolites in an acidic environment. The activated drug covalently bonds to the luminal proton pump of gastric parietal cells and irreversibly inhibits proton secretion [Lorentzon 1985, Lorentzon 1987, Shin

2013, Shin 2008]. Thus, the stomach pH is increased and the mucosa protected from acid detriment [Fryklund 1988].

1.2 BMUs

Through close interaction of osteoblasts and osteoclasts, which together form the so-called basic multicellular units (BMUs), an exact equilibrium between the formation of new bone and the disassembly and resorption of obsolete bone matrix is realised [Frost 1964, Rucci 2008].

1.2.1 Osteoblasts

Osteoblasts originate from mesenchymal stem cells that differentiate into preosteoblasts, mature osteoblasts, and finally osteocytes. An important element in controlling and regulating osteoblast differentiation is the wingless type (Wnt) pathway. Operating downstream of the bone morphogenetic protein and hedgehog signalling paths, it leads to the differentiation of mesenchymal stem cells into mature osteoblasts and blocks the pathways that alternatively lead to adipocytic and chondrocytic differentiation. This cascade is inhibitorily controlled by the Dickkopf-1 glycoprotein. Osteoprotegerin (OPG), which is a decoy receptor for receptor

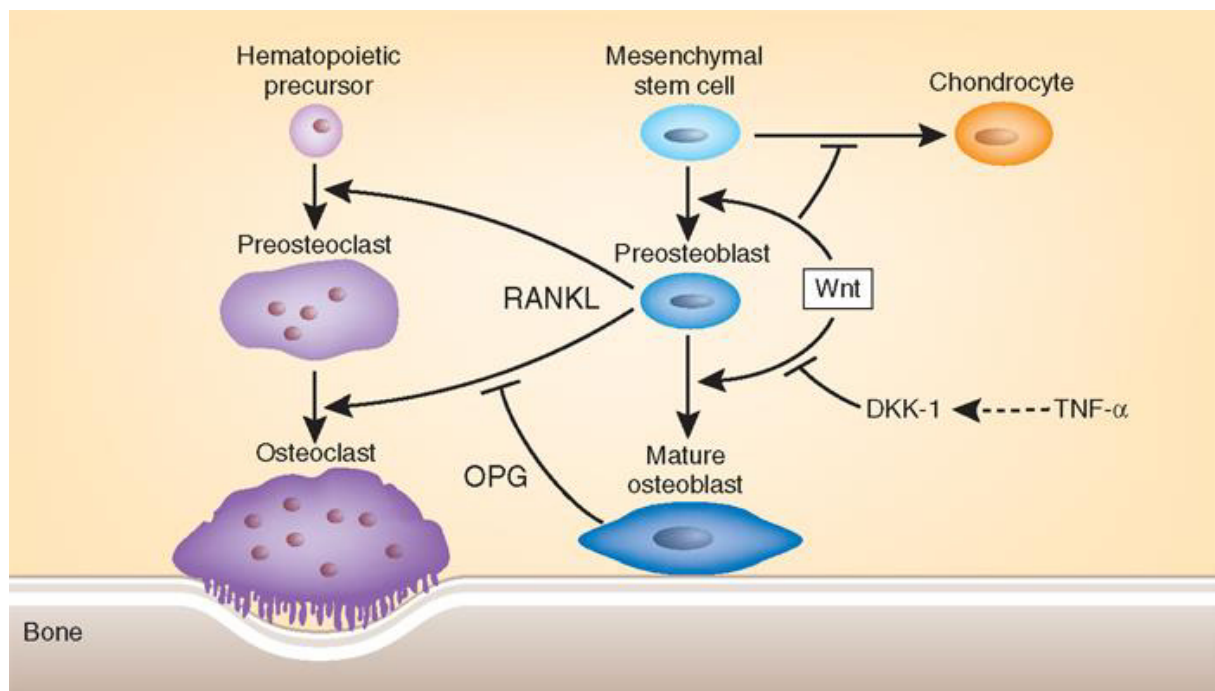


Figure 2: The Wnt pathway and its role in the differentiation of mesenchymal stem cells into mature osteoblasts, which in turn influence the differentiation of hematopoietic precursors into active osteoclasts. OPG=osteoprotegerin, RANKL=receptor activator of nuclear factor κ B ligand, Wnt=wingless type [Goldring 2007]

activator of nuclear factor κ B ligand (RANKL) synthesised by osteoblasts, acts as the functional inhibitor to osteoclastogenesis (*Figure 2*) [Goldring 2007]. Osteoblasts possess the ability to synthesise collagen I-rich extracellular matrix (ECM), which is mineralised over time with inorganic compounds, mostly hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). This leads to the progressive encapsulation of osteoblasts that ultimately become osteocytes. Through connections to adjacent osteocytes via canaliculi and signalling via gap junctions, they uphold ECM integrity [Hambli 2014].

1.2.2 Osteoclasts

Osteoclasts, the functional counterpart to osteoblasts, differentiate from hematopoietic stem cells. Through signalling substances of the tumour necrosis factor (TNF) group, surface receptors of progenitor cells are activated and intracellular signal cascades stimulate cell differentiation (*Figure 3*). In their work with murine bone marrow macrophages (M-BMM),

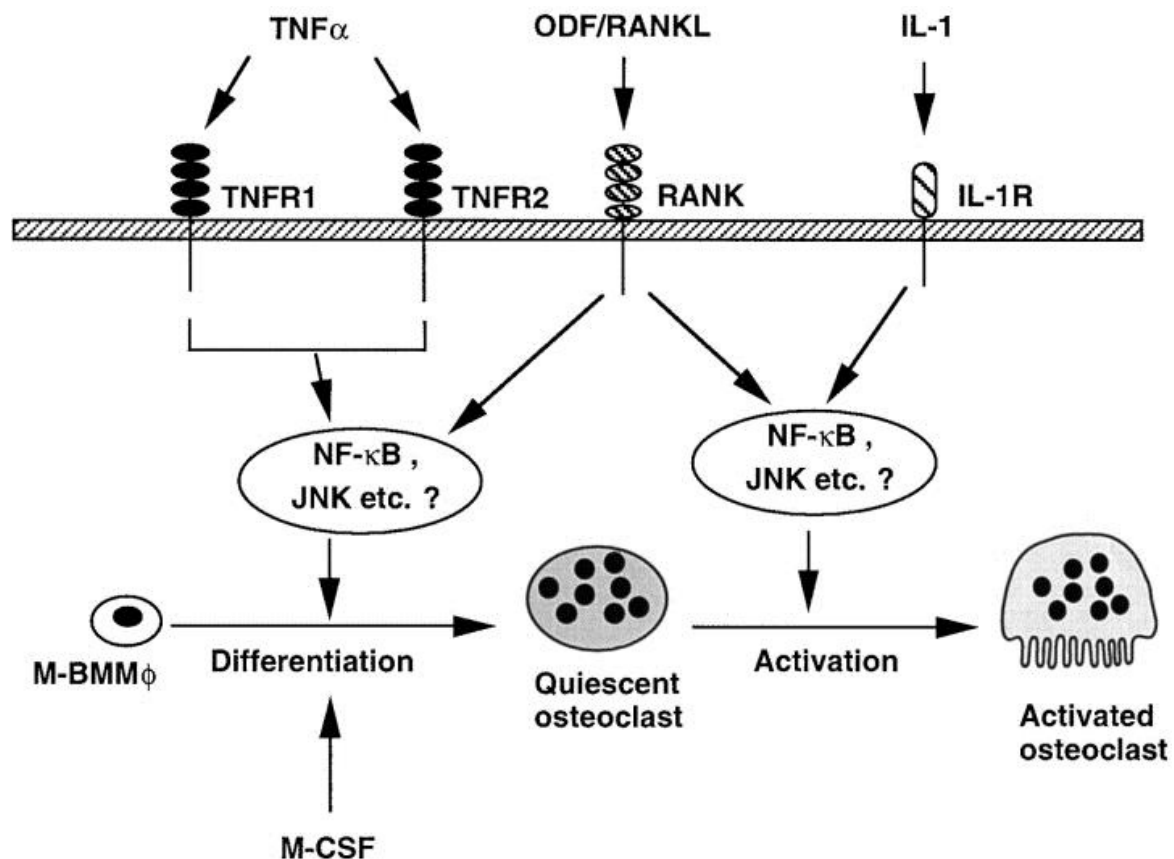


Figure 3: The regulation of osteoclast differentiation and activation is closely controlled through RANK, NF- κ B and JNK activation. First, under the additional influence of M-CSF, multinucleated quiescent osteoclasts are formed that are then activated based on demand. JNK=c-Jun N-terminal kinases, M-CSF=macrophage colony-stimulating factor, NF- κ B=nuclear factor kappa-light-chain-enhancer of activated B cells, RANK=receptor activator of nuclear factor κ B, $\text{TNF}\alpha$ =tumour necrosis factor α , TNFR=tumour necrosis factor receptor [Kobayashi 2000]

Kobayashi *et al.* showed that differentiation and activation of osteoclasts occur in several steps that include TNF-dependent activation of tumour necrosis factor receptor (TNFR) and RANKL-dependent receptor activator of nuclear factor κ B (RANK) activation [Boyce 2010, Kobayashi 2000]. This leads to downstream activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and c-Jun N-terminal kinases (JNK) and subsequently to an alteration of gene expression.

Activated mature osteoclasts are multinucleated cells that can actively degrade and absorb bone. To do this, osteoclasts bind to bone matrix via integrins and form a tight seal. This forms a compartment, which is subsequently acidified through the activity of vacuolar proton pumps. Interestingly, these proton pumps are potentially influenced by PPIs [Mattsson 1991]. In this acidic microenvironment, special enzymes, such as cathepsin K, then break down the extracellular matrix [Gruber 2015].

1.2.3 Cell-cell interaction and bone metabolism

Bone metabolism is finely regulated to ensure adequate adaptation to the constantly changing demands of the musculoskeletal system [Martin 2008]. This requires constant intercellular communication and signalling (*Figure 4*) [Sims 2014a, Sims 2014b].

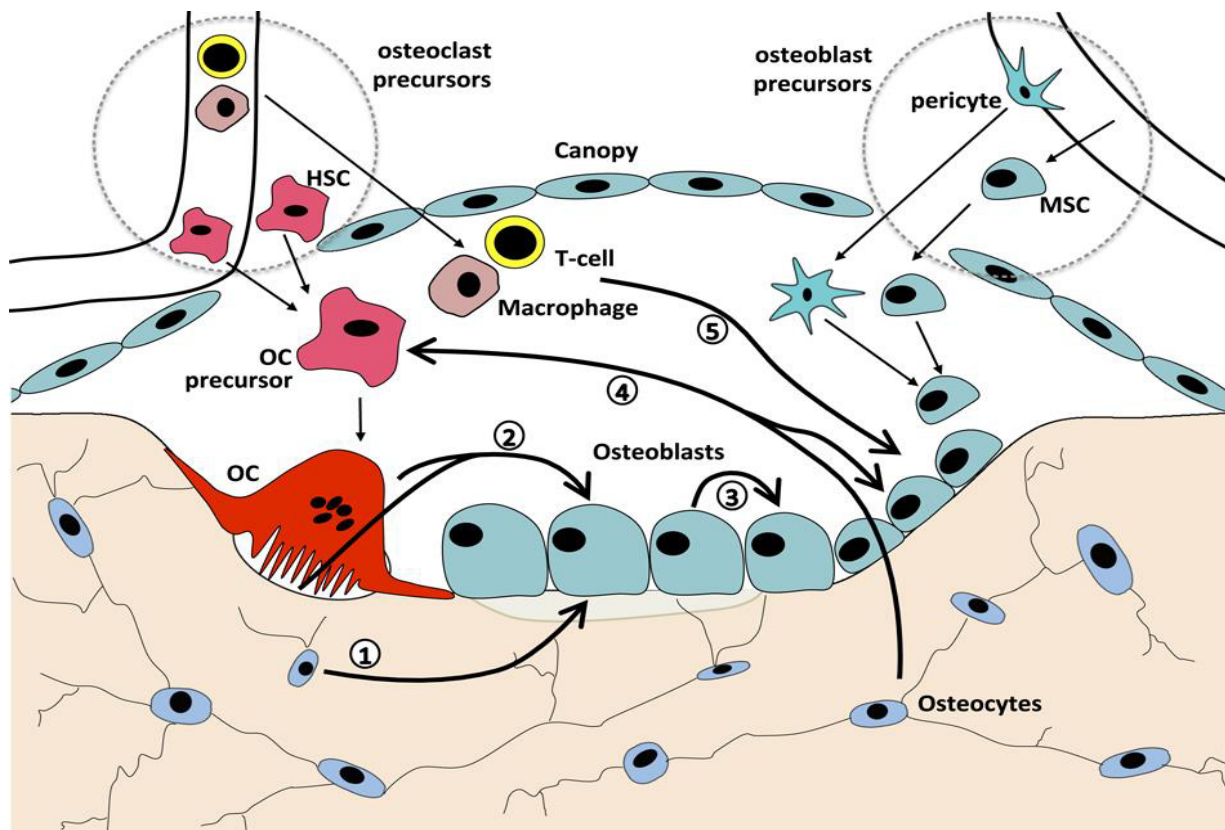


Figure 4: Intercellular signalling within the BMU. Osteocytes signal to osteoblasts and osteoclasts. Osteoclasts, in turn, signal to osteoblasts and *vice versa*. Intercellular signalling induces cell migration, proliferation and differentiation. HSC=hematopoietic stem cell, MSC=mesenchymal stem cell, OC=osteoclast [Sims 2014a]

Paracrine secretion of RANKL by osteoblasts and osteocytes directly affects osteoclastogenesis through binding the RANK receptor, which is an integral transcription factor of the TNF group. In contrast, osteoprotegerin, also known as osteoclastogenesis inhibitory factor, is a decoy receptor synthesised by osteoblasts that is able to bind RANKL and thus prevent the maturation of precursor cells into active osteoclasts. Additionally, osteoblasts are also able to synthesise and secrete macrophage colony-stimulating factor (M-CSF), an important factor in the differentiation of osteoclast precursor cells [Matsuo 2008]. Through the alternating activation and deactivation of BMUs, physiological bone metabolism is always well adapted to the current specific requirements [Rosenberg 2012].

1.3 Clinical use and detrimental effects of PPIs

Since their introduction, PPIs have quickly gained a high popularity and prescription for having a favourable safety profile [Lodato 2010]. The most common adverse effects are mild and include gastrointestinal distress, headaches and allergic reactions. Over the years, the prescription of PPIs has inflationarily increased, without the critical evaluation of the respective indication. In recent years, this prompted the assessment of potential detrimental effects of PPI administration in many clinical, as well as experimental studies.

Unwanted consequences of PPI use are multifaceted. Omeprazole, for instance, strongly interacts with the cytochrome P450 system and thus interacts with the efficacy of other drugs, such as platelet inhibitors [Arbel 2013]. This effect is less predominant in other PPIs, such as pantoprazole. Furthermore, PPI administration is an independent risk factor for infectious pathologies, such as pneumonia and gastrointestinal complications, especially in ICU patients [Buendgens 2014, Eom 2011, Herzig 2014]. Even acute hepatotoxic and nephritic reactions have been described [Aslan 2014, Klassen 2013]. Interestingly, there are also studies that refute the hypothesis of PPIs causing many of such complications [Filion 2014].

Most interesting for our experiments were obviously studies concerning PPI use and fracture risk and loss of bone mineral density (BMD). Curiously, the results from the different studies are very diverse. Some of these studies found increasing fracture risk and BMD loss others did not. In any case, the causal relation is not yet proven [Adams 2014, Cea Soriano 2014, Ding 2014, Gray 2010, Ho 2014, Insogna 2009, Kaye 2008, Maggio 2013].

1.4 Experimental approach

The strong heterogeneity in clinical, as well as experimental studies concerning detrimental effects of PPIs on bone salubrity and the potential underlying pathomechanisms, prompted us to design a string of experiments that investigate the effects of PPI administration, in this case pantoprazole, on a cellular level. As mentioned before, bone metabolism, regulated in its core through BMU activity, is reliant on the appropriate regulation of their cellular subunits. Unlike other studies that focused on experimenting with murine or human precursor cells, we decided to concentrate on the isolation of primary human osteoblasts and peripheral blood mononuclear cells (PBMCs) and their respective differentiation into active cells. Since the administration of PPIs and subsequently the described adverse effects are mostly occurring

in older patients, we tried to select donors of higher age. This was easily achieved for the isolation of osteoblasts, since the bone samples from which the cells were isolated, were cancellous bone harvested after hip replacement surgery, a procedure that is in itself predominant in patients of higher age. Thus, patient age ranged from 75 to 91 years. Since the isolation of PBMCs depended on donated buffy coats, the highest possible age range was 50 to 67 years.

When assessing the effects of a drug on cellular level, several components need to be taken into account. Firstly, cytotoxicity plays a key role in evaluating the safety, or in this case, potential adversity of a drug. If a drug is cytotoxic to a specific cell line *in vitro* in concentrations that it is clinically used in, adverse effects on the *in vivo* organism are possible. We evaluated the extent of cytotoxicity by quantifying cell viability as well as relative cell count. The second essential component of cellular function is gene expression. In this case, we examined corresponding genes of essential functional proteins of each of the two cell types, respectively. For osteoblasts, those included runt-related transcription factor 2 and activating transcription factor 4, two major transcription factors of osteogenic differentiation. The matrix proteins $\alpha 1$ (I) collagen, osteocalcin, and osteopontin and finally, RANKL and osteoprotegerin expression, were analysed to illustrate effects on cell-cell interaction, these two factors having antagonistic function with respect to osteoclast activation and inhibition. Accordingly, the transcription factors NF- κ B and nuclear factor of activated T-cells, cytoplasmic 1 (NFATC1) were analysed in osteoclasts, as well as the functional protein tartrate resistant acid phosphatase (TRAP) and a subunit of the vacuolar adenosine triphosphatase (VATP). Furthermore, synthetic, as well as cellular functional activity were assessed. This included the ability of osteoblasts to synthesise protein versus the ability of osteoclasts to resorb complexed calcium.

Pantoprazole is most commonly prescribed in the doses of 20 mg or 40 mg *per diem*. These doses correlate to serum concentrations of 1 – 1.5 μ g/ml and 2 – 3 μ g/ml, respectively. Since these are the concentrations that are most relevant to the clinical application of the drug, they were at the centre of our experimental focus. Additionally to examining effects of the most common serum concentrations, we added a stimulation concentration of one decimal power above, as well as below. The corresponding concentrations are 0.1 μ g/ml and 10 μ g/ml, respectively. These additional measurements were performed to evaluate the consequence of fluctuations in serum concentrations that may quickly occur when drug ingestion is carried out erratically, or irregularly. Finally, a negative control that contained 0 μ g/ml pantoprazole was included.

2 Methods

2.1 Cell isolation and culture

2.1.1 Osteoblasts

Primary human osteoblasts were isolated from cancellous femur heads after total endoprosthetic hip replacement surgery. All of our research was performed after a positive vote from the ethics committee of Technical University Munich and in accordance with the Declaration of Helsinki. Patient consent was obtained prior to collection. The bone samples were collected in sterile containers and processed strictly under a sterile bench to minimise the risk of contamination. In order to isolate osteoblasts, small bone fragments were taken using sterile gloves and a Stille-Luer bone rongeur. The fragments were gathered into 50 ml Falcon tubes (depending on the amount of fragments, more than one Falcon tube was required) and washed repeatedly with Dulbecco's phosphate buffered saline (DPBS). When the residue was mostly blood- and fat-free, the now clean bone fragments were transferred into 175 cm² culture flasks with about 10 ml bone fragments per flask. Now the culture medium, which consisted of 500 ml low glucose Dulbecco's modified eagle medium, 0.05 M L-ascorbic acid, 10% foetal calf serum (FCS) and 100 U/ml penicillin and 10 mg/ml streptomycin was added. Initially, the bone fragments were incubated at 37°C and 5% CO₂ over a week and remained untouched during that period, as to not disturb the initial cell proliferation. The first medium replacement was then performed with considerable care, as to not dislodge any bone fragments. This process was repeated after an additional seven days. Cell proliferation could be observed after approximately 7 days and reached satisfactory confluence after 2 to 3 weeks. Media replacements took place twice a week. For cell passaging, the bone fragments were removed and the residue was washed away with DPBS. After incubation for roughly 7 to 10 minutes with trypsin-ethylenediaminetetraacetic acid (EDTA) solution that caused cell detachment, the cells were resolved in culture medium and distributed evenly into new culture flasks. After 3 passages, the cells reached the desired quantity and purity for our experimental setup. Here, cells were plated at a density of 1·10⁴/cm² (*Figure 5*) and stimulated with a solution of pantoprazole (as mentioned above) in differentiation medium that contained 500 ml low glucose Dulbecco's modified eagle medium, 10 mM β-glycerol phosphate, 1.56 mM calcium chloride·2H₂O, 5% FCS, 100 U/ml penicillin and 10 mg/ml streptomycin, 0.2 mM L-ascorbic

acid, 100 nM dexamethasone and 0.025 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer.

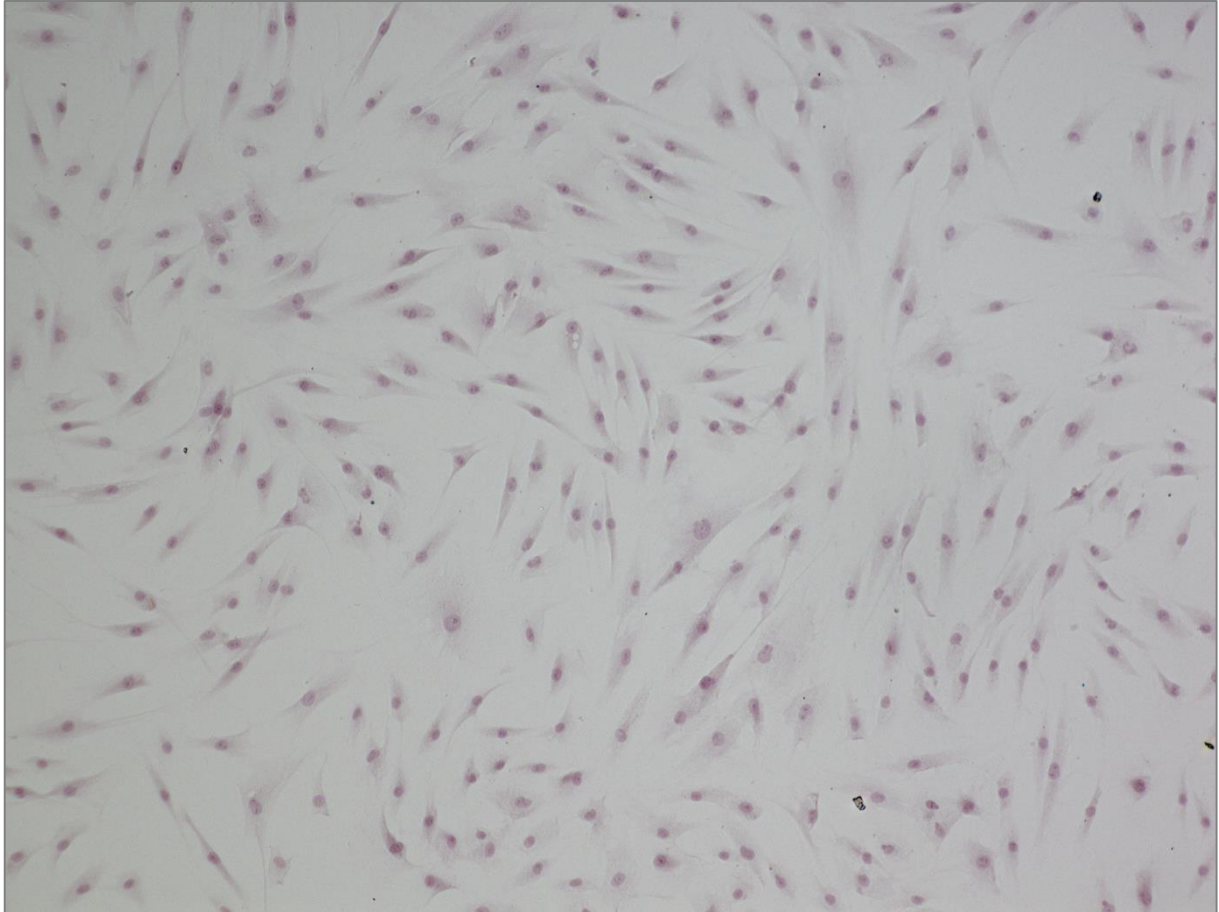


Figure 5: Depicted are primary human osteoblasts 12 hours after plating at 10x magnification. The applied alizarin red stain has little to no effect since extracellular matrix mineralisation has not yet taken place.

2.1.2 Osteoclasts

In order to obtain a sufficient amount of osteoclasts for our experimental setup, we obtained so-called buffy coats from the Bayerisches Rotes Kreuz Blutspendedienst (Bavarian Red Cross Blood Donation Centre, Ulm, Germany). Buffy coats form during the centrifugation of whole blood as a layer between the plasma and erythrocytes and are especially rich in leucocytes and thrombocytes. For the separation of PBMCs, the buffy coats were layered upon lymphocyte separation medium (LSM) with a density of 1.077 g/ml (Biowest, Nuaille, France). Following centrifugation, the developed interphase that contains lymphocytes and monocytes was pipetted off and transferred into a falcon which contained 40 ml DPBS. After further centrifugation, the

PBMCs formed a pellet in the bottom of the Falcon tube which was resuspended in DPBS and centrifuged once more for additional purification. The cells were then resuspended in culture medium, counted, and plated at a concentration of $3 \cdot 10^6$ cells per well on 48-well plates and $6 \cdot 10^6$ million cells per well on 24-well plates. The culture medium contained 500 ml alpha modification minimum essential medium, 10% FCS, 100U/ml penicillin, and 10 μ g/ml streptomycin. For the first 6 days, 25 ng/ml M-CSF were added to the culture medium. Starting on day seven, half the culture medium was replaced with culture medium containing 20 ng/ml RANKL. Subsequent media replacements were performed with only the RANKL-supplemented medium until after 24 days, when the stimulation experiments were initiated.

2.2 Gene expression analysis on mRNA level

2.2.1 RNA isolation

Semi-quantitative gene expression analyses on messenger ribonucleic acid (mRNA) level were performed after reverse transcription polymerase chain reaction (RT-PCR). In order to gather the total ribonucleic acid (RNA), the medium supernatant was pipetted off and replaced with Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA). The samples were then frozen at -80°C . Afterwards, the cells were detached and harvested from the wells using a cell scraper and transferred into 2 ml tubes. After 5 minutes of incubation on ice, chloroform was added and the solution was vortexed shortly. After further 10 minutes of incubation on ice, centrifugation at 14,000 g and 4°C , the aqueous phase that contained the RNA was pipetted into prepared tubes containing isopropanol. Here, the RNA formed a pellet in the bottom of the tube after further centrifugation. The isopropanol supernatant was decanted and two washing steps with ethanol followed. After the final washing step, the ethanol was again decanted and residual ethanol was left to evaporate. The isolated RNA was resuspended in *aqua ad iniectabilia* and stored at -80°C .

2.2.2 Transcription of RNA to cDNA

The transcription of the isolated RNA into complementary deoxyribonucleic acid (cDNA) was executed using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). In short, the RNA was mixed with primers and denatured at 65°C to allow primer annealing. Afterwards, an abundance of deoxynucleoside triphosphates (dNTPs) was added to ensure

sufficient elongation of the annealed primers by the simultaneously added reverse transcriptase. After completing the transcription cycles at 37°C in the Eppendorf MasterCycler S (Eppendorf, Hamburg, Germany), the resulting cDNA was diluted in *aqua ad iniectabilia* and stored at -20°C.

2.2.3 RT-PCR

Our RT-PCRs were performed in accordance with an established standardised protocol. Essentially, the addition of specific forward and reverse primers (Eurofins, Ebersberg, Germany) and dNTPs to our cDNA samples allowed the enzyme Taq deoxyribonucleic acid (DNA) polymerase to elongate the primers (for specific primer sequences, please refer to the attached research articles), which had annealed to their respective loci on the cDNA (PCR reagents: Axon Labor Technik, Kaiserslautern, Germany). Each of the 35-40 cycles consisted of each of these individual steps: denaturation (preformed double-stranded DNA segments are separated at a temperature of 95°C), annealing (the primers bind to their corresponding cDNA segment; the optimal temperature for this process is unique to each primer), and extension (DNA polymerase binds to the primer and elongates the DNA complementary to its cDNA counter strand). Finally, after all cycles had been completed, the samples were cooled down to 4°C and were immediately used for further analyses.

2.2.4 Semi-quantitative analysis of PCR products

The gel used to separate and visualise DNA boundaries consisted of 1.8% w/v agarose and 7 µg ethidium bromide. After the cast of the gel was cooled down to room temperature, the plasmid cloning vector pUC19 was added to each row of loading pockets in order to mark the boundaries of different lengths of DNA strands. The remaining pockets were then loaded with the PCR products. Electrophoresis was run at 90 V for 40 minutes. After the separation of the respective PCR products, pictures were taken. Semi-quantitative analysis was performed using the ImageJ software (NIH, Bethesda, MD, USA), through measuring the signal intensity over each individual boundary. The signal intensity of each of the investigated genes was afterwards normalised to the housekeeping gene β -actin.

2.3 MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay measures the ability of cells to reduce MTT to its formazan, which corresponds to a change of colour from yellow to purple. The samples were incubated with MTT solution over two hours at 37°C and 5% CO₂. Over this time period, the reduction of the yellow MTT to the purple formazan took place. The formazan was then dissolved in a solution of 5 g sodium dodecyl sulphate (w/v), 49.7 ml dimethyl sulfoxide, and 0.3 ml acetic acid and its concentration was subsequently photometrically detected and quantified.

2.4 Osteoblast-specific assays

2.4.1 EdU fluorescence staining

The proliferation rate of our cell samples was evaluated through fluorescence staining. Basically, the 5-ethynyl-2'-deoxyuridine (EdU) stain is a thymidine analogue that is integrated into the replicating genome. In combination with Hoechst 33342, which stains all DNA, the relative number of replicating cells, *i.e.* the proliferation rate, could be ascertained by dividing cells that emitted both fluorescence patterns by all cells that were affected by the Hoechst stain (*Figure 6*). In order to allow sufficient integration into the replicating genome, the samples were incubated with the EdU stain from the Click-iT®EdU Alexa Fluor® 488 Imaging Kit (Life Technologies, Carlsbad, CA, USA) for a period of 24 hours over night. After fixation and Hoechst staining over 30 minutes, fluorescence pictures were taken of representative fields of view of both the EdU and the Hoechst stain, respectively, with the BZ-9000 fluorescence microscope (Keyence, Osaka, OSK, Japan). In order to analyse the proliferation rate, a cell counting template was created using the BZ-II Analyzer (Keyence, Osaka, OSK, Japan) that could overlay the two stained pictures, differentiate signals according to size and shape and thus discern dyed cells from cell fragments and dye artefacts.

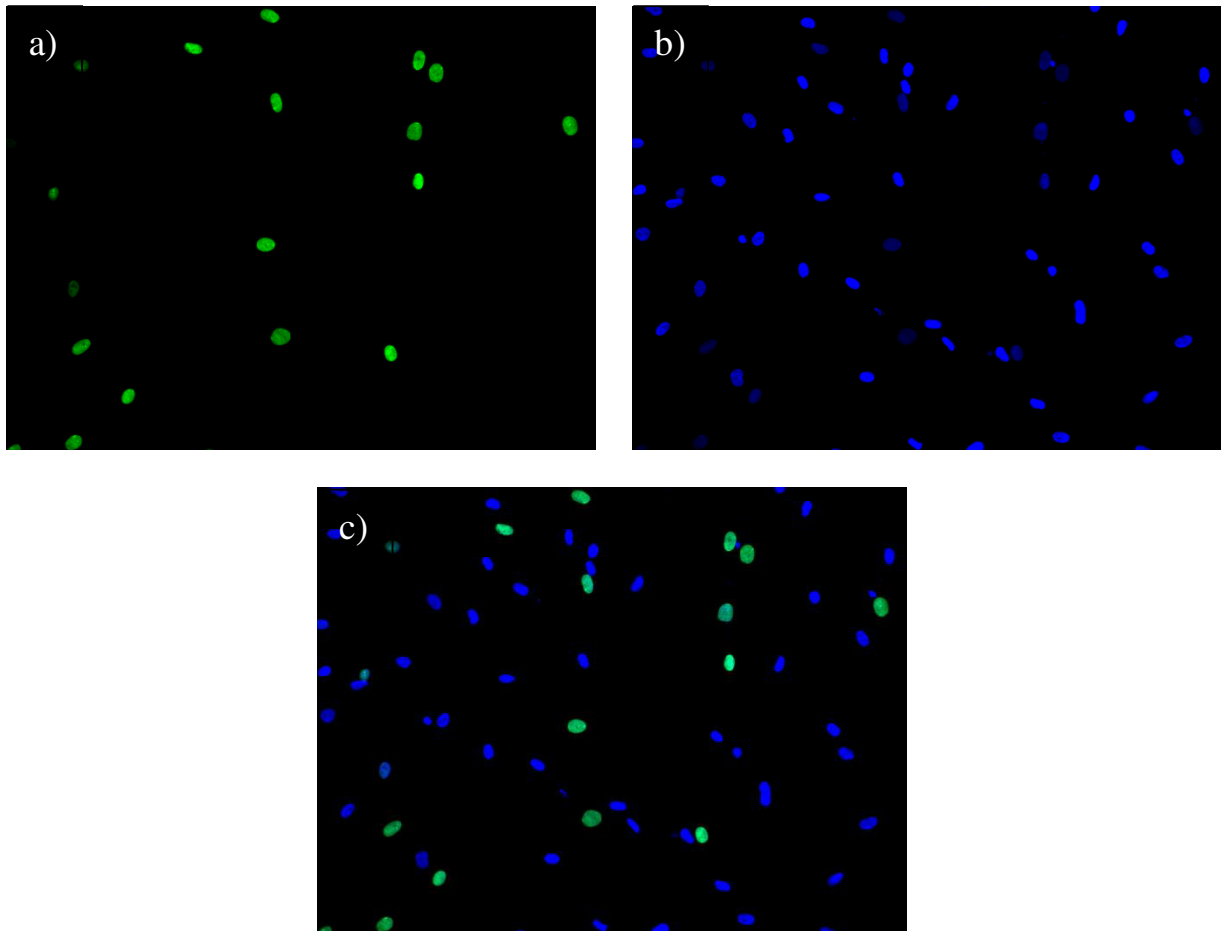


Figure 6: Depicted are the EdU stain a), the Hoechst DNA stain b), and the overlay of the two stainings that was used to calculate the proliferation rate c) 12 hours after plating at 20x magnification.

2.4.2 Relative cell count measurement

The CyQuant® NF Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA, USA) is a fluorescence dye that binds to DNA. Since the DNA content is closely proportional to the cell number, the fluorescence signal of a DNA stain can be taken as a surrogate parameter to measure the cell number. In this case, we related the absolute fluorescence signal of each sample to the value of the control sample to receive the relative cell count. The assay was performed according to the manufacturer's protocol that included washing the cells with DPBS and incubating them with the dye reagent over 60 minutes at 37°C. The fluorescence intensity was photometrically quantified and the cell count was expressed relative to the control.

2.4.3 SRB staining and quantification

Sulforhodamine B (SRB) is a dye that unselectively binds cellular protein. Through photometric measurement of the resuspended dye, quantitative analysis of the protein content in stimulated samples relative to the control can be performed. Briefly, the stimulation medium was removed and the cells were washed with DPBS and fixated over 15 minutes with methanol. Afterwards, the SRB solution, containing 200 mg SRB and 50 ml acetic acid, was added and incubated for 30 minutes on a shaker to ensure even distribution of the dye. Then, the SRB solution was removed and the samples were washed once with 1% acetic acid solution. The bound SRB was dissolved in 10 mM unbuffered tris(hydroxymethyl)aminomethane solution and the SRB content was photometrically quantified.

2.4.4 AP activity measurement

Alkaline phosphatase (AP) is an enzyme that is found in, yet not specific to, osteoblasts and is used as a marker for osteogenic differentiation. The ability of the enzyme to dephosphorylate molecules enables us to use it in a colour-changing reaction of para-nitrophenylphosphate to para-nitrophenol. Therefore, the samples were washed twice with DPBS and covered with freshly prepared AP substrate solution, which consisted of 1.3 mg 4-nitrophenyl phosphate disodium salt hexahydrate in a buffer of 2-amino-2-(hydroxymethyl)-1,3-propanediol, glycine and magnesium chloride. After 30 minutes, triplicates of each sample were transferred into 96-well plates and the concentration of para-nitrophenol was photometrically quantified and the AP activity was calculated according to a standard curve that was freshly prepared and measured each day.

2.5 Osteoclast-specific assays

2.5.1 Von Kossa staining

The ability to degrade mineralised bone matrix is one of the core functions of fully differentiated osteoclasts. Therefore, we used von Kossa staining on calcium phosphate-coated wells (Corning, Tewksbury, MA, USA). The von Kossa stain dyes calcium and calcium salts and is thus able to reveal loci of osteoclast activity, which are in this case represented by the absence of the stain (*Figure 7*). In order to fully reveal resorption pits, the medium was removed

and the samples were incubated with sodium hypochlorite in order to remove the remaining osteoclasts. After two washing steps with distilled H₂O, the 3% silver nitrate in H₂O dye was added and incubated for 30 minutes. After the staining solution was removed, the samples were washed repeatedly with distilled H₂O over 10 minutes. Then, incubation with 1% pyrogallol in H₂O was carried out, followed again by two washing steps with distilled H₂O. Finally, the samples were fixated by incubating them with a 5% solution of sodium thiosulphate in H₂O. After multiple final washing steps with distilled H₂O, representative pictures were taken with the BZ-9000 fluorescence microscope.

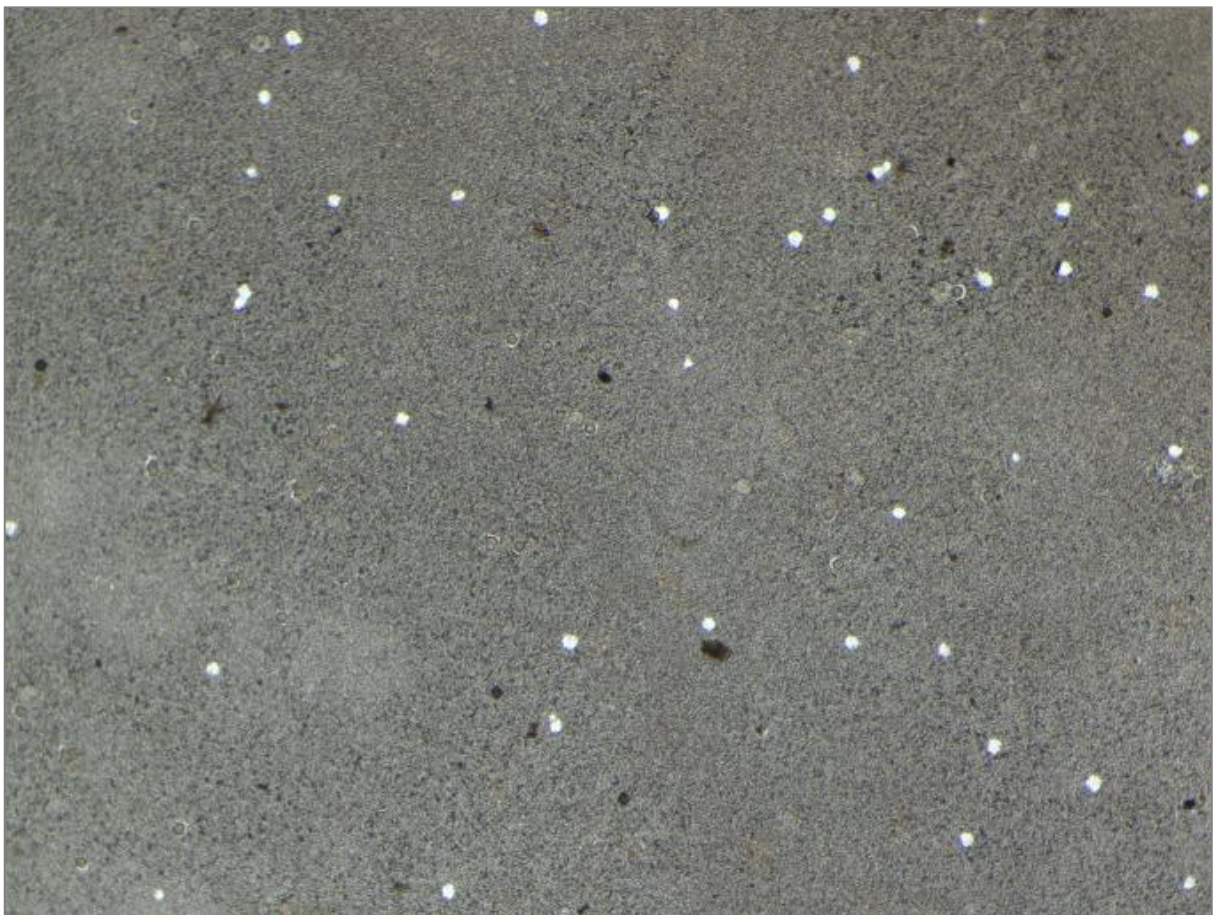


Figure 7: The image shows a calcium phosphate-coated, von Kossa-stained plate, 7 days after differentiation and at 20x magnification. The white spots represent former locations of osteoclasts, which have resorbed the local calcium phosphate. The formerly present osteoclasts have been lysed and removed during the staining process.

2.5.2 Toluidine blue staining

Another way to demonstrate the ability to resorb bone matrix is the visualisation of Howship's lacunae on dentine chips. This was realised by plating PBMCs on dentine chips immediately after isolation. The cells were differentiated on the dentine chips and over time formed the specific resorption pits, which can be stained with toluidine blue (*Figure 8*). Therefore, the cells were lysed with sodium hypochlorite and wiped off with paper towels. The chips were then shortly immersed in 80% ethanol and again dried off with paper towels. Then, the samples were shortly immersed in 1% toluidine blue in DPBS solution and immediately rinsed with tap water. The pictures were taken with the BZ-9000 fluorescence microscope.

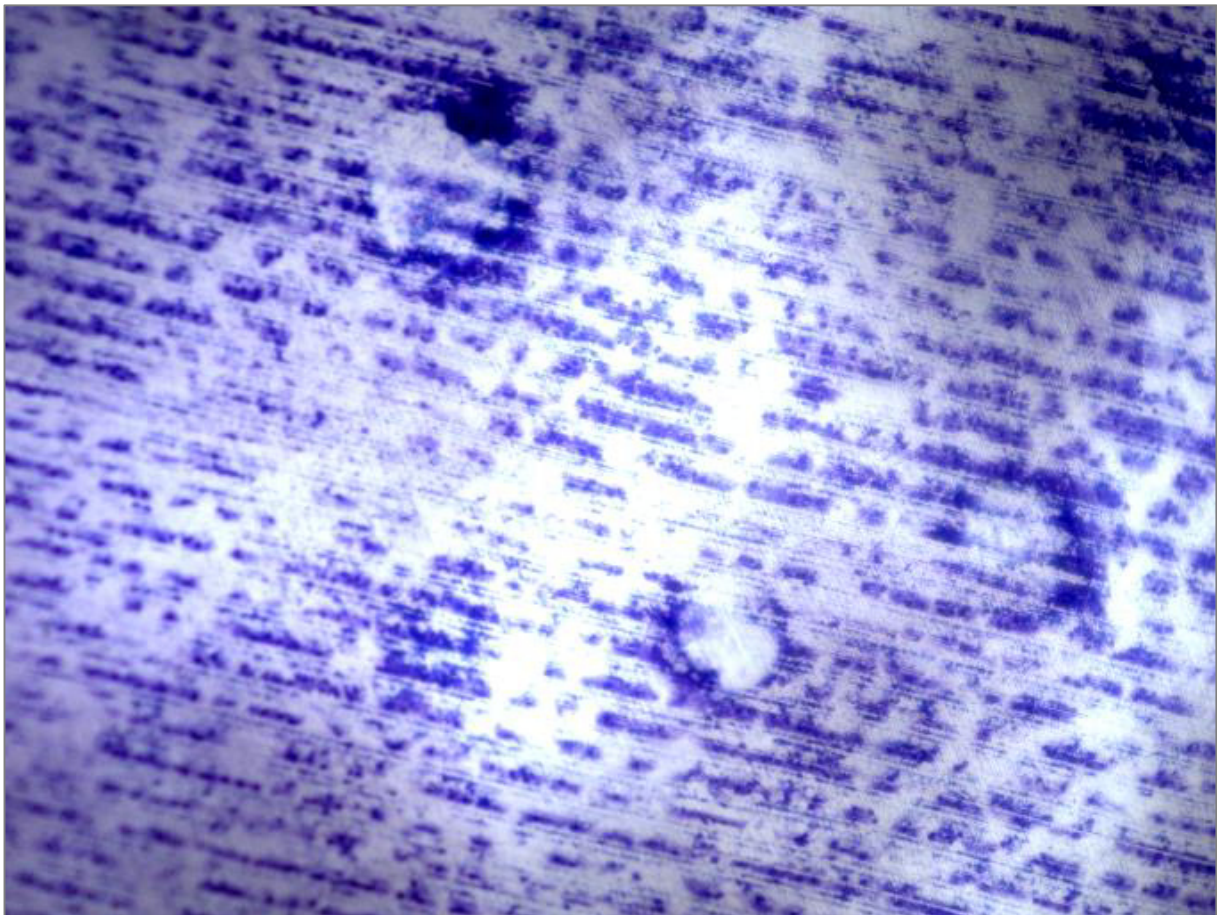


Figure 8: In this image, several Howship's lacunae, visualised by toluidine blue staining of a previously osteoclast-laden dentine chip, can be observed. The picture was taken 7 days after differentiation at 10x magnification.

2.5.3 TRAP staining

TRAP is an enzyme that is highly expressed by differentiated osteoclasts. By selectively staining this enzyme, the formation of multicellular osteoclasts from PBMCs can be visualised (*Figure 9*). The staining is based on a dephosphorylation reaction that is catalysed by TRAP. Therefore, after fixation with a solution of formaldehyde and triton-X-100 in phosphate buffered saline (PBS) over 2 minutes, naphthol AS-MX phosphate disodium salt (Sigma Aldrich, St. Louis, MO, USA) and fast red violet LB salt (Sigma Aldrich, St. Louis, MO, USA) were added and incubated for 20 minutes at 37°C and 5% CO₂. Before taking pictures, the samples were carefully washed with DPBS. The pictures were then taken with the BZ-9000 fluorescence microscope.

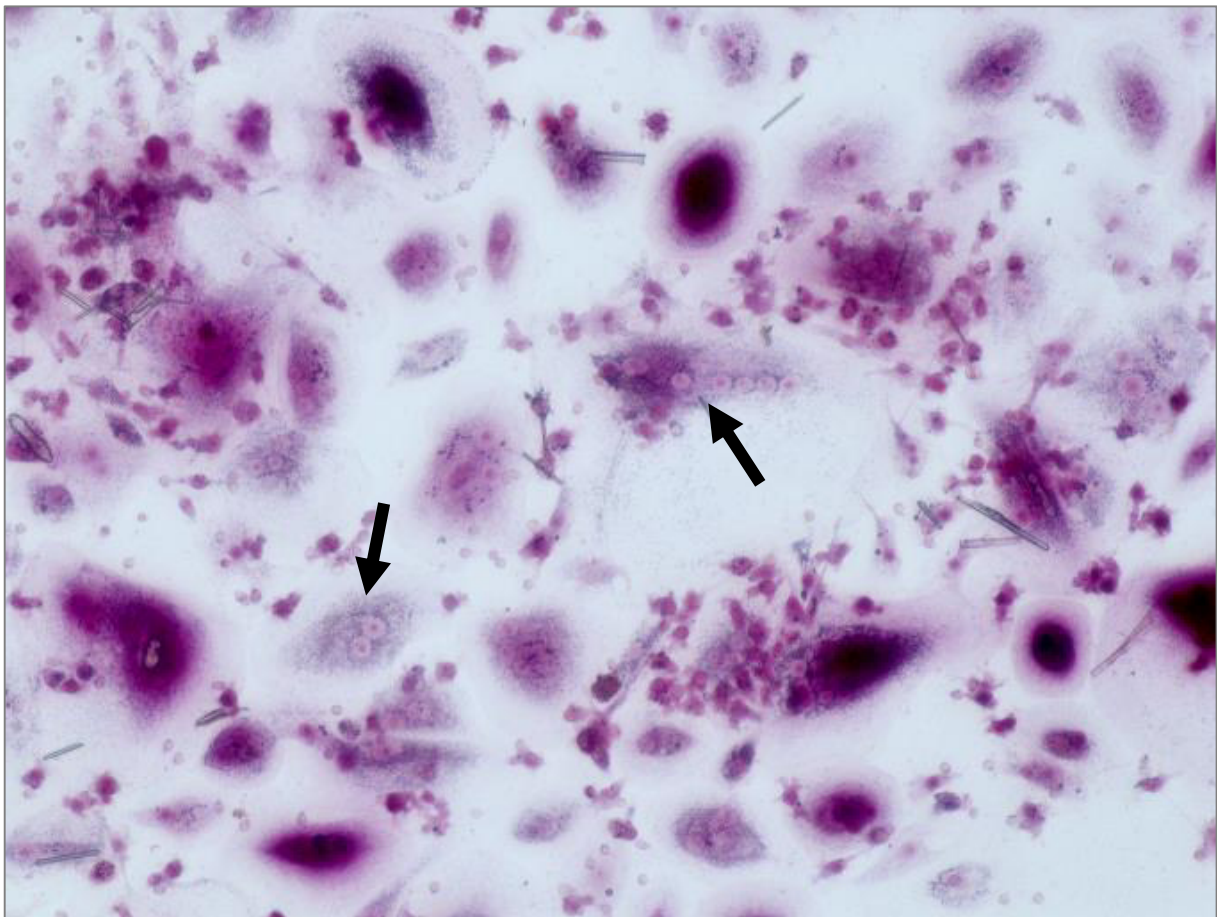


Figure 9: The image shows the formation of multinucleated cells and the heavy accumulation of the TRAP stain in said cells after 24 days of differentiation at 20x magnification. The black arrows indicate two exemplary multinucleated cells.

2.5.4 TRAP activity measurement

According to TRAP staining, the enzyme's activity can be measured to illustrate osteoclast function. Just as AP activity, TRAP activity can be quantified through photometrically detecting the formation of a dephosphorylation product over time. Therefore, triplicates of each sample's medium supernatant were collected and the substrate solution was added, which consisted of 1.9 mg 4-nitrophenyl phosphate di-sodium salt hexahydrate in a buffer of sodium acetate, sodium tartrate dibasic dehydrate and doubly distilled H₂O. After incubation at 37°C and 5% CO₂ over 60 minutes, the reaction was stopped by adding 3 M sodium hydroxide and the photometric measurements were performed. Finally, TRAP activity was calculated according to a freshly prepared standard curve.

2.5.5 Statistical analysis and depiction of data

GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used to statistically analyse the obtained data. Data comparison was performed using the non-parametric Kruskal-Wallis test and Dunn's multiple comparisons test. The minimum level of significance was defined as $p < 0.05$. Graphs were depicted as mean values \pm standard error of the mean (SEM).

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3.1 Summary of research article “Pantoprazole increases cell viability and function of primary human osteoblasts *in vitro*”

The aim of the research project was to prove or disprove adverse effects of the PPI pantoprazole on primary human osteoblasts in an *in vitro* environment. This is interesting, as there are several studies that found a potential correlation between the intake of PPIs and an increased fracture risk. Accordingly, the first sensible step for us was to determine in an *in vitro* environment whether or not the function and viability of osteoblasts and thus the formation of new bone is negatively influenced by the drugs.

In order to obtain primary osteoblasts, bone fragments were isolated from cancellous femur heads that were extracted during total hip replacement surgery. After several washing steps with DPBS and subsequent administration of differentiation medium, the proliferation of primary osteoblasts was initiated. After cultivation up to the 3rd passage to ensure sufficient purity, the cells were plated at a density of $1 \cdot 10^4/\text{cm}^2$. Our experimental setup included the administration of pantoprazole in concentrations ranging from 0.1 $\mu\text{g}/\text{ml}$ to 10 $\mu\text{g}/\text{ml}$, as well as a negative control of 0 $\mu\text{g}/\text{ml}$. The drug was administered every other day in a freshly prepared solution with differentiation medium over the course of one week. Experimental assays were performed after 1, 3, and 7 days, respectively. In order to adequately reveal any effects on the cells, experiments measuring cell count, viability/metabolic activity, protein synthesis, AP activity, and gene expression were conducted.

Cell viability, as well as total protein synthesis and AP activity assays, yielded slightly increased values on day 3 that were further increased on day 7 and were most remarkable in samples with high pantoprazole concentrations. Additionally, the cell count and proliferation rate showed no significant differences to the control, as well as between stimulated samples. Finally, gene expression rates showed a trend to slightly increased levels in stimulated samples on day 3 that returned to baseline levels on day 7.

Our findings suggest that, after short-term administration of pantoprazole in an *in vitro* environment, the ability of osteoblasts to remain metabolically active and synthesise protein,

rather than decreasing, was increased time- and dose-dependently. As far as such a statement can be made at this stage of experimental research, osteoporosis, as suggested by some authors to be one of the potential causes of the increased fracture risk, seems unlikely. Enhanced osteoblast activity in itself would consequently lead to higher bone density and not osteoporosis. Since no adverse effects on a cellular level could be found in our research, further studies are needed to reveal underlying pathomechanisms. The effects on other cells, most importantly osteoclasts, cell groups, and whole organisms should be evaluated. Also the existence of hitherto unknown confounding factors is a possible explanation for the alleged correlation between PPI use and fracture risk.

The design and execution of this research project was a group effort of all associated authors. As doctoral candidate and first author of this research article, it was my duty to do the majority of the work. Initially, I was elaborately introduced into each specific field, *e.g.* cell isolation and culture, the different functional assays, and the evaluation and statistical analysis of obtained data, by the respective specialist at our laboratory. This enabled me to perform the experiments either by myself, or, if necessary, with the help of one of the associated authors. A rough outline of the article was devised by me and was then brought to completion in consultation with all authors.

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M. Prause, C. Seeliger, M. Unger, M. van Griensven, and A. T. Haug, "Pantoprazole increases cell viability and function of primary human osteoblasts in vitro," *Injury*, vol. 45, pp. 1156-64, Aug 2014.

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3.3 Summary of research article “Pantoprazole decreases cell viability and function of human osteoclasts *in vitro*”

Following our first string of experiments with osteoblasts, it was the logical next step to examine the influence of pantoprazole on the osteoblast counterpart in the BMU, namely osteoclasts. As previously stated, a potential correlation between PPI use and increased fracture risk has been hypothesised. Thus, it was our intention to reveal detrimental effects on a cellular level, specifically on the cells responsible for bone formation and resorption.

In order to be able to collect enough cells of each donor, buffy coats were acquired and used for the isolation of PBMCs. After isolation and purification, the cells were differentiated into osteoclasts using differentiation medium, M-CSF, and subsequently RANKL. Corresponding to the osteoblast experiments, stimulation with 0, 0.1, 1, 3, and 10 µg/ml pantoprazole was carried out on alternating days with freshly prepared solutions. Furthermore, the experiments were performed after 1, 3, and 7 days. To confirm the osteoclast phenotype, several specific stains were performed: TRAP staining that directly depicts the presence of its eponym, which is a highly expressed protein in osteoclasts. Additionally, von Kossa and toluidine blue staining were performed to indirectly reveal osteoclast activity by visualising resorption pits and Howship’s lacunae. Functional analyses included TRAP activity measurements, MTT tests, and conventional RT-PCR analyses.

The aforementioned staining assays confirmed the successful differentiation of the isolated PBMCs into active osteoclasts after 1, 3, and 7 days, respectively. TRAP activity showed no significant differences until day 7, where a significant decrease in samples stimulated with 0.1 µg/ml and 1 µg/ml was apparent. Cell viability was significantly decreased on day 1 at 3 µg/ml pantoprazole and on day 7 at 10 µg/ml pantoprazole. The gene expression levels did not change significantly over the course of stimulation.

Overall, osteoclast activity seems to be slightly inhibited by the administration of pantoprazole. Decreased viability and TRAP activity ultimately lead to decreased bone resorption. When also taking into consideration the results from our previous studies that showed increased activity of osteoblasts after stimulation, it seems that the administration of pantoprazole would lead to decreased bone turnover, if *in vitro* findings can be extrapolated that far. In accordance with the conclusion of our previous publication, we think that osteoporosis as a cause of the increased fracture risk after the administration of PPIs is unlikely, at least through direct cellular effects.

Either a different pathomechanism, such as decreased mineralisation subsequent to impaired calcium absorption, as suggested by some authors, is responsible, or some underlying confounding factors distort the actual correlation between proton pump inhibitor use and the increased fracture risk.

Just as the previous research article, the design and execution of this project was a team effort. The isolation of PBMCs was performed by Alexander Tobias Haug and myself, the following purification and differentiation as well as the execution of the majority of the experiments was my task. PCR and gene expression analysis was performed with the help of my co-authors. After careful collection and evaluation of all data, I, once again, created the rough draft of our research article, which was then completed in coordination with the other authors.

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4 Results and Discussion

4.1 Osteoblast research

Overall, proliferation and cell count assays revealed no major differences between stimulated samples and the control over the course of our experiments. Solely, the highest stimulation dose of 10 µg/ml pantoprazole caused a slight decrease in relative cell number after 7 days of stimulation. Cell viability displayed a time- and dose-dependent increase that was significant at doses of 1 µg/ml and above, after 7 days. Total protein synthesis and AP activity increased in a corresponding manner, yielding significant differences after 3 and 7 days of stimulation, especially in concentrations of 1 µg/ml and above. Gene expression amplified dose-dependently after 3 days. However, this trend remained insignificant and returned to baseline values after 7 days of stimulation.

4.2 Osteoclast research

Osteoclasts seemed to react in an opposite manner. Cell viability decreased, even significantly after 3 days at 1 µg/ml and 7 days at 10 µg/ml pantoprazole. TRAP activity decreased significantly after 7 days at 0.1 µg/ml and 1 µg/ml pantoprazole, respectively. Gene expression showed great variability and no trend or significant differences were observed.

4.3 Discussion

The safety, efficacy, and detriments of PPI administration have been a common cause of discussion, especially in recent years. Even the latest published research and review articles reveal that the heterogeneity in the results and varying hypotheses of pathomechanisms still do not permit generally valid statements about the drugs' adverse effects in certain regards. In respect to effects on bone metabolism and fracture risk, published literature is likewise discordant. While an increased fracture risk in patients undergoing PPI therapy was found in meta-analyses, no causal relation has been proven and confounding factors remain a potential explanation for the correlation [Leontiadis 2014, Targownik 2012].

A prospective study by Ozdil *et al.* showed that PPI therapy leads to a decreased BMD, especially after long-term administration [Ozdil 2013]. Contrastingly, a review by Lau *et al.*

state that most research does not support the notion of decreasing BMD under PPI therapy and a review by Targownik *et al.* only states differing baseline BMD [Lau 2015, Targownik 2012]. In a review and meta-analysis by Ngamruengphong *et al.* the dose and duration dependence of fracture risk after PPI therapy was evaluated. Interestingly, detrimental effects were more apparent in short-term application, albeit there was, as many other authors also describe, significant heterogeneity between the surveyed studies [Ngamruengphong 2011].

Declining BMD, respective osteoporosis, independently of the explicit underlying pathomechanism and despite some studies arguing against it, remain the most common theories in regard to the cause of increased fracture risk after PPI administration. Thus, it was our goal to determine cellular short-term effects of pantoprazole *in vitro*. When evaluating fracture risk, or rather potential underlying pathomechanisms, the effects on the cells that are responsible for bone homeostasis are crucial. Hence, our experimental focus was on the differentiated human BMU subunits: osteoblasts and osteoclasts. In respect to fracture healing, short-term effects are especially important, since the regularly prescribed combination of NSAIDs and PPIs for the treatment of posttraumatic pain is usually not administered over extended periods of time. The investigation of both the bone synthesising, as well as degrading cell lines allow us to postulate differentiated hypotheses about effects on cellular bone homeostasis, as far as these can be extrapolated from *in vitro* findings.

In previous studies, Hyun *et al.* revealed in a murine *in vitro* model that PPIs, in their case represented by omeprazole, influence osteoblast and osteoclast activation [Hyun 2010]. Specifically, osteoblasts are moderately more active when exposed to omeprazole, the opposite being the case for osteoclasts. Subsequently, Costa-Rodrigues *et al.*, who worked with human osteoblast and osteoclast precursor cells, researched the effects of omeprazole, esomeprazole, and lansoprazole at varying stimulation doses [Costa-Rodrigues 2013]. Interestingly, they revealed detrimental effects on the viability and activity on both cell lines, albeit these effects were only present in supraphysiological doses of PPIs.

The varying theories as to how PPIs cause fractures or impair bone homeostasis, seem to be mostly based upon effects on the macroorganism and its hormonal balance, rather than direct cellular compromising. Even though some direct cellular effects, such as the inhibition of VATP that results in reduced bone resorption have been proven [Mizunashi 1993], these do not seem to affect the fracture risk significantly. As mentioned before, the formation of osteoporotic bone structure may be a major factor in the development of an increased fracture risk, but since

the administration of pantoprazole leads to increased osteoblast and decreased osteoclast activity, according to our studies, this seems unlikely. In accordance with our findings, which suggest only mildly impaired cellular function, the development of inferior bone matrix has to originate from BMU superordinate mechanisms.

When discussing the applicability of our research to *in vivo* organisms, one has to take into account that *in vitro* experiments take place under very specific conditions. For one, superordinate hormonal regulating systems that can potentially counteract, but also further impair detrimental effects on bone homeostasis are a nonfactor here. Thus, any and all effects that are in part, or entirely, due to the organism reaction to drug application and not direct cellular effects of the drug, cannot be detected. For instance, Schinke *et al.* showed in a cholecystokinin B receptor-deficient murine model that decreased gastric acidification subsequently leads to secondary hyperparathyroidism, osteoporosis and hypocalcaemia [Gerson 2013, Schinke 2009], an effect that was also described in humans [Gerson 2013, Targownik 2012]. Additionally, hypoacidity impairs the resorption of other substances, such as iron, magnesium, and vitamin B12, which are important for healthy bone metabolism [Clemens 2014, Ito 2010]. Notably, Abrahamsen *et al.* found a possible interaction between histamine homeostasis and osteoclast function [Abrahamsen 2013]. According to them, long-term gastrin stimulation under PPI therapy induces enterochromaffin cell hyperplasia, which leads to overexpression of histamine and subsequently osteoclast stimulation. Interestingly, the negative effects of PPI could be compensated by administering histamine receptor antagonists, which even led to overall decreased fracture risk. This suggests that histamine receptor antagonists may act as a confounding factor in the correlation of PPIs and fracture risk.

PPIs are converted into their active metabolites in acidic environments. This transformation also takes place at neutral pH, albeit at a slower rate [Mattsson 1991, Pytlik 2012]. Potentially, detrimental effects are stronger when the active metabolite itself affects the cells, which would lead to their underestimation under neutral *in vitro* conditions. Another important factor of BMU function is intercellular signalling between the different subunits. Since osteoblasts can directly alter osteoclast function and *vice versa*, these interactions must also be taken into consideration in future research [Kular 2012]. As some authors describe, the cause of an increased fracture risk might not even be metabolic disorder, but due to the patients' increased risk of falls. For instance, Lewis *et al.* describe in a prospective study in postmenopausal women that bone structure itself is not affected by PPI therapy [Lewis 2014]. Rather, a significantly

increased risk of falls due to self-reported subjective dizziness and numbness of the feet, as well as impaired results in the objective “Timed Up and Go” test were assessed.

4.4 Summary and outlook

In summary, it is yet unclear whether PPIs directly cause increased fracture risk or if confounding factors play a major role in the correlation of the two. Our studies revealed no major cellular detriment or toxicity at physiological drug doses and through its specific effects on the BMU cells, decreasing BMD and osteoporosis seem unlikely pathomechanisms for the increased fracture risk. Consequently, further experimental, as well as observational studies are needed to adequately assess PPI safety. Nevertheless, the inflationary use of PPIs without clear indication is proving to be more hazardous than initially anticipated. As stated above, varying detrimental effects have been found and when prescribing PPIs, one should carefully evaluate the risks and benefits of drug administration. Nonetheless, if a clear indication is given, the benefit of the correct use of PPIs still, as of now, outweighs the potential hazards [Reimer 2013].

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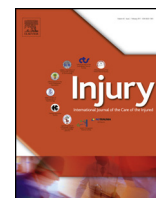
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Appendix



Pantoprazole increases cell viability and function of primary human osteoblasts *in vitro*



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ABSTRACT

Proton pump inhibitors (PPIs) are a class of drugs that irreversibly inhibit the H^+/K^+ -ATPase in gastric parietal cells. Since an association between PPI use and increased fracture risk has been found, the aim of this study was to detect potential adverse effects of pantoprazole, a representative of the PPIs, on primary human osteoblasts *in vitro*. The isolated cells were stimulated with pantoprazole concentrations ranging from 0 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$. Changes in proliferation, total cell number, viability, cytotoxicity, alkaline phosphatase activity, total protein synthesis and gene expression on mRNA level were determined over a period of 7 days. Pantoprazole stimulation resulted in increased viability and decreased cytotoxicity in the osteoblasts. The proliferation rate was stable and so was the relative cell number. Only at the highest pantoprazole concentration on day 7, a slight decrease of the cell number was detected. Alkaline phosphatase activity increased over the tested period under exposure to pantoprazole ($p < 0.05$ at 3 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ pantoprazole). Osteoblast-specific gene expression was increased through pantoprazole stimulation compared to the control on day 3. Towards day 7, gene expression returned to baseline levels or decreased slightly compared to unexposed cells. Interestingly, this *in vitro* experiment detected no evidence of adverse effects of PPIs on primary human osteoblasts. Osteoblasts were rather more viable with increased mitochondrial activity, gene expression and protein synthesis under pantoprazole stimulation. Therefore, these *in vitro* results do not suggest that impaired osteoblast function is the cause of an increased fracture risk in patients under PPI therapy.

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Introduction

Proton pump inhibitors (PPIs) are a group of drugs that irreversibly inhibit the H^+/K^+ -ATPase (proton pump) in gastric parietal cells and thus result in reduced stomach acidity. They are commonly used to treat gastroesophageal reflux disease and to counteract the adverse effects of non-steroidal anti-inflammatory drugs (NSAID) on the gastric mucosa [1,2]. After their introduction, PPIs were deemed almost risk-free which led to a rapid increase in their prescription. Consequently, today's use of PPIs is inflationary and in numerous cases PPIs are administered prophylactically for no evident reason.

The potentially detrimental effects of PPIs on bone metabolism and the resulting changes in bone density with increased fracture

risk have been discussed before, leading to contradicting conclusions. Yu et al. performed a meta-analysis of 11 observational studies that evaluated a possible association between the use of PPIs and the risk of bone fracture in humans, finding an increased fracture risk in both male and female patients [3]. However, the authors could not completely exclude the possibility of unnoticed confounding factors. In contrast, Kaye et al. found no significant correlation between the use of PPIs and increased fracture risk without concomitant major risk factors [4]. A potential explanation for the detrimental effects of PPIs on bone density could be decreased calcium absorption due to less acidic stomach fluids as hypothesised by Insogna et al., although long-term studies regarding this matter are yet to be performed [5].

Additionally to their effects on gastric parietal cells, PPIs seem to inhibit a proton pump (V-ATPase) in osteoclasts [6,7]. This could potentially lead to decreased bone resorption and therefore have a positive effect on bone density [8]. Furthermore, Hyun et al. found an osteopetrosis-like effect on bone density through increased activity of osteoblasts (OBs) and decreased activity of osteoclasts in

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a murine cell model [9]. Targownik et al. used a multivariate linear regression model on a set of data from the Canadian Multicentre Osteoporosis Study to determine whether there is a relevant decrease in bone mineral density (BMD) after administration of PPIs [10]. The analysis revealed that there was no association between PPI usage and accelerated BMD loss over a ten-year period, although PPI users had a lower average baseline BMD.

Bone metabolism is ultimately regulated through two interacting types of cells, namely osteoclasts and osteoblasts. Together they form so-called “basic multicellular units”, which are responsible for bone homeostasis [11].

Osteoclasts are multinucleated, bone-specific macrophages that derive from hematopoietic stem cells. Once activated, osteoclasts bind to bone matrix and form so-called Howship's lacunae, which are the site of active bone resorption. Contrary, OBs derive from mesenchymal stem cells, synthesise bone matrix and ultimately differentiate into osteocytes, which sustain matrix integrity.

As stated above, an association between PPI use and fracture risk has been hypothesised. Since the underlying mechanisms remain unknown, it was our goal to investigate the effects of pantoprazole (PP) on primary human OBs regarding their metabolic activity, proliferation potential and specific gene activity *in vitro*.

Materials and methods

Plastic labware was purchased from Sarstedt (Nümbrecht, Deutschland). Culture plates were purchased from GE Healthcare (Little Chalfont, United Kingdom). Chemicals, unless stated otherwise, were purchased from Sigma–Aldrich (St. Louis, MO, United States).

Isolation and culture of primary human OBs

Femoral heads, which otherwise would have been disposed as residue after surgery, were obtained from patients undergoing total endoprosthetic hip replacement ($N=4$). The age of the recruited patients ranged from 75 to 91 years and the cohort consisted of three female and one male donor. Patient recruitment as well as realisation of the experiments was conducted in accordance with the Declaration of Helsinki and the principles of good clinical practice. Informed consent was obtained from each of the patients. The study was approved by the ethics committee of the Technical University of Munich. Neoplasia and infection were defined as exclusion criteria in this study.

Briefly, cancellous bone fragments were collected from the centre of the femoral heads using Luer's pliers and subsequently washed in Dulbecco's phosphate buffered saline (DPBS) in order to remove unwanted tissue fractions (e.g. blood and fat). The fragments were transferred into 175 cm² culture flasks. The culture medium consisted of 500 ml low glucose Dulbecco's modified eagle medium, 0.05 M L-ascorbic acid, 10% foetal calf serum (FCS) and 100 U/ml penicillin and 10 µg/ml streptomycin. The cells were incubated at 37 °C and 5% CO₂. The isolated cells were expanded until reaching the third passage to ensure the desired purity. The osteoblastic phenotype was confirmed by alkaline phosphatase (AP) activity measurement and characteristic gene expression (i.e. osteocalcin, among others). Finally, the cells of each individual donor were plated separately at a density of 10,000/cm² for the experiments.

Experimental setup

Primary human OBs were cultivated using a differentiation medium containing 500 ml low glucose Dulbecco's modified eagle

medium, 10 mM β-glycerol phosphate, 1.56 mM CaCl₂·2H₂O, 5% FCS, 100 U/ml penicillin and 10 µg/ml streptomycin, 0.2 mM L-ascorbic acid, 100 nM dexamethasone and 0.025 M HEPES buffer. After 24 h, detached cells were removed. Subsequently, the cells were incubated with PP (Sigma–Aldrich, St. Louis, MO, United States). In addition to a negative control containing 0 µg/ml PP, the following concentrations of the drug were administered: 0.1 µg/ml, 1 µg/ml, 3 µg/ml and 10 µg/ml. The medium was changed every other day including freshly prepared PP. The effects of PP on the metabolism, proliferation and gene expression of primary human OBs were analysed after 1, 3, and 7 days.

5-Ethynyl-2'-deoxyuridine (EdU) fluorescence stain

Proliferation was analysed using the thymidine analogue EdU in order to label the DNA of dividing cells in our cell culture experiments. The fluorescence stain was performed according to the manufacturer's protocol, using the Click-iT[®] EdU Alexa Fluor[®] 488 Imaging Kit (Life Technologies, Carlsbad, CA, United States). Briefly, the cells were incubated with medium containing EdU at 37 °C for 24 h. Afterwards, the cells were fixated and the click reaction initiated. Finally, a DNA stain was performed by incubating the cells with Hoechst 33342 for 30 min. The fluorescence signals were documented at room temperature using the BZ-9000 fluorescence microscope (Keyence, Osaka, OSK, Japan). For analysis, a cell counting template was created using the BZ-II Analyzer (Keyence, Osaka, OSK, Japan). Fluorescence signals that were to be counted as cells were defined according to size and shape of OBs. For each representative field of view, all cells that exhibited proliferation activity (EdU stain, green fluorescence) were counted. Then the total cell number (Hoechst stain, blue fluorescence) in each field of view was determined. The proliferation rate was calculated by dividing the proliferating cells by the total amount of cells.

Cell count

The relative cell count was obtained using the CyQuant[®] NF Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA, United States). The cells were washed once with DPBS and then incubated with the dye reagent for 60 min at 37 °C. The reagent was prepared according to the manufacturer's protocol. The fluorescence signal was photometrically quantified at $\lambda = 530$ nm. The results were expressed relative to the control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was used to determine the effects of stimulation with PP on cell viability and metabolic (mitochondrial) activity. In short, the plated cells were washed twice with DPBS and then incubated with MTT solution containing 1.2 mM thiazolyl blue for 120 min at 37 °C and 5% CO₂. The formazan production was determined photometrically at $\lambda = 570$ nm. The measurements were normalised with the optical density at $\lambda = 690$ nm. The results were expressed relative to the control.

Sulforhodamine B (SRB) staining

In order to obtain the complete amount of cellular protein, SRB staining was performed. Bound SRB was resuspended in unbuffered Tris(hydroxymethyl)aminomethane (TRIS) solution and photometrically quantified at $\lambda = 565$ nm. The protein content was then calculated according to a standard curve that was obtained by plotting known protein quantities to their respective absorptions. The results were expressed relative to the control.

Table 1
Primers used for RT-PCR analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ACTB	ACAGAGCTCGCCTTTGCCGAT	GCGAAGCCGGCCTTGACAT
RUNX2	TGCCTAGGCGCATTTACAGGTGC	TGAGGTGACTGGCGGGTGT
ATF4	TCCCCCTTCGACCAGTCCGG	TTCTGGGAGATGGCCAATTGGGT
COL1A1	AGCGGACGCTAACCCCTCC	CAGACGGGACAGCACTCGCC
BSP2	TGACTCATCCGAAGAAAATGGAG	CTGGATTGCAGCTAACCCCTGT
OC	CCAGCGGTGCAGAGTCCAGC	GACACCCTAGACCGGGCCGT
OP	CTCCATTGACTCGAACGACTC	CGTCTGTAGCATCAGGGTACTG
OPG	CCGGAACAGTGAATCAACTC	AGGTTAGCATGTCCAATGTG

AP activity

In order to verify the osteoblast (OB) function, AP activity was measured. The cells were washed twice with DPBS and incubated with AP substrate solution (0.2% 4-nitrophenyl phosphate, 50 mM glycine, 1 mM MgCl₂, 100 mM TRIS, pH 10.5) for 30 min at 37 °C. AP catalyses the formation of 4-nitrophenol, which was then photometrically quantified at $\lambda = 405$ nm. AP activity was calculated according to a standard curve. The results were expressed relative to the control.

Gene expression

Gene expression activity was determined through semiquantitative analysis of reverse transcription polymerase chain reaction (RT-PCR). Briefly, the total RNA was extracted from the cells using Tri Reagent (Sigma–Aldrich, St. Louis, MO, United States) and isolated through phenol chloroform extraction and ethanol precipitation. Subsequently, cDNA was transcribed using the First-Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Primer information for the OB genes under investigation is summarised in Table 1. RT-PCRs were run according to a standard protocol. The PCR products were separated using 1.8% (w/v) agarose gels containing ethidium bromide for visualisation. A densitometric analysis of the obtained signals was performed using the ImageJ software (NIH, Bethesda, MD, USA) and normalised to the housekeeping gene β -actin (ACTB).

Statistics

The results were expressed as the mean \pm SEM of at least two independent experiments ($N \geq 2$) measured as duplicates or more ($n \geq 2$). The experiments were performed individually for each donor ($N = 4$). The data sets were compared using the non-parametric Kruskal–Wallis test and Dunn's multiple comparisons test (GraphPad Prism Software, El Camino Real, USA) with $p < 0.05$ as the minimum level of significance.

Results

Proliferation

No differences in proliferation were detected using the EdU labelling method after 3 and 7 days of stimulation with PP (Figs. 1 and 2). The percentage of proliferating cells remained stable at approximately 15% irrespective of the used concentration of PP.

Cell count

The relative cell number, i.e. the cell number in stimulated OB cultures in relation to the cell number of the untreated control cultures, remained stable throughout all stimulated samples on day 3 (Fig. 3A). Merely the OBs stimulated with the highest concentration of PP (10 μ g/ml) showed a decrease of 11.7% ($p < 0.0001$) on day 7 compared to the control (Fig. 3B).

Viability/metabolic activity

The MTT assay performed on day 1 showed no difference in viability between the control and stimulated cells (Fig. 4A). On day 3, a slight concentration-dependent increase in cell viability was detected, albeit statistically not significant (Fig. 4B). This increase in cell viability amplified towards day 7, when a significant difference relative to the control was observed (Fig. 4C). At a concentration of 1 μ g/ml PP, cell viability increased by 22.6% ($p < 0.01$), at 3 μ g/ml PP, viability increased 18.7% ($p < 0.05$) and at 10 μ g/ml PP, it increased 34.2% ($p < 0.0001$). PP concentrations of 100 μ g/ml and more caused extensive cytotoxicity (data not shown) and were therefore excluded from further experiments.

SRB staining

SRB staining yielded no significant differences in total protein content between the control and cultures stimulated with PP on day 1 (Fig. 5A). On day 3, protein content in stimulated cultures slightly rose above control levels. At 10 μ g/ml PP, the total protein content was increased by 16.4% ($p < 0.05$) (Fig. 5B). On day 7, a dose-dependent rise in total protein content was apparent; protein content increased by 30.5% at 1 μ g/ml PP, 48.3% at 3 μ g/ml PP ($p < 0.001$) and by 69.9% at 10 μ g/ml PP ($p < 0.0001$) (Fig. 5C).

AP activity

AP activity was increased in stimulated samples in relation to the control. On day 3, a significant increase of 12.4% ($p < 0.05$) at 3 μ g/ml was detected (Fig. 6B). The difference between control and sample AP activity increased further on day 7 (Fig. 6C). Especially at

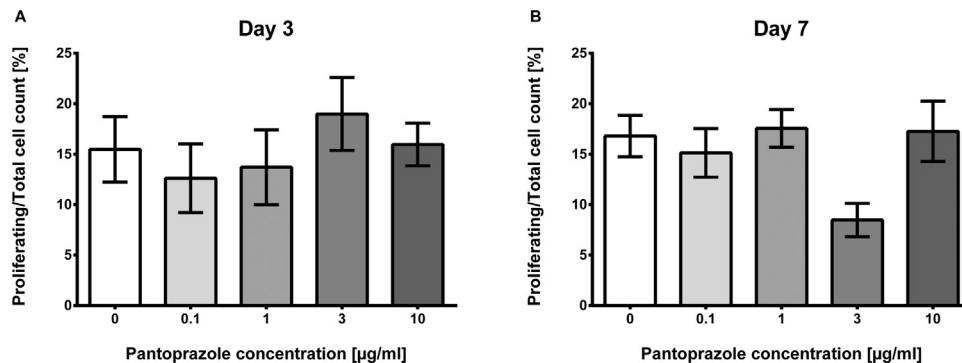


Fig. 1. Proliferation analysis in primary human OB cultures after stimulation with PP ($N = 2$ and $n = 3$). The proliferation rates were determined using EdU fluorescence staining. The cells were counted using a software template optimised to OB cell size and shape. Proliferating cells were related to the total cell number. Accordingly, the results were expressed as a percentage and depicted as column bar graphs (mean \pm SEM).

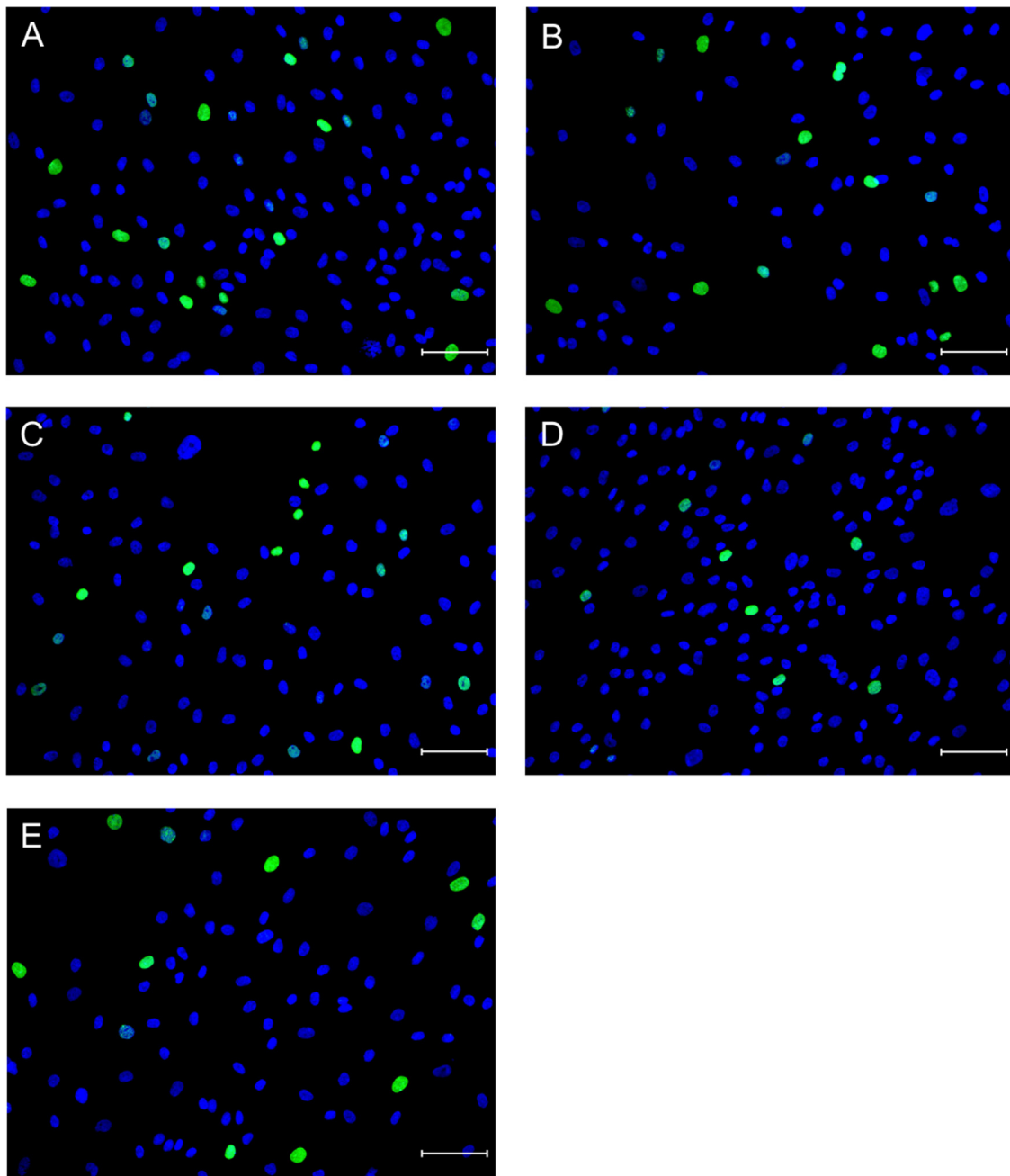


Fig. 2. EdU stain of primary human OBs after 7 days of stimulation with PP ($N = 2$ and $n = 3$). One representative field of view (Objective: PlanFluor_ELWD_DM; magnification: 20 \times ; numerical aperture: 0.45) of the negative control (A) and each stimulated culture ((B) 0.1 $\mu\text{g/ml}$, (C) 1 $\mu\text{g/ml}$, (D) 3 $\mu\text{g/ml}$, (E) 10 $\mu\text{g/ml}$) is depicted. EdU-stained proliferating cells (green stain) are overlaid with the Hoechst DNA stain of all cells (blue stain). The white bar in each lower right corner represents 100 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

higher concentrations of PP, AP activity rose significantly, i.e. by 24.3% ($p < 0.01$) at 3 $\mu\text{g/ml}$ PP and 24.6% ($p < 0.05$) at 10 $\mu\text{g/ml}$ PP.

Gene expression

OB-related gene expression analysis on mRNA level showed a similar trend in all tested genes: gene expression increased with rising concentrations of PP on day 3 and returned to baseline on day 7. The two analysed transcription factors, runt-related transcription factor 2 (*RUNX2*) and activating transcription factor 4 (*ATF4*), as well as the non-collagenous matrix protein osteocalcin (*OC*) and the receptor activator of nuclear factor κB ligand (*RANKL*), decoy receptor osteoprotegerin (*OPG*) are shown

in Fig. 7, with the most prominent increase at 10 $\mu\text{g/ml}$ PP on day 3. $\alpha 1$ (I) collagen (*COL1A1*), bone sialoprotein 2 (*BSP2*) and osteopontin (*OPN*) are not shown in the graphs, but yielded similar results. Although the described trend was apparent in all analysed genes, it remained statistically insignificant throughout the tested period.

Discussion

PPIs are regularly used in combination with NSAID for pain management in a wide variety of patients including patients with fractures, a circumstance where unimpaired bone homeostasis is crucial for proper convalescence. In general, NSAID inhibit the

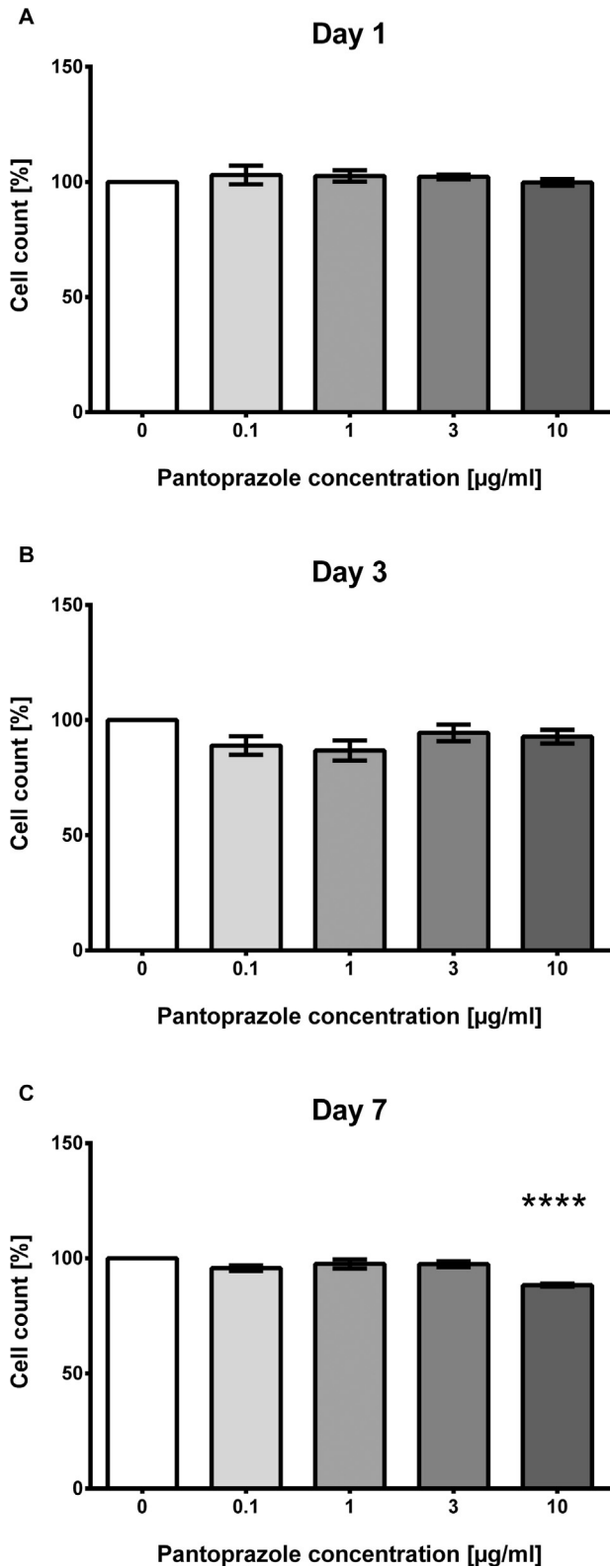


Fig. 3. Cell count in primary human OB cultures after stimulation with PP ($N = 3$ and $n = 3$). The total cell count was measured using the CyQuant[®] NF Cell Proliferation Assay Kit and was related to the untreated controls. Accordingly, the results were expressed as a percentage of the control and depicted as column bar graphs (mean \pm SEM). **** $p < 0.0001$ versus the untreated controls (0 μ g/ml PP).

synthesis of prostaglandins which, in the physiological environment, shield the gastric mucosa from excessive acidic stress. PPIs specifically counteract that effect by irreversibly inhibiting proton

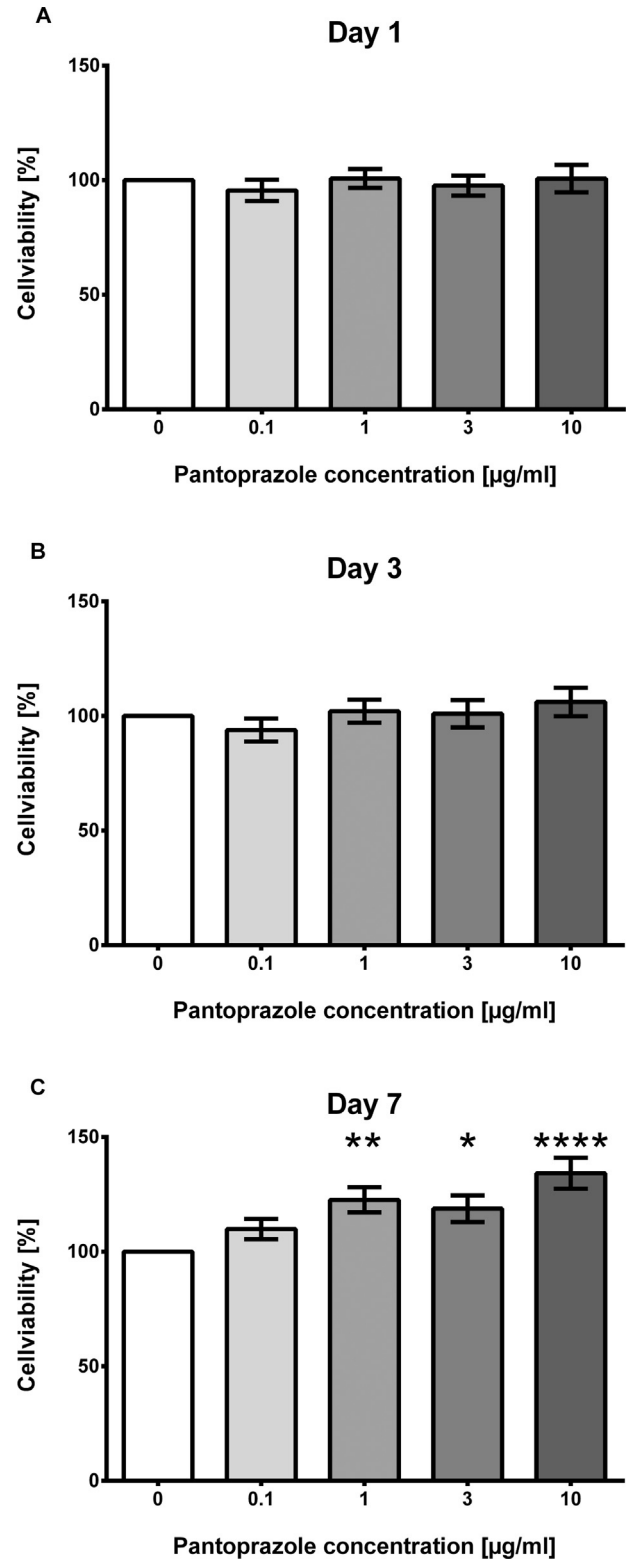


Fig. 4. Cell viability in primary human OB cultures after stimulation with PP ($N = 4$ and $n = 4$). The cell viability was measured using the MTT assay and was related to untreated controls. Accordingly, the results were expressed as a percentage of the control and were depicted as column bar graphs (mean \pm SEM). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ versus the untreated controls (0 μ g/ml PP).

pumps and thereby reducing the acid production of parietal cells in the stomach. Additionally to the effect on parietal cells, the inhibition of proton pumps in osteoclasts has been described [6,7].

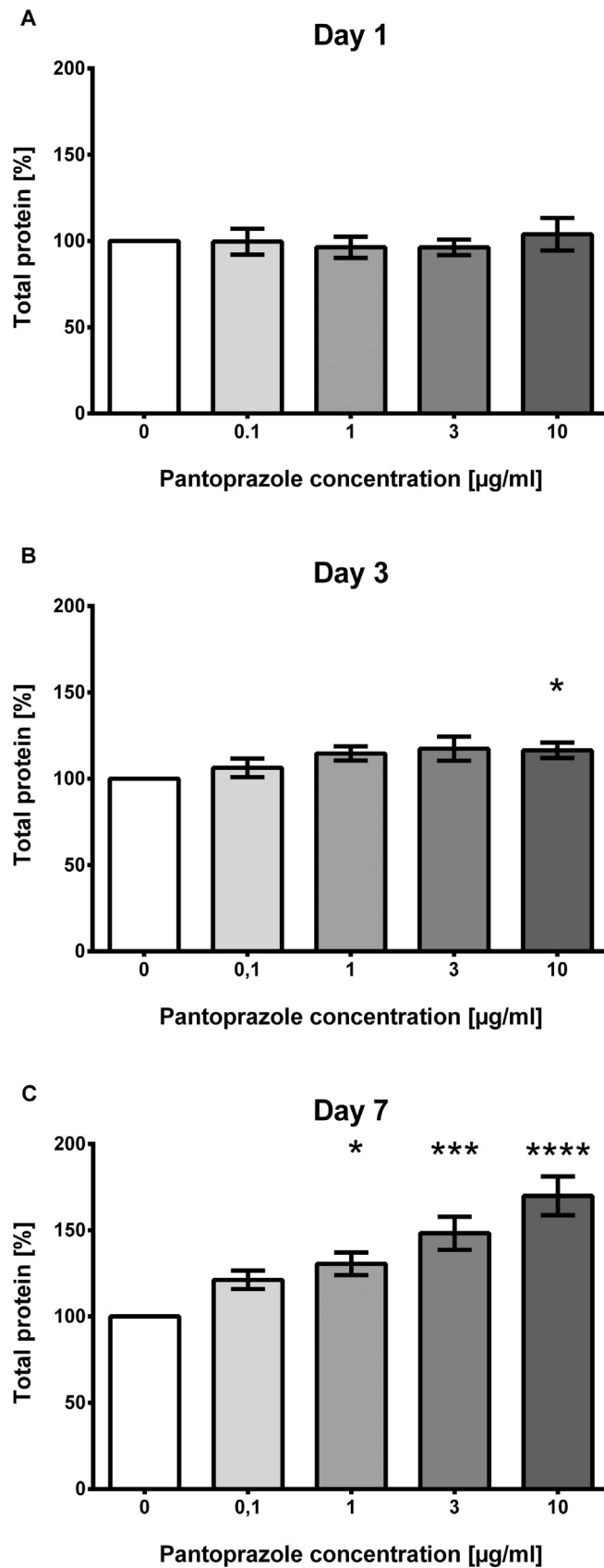


Fig. 5. Total protein content in primary human OB cultures after stimulation with PP ($N = 4$ and $n = 3$). The total amount of protein was measured using the SRB staining method and was related to the untreated controls. Accordingly, the results were expressed as a percentage of the control and depicted as column bar graphs (mean \pm SEM). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ versus the untreated controls (0 $\mu\text{g/ml}$ PP).

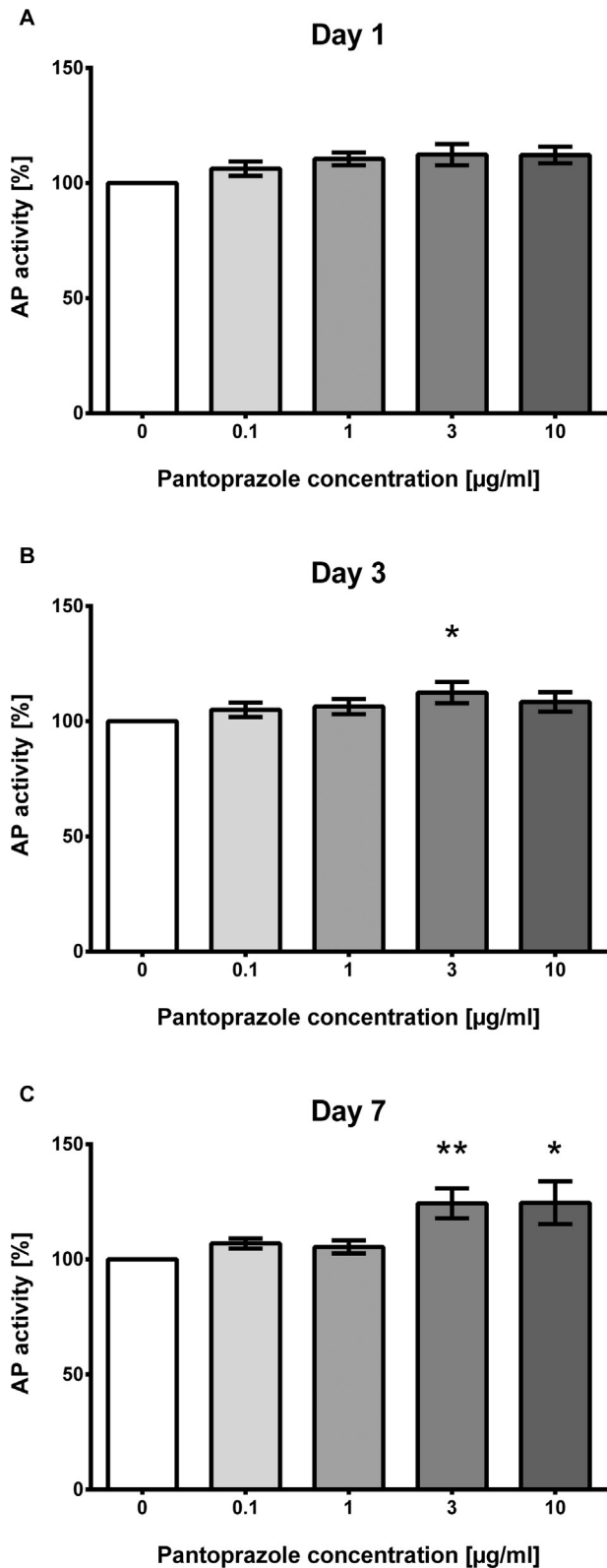


Fig. 6. AP activity of primary human OBs after stimulation with PP ($N = 4$ and $n = 3$). The AP activity of OBs stimulated with PP was related to untreated controls. Accordingly, the results were expressed as a percentage of the control and depicted as column bar graphs (mean \pm SEM). * $p < 0.05$, ** $p < 0.01$ versus the untreated controls (0 $\mu\text{g/ml}$ PP).

The reduced stomach acidity, which influences the resorption of minerals such as calcium and other hitherto unknown effects could potentially affect bone homeostasis even further [5]. Physiologically,

new bone matrix is synthesised by OBs which first form osteoid, an organic, collagen type 1-rich matrix, which is subsequently mineralised with inorganic hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$)₂ crystals. During the process of bone formation, OBs are enclosed in the formed extracellular matrix (ECM). These cells, now referred to as osteocytes, form processes that interconnect *via* gap junctions and maintain matrix integrity [12]. The so-called “master gene of osteoblast differentiation” [13], RUNX2, is the key regulator of OB function and differentiation. In collaboration with ATF4, it promotes the expression of many crucial components of the organic matrix such as COL1A1, BSP and OC. OC is a specific classical marker of late OB differentiation and the most abundant non-collagenous protein of the ECM in bone. It is significantly involved in the regulation of bone formation. OPN plays an important role in stem cell differentiation towards an osteoblastic phenotype [14]. It also binds to bone ECM and is able to anchor osteoclasts, which can in turn begin bone resorption at the binding site [15]. OPG, another protein synthesised by OBs is a decoy receptor, binding to RANKL. Thus, OPG inhibits the binding of RANKL to receptor activator of nuclear factor κB (RANK) on osteoclasts and therefore the activity of this cell type. Therefore, OPG is able to inhibit a major axis of osteoclast differentiation and ultimately bone resorption. As far as our gene expression analysis goes, none of the major transcription factors and ECM proteins was negatively affected by the stimulation with PP.

This study investigated short-term effects of PP on primary human OBs *in vitro* and revealed no detrimental effects. The used concentrations of PP were chosen according to the maximum serum concentrations of PP at the two most commonly prescribed drug doses (20 mg and 40 mg), which correspond to serum levels/stimulation doses of 1 $\mu\text{g/ml}$ and 3 $\mu\text{g/ml}$, respectively. Additionally, a negative control, as well as supra- and sub-physiological concentrations (0.1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$), was administered, since the de facto concentration of PP that takes effect on OBs *in vivo* is unknown.

PP did not influence cell proliferation at any given point in time during the experiment. This was confirmed by the additionally performed analysis of relative cell count that revealed equal levels in all stimulated samples, with the exception of the highest concentration of PP (10 $\mu\text{g/ml}$) on day 7. Here, a decrease in cell count was observed. Thus, we conclude that any changes observed in the stimulated samples in other assays result from changed cellular activity and not from differences in cell number. The performed MTT assay analysis revealed that PP increases the viability and activity of cells under stimulation with PP in a dose-dependent manner. This effect became more obvious over time and corresponded well to low levels of cytotoxicity, which we quantified through lactate dehydrogenase activity measurement (data not shown).

In a recently performed study by Costa-Rodriguez, the effects of the three PPIs (omeprazole, esomeprazole and lansoprazole) on osteoblast and osteoclast precursor cells were examined [16]. Their findings suggested that PPI concentrations of 10^{-5} M and above (the highest administered concentration of PP in our study, 10 $\mu\text{g/ml}$, corresponds to roughly 2.5×10^{-5} M) led to significantly reduced total protein content and AP activity in OBs, as well as reduced ability to synthesise mineralised matrix. At PPI concentrations that correspond to the physiological serum concentrations, no significant changes were detected. These findings could not be confirmed by our study. Our results are consistent with the study of Hyun et al. that was performed in a murine *in vitro* model, and also showed no adverse effects of PPIs on OB cell viability and rather increased cell activity, as far as protein synthesis and gene expression was concerned [9].

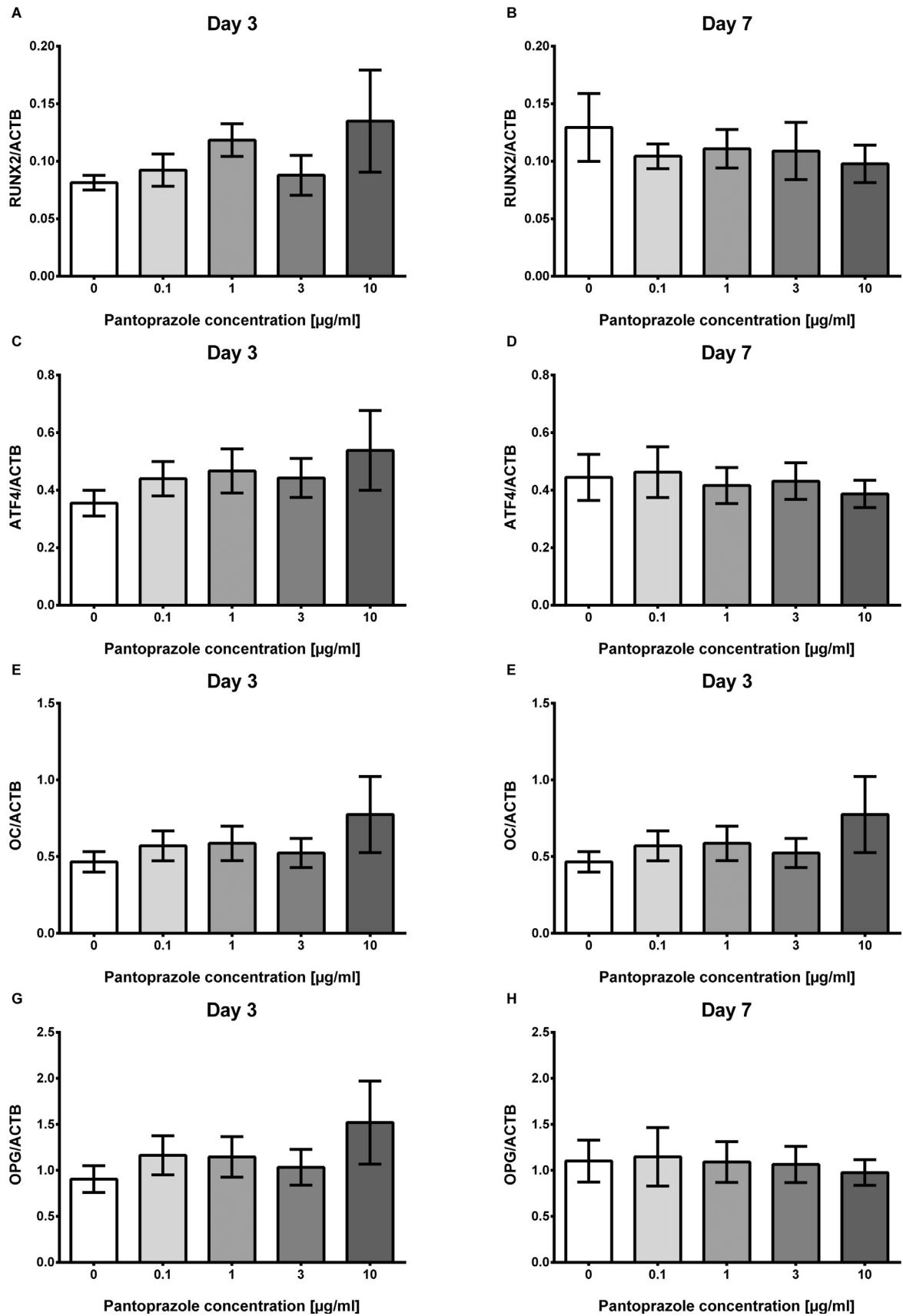


Fig. 7. Gene expression analyses of transcription factors controlling OB differentiation after stimulation with PP ($N = 4$ and $n = 2$). Gene expression analyses of target genes in OBs: (A) *RUNX2* day 3, (B) *RUNX2* day 7, (C) *ATF4* day 3, (D) *ATF4* day 7, (E) *OC* day 3, (F) *OC* day 7, (G) *OPG* day 3 and (H) *OPG* day 7. The analyses were performed using RT-PCR and subsequently the results were analysed semi-quantitatively and compared to untreated controls (0 µg/ml PP). The results were expressed as column bar graphs (mean ± SEM).

The increased fracture risk that has been found in patients under PPI therapy cannot be explained through negative effects of PPIs on OBs, as far as data obtained in our study is concerned. Thus, the mechanism of potential adverse effects of PPIs on bone metabolism *in vivo* remains unknown. An osteopetrosis-like effect as brought forth by Hyun et al. remains a conceivable explanation for an increased fracture risk, yet must still be confirmed in further studies [9].

The limits of our study were those of any *in vitro* experiment. The specific *in vitro* conditions only allow conclusions regarding effects on *in vivo* organisms to a certain extent. For instance, the conversion of PP into active metabolites usually takes place at around pH 1 [17]. This reaction slows with rising pH. Thus, the activation under optimal *in vitro* conditions cannot be taken for granted and it must be considered that the observed effects do not result from activated metabolites, but from PP itself and may therefore differ from *in vivo* effects.

Conflict of interest statement

None declared.

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Research Article

Pantoprazole Decreases Cell Viability and Function of Human Osteoclasts *In Vitro*

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Proton pump inhibitors (PPIs) are commonly prescribed drugs that decrease stomach acidity and are thus often used to treat gastroesophageal reflux disease and as a preventative agent for the adverse effects of nonsteroidal anti-inflammatory drugs on the stomach mucosa. In recently published literature, an association between proton pump inhibitor administration and increased fracture risk has been stated. In order to reveal the underlying pathomechanisms of these observations, the effects of pantoprazole, a representative of the proton pump inhibitors, on human osteoclasts *in vitro* were evaluated in this study. Osteoclasts were stimulated with increasing concentrations of pantoprazole ranging from 0 $\mu\text{g}/\text{mL}$ to 10 $\mu\text{g}/\text{mL}$ over a period of seven days. Cell viability and tartrate-resistant acid phosphatase (TRAP) activity assays were performed after 1 day, 3 days, and 7 days, respectively. Here, stimulated osteoclasts presented a significantly lower viability and TRAP activity than the negative controls. Osteoclast-specific gene expression was evaluated after seven days and revealed no significant differences between all samples. Overall, the bone degrading and resorptive function of osteoclasts is inhibited by the administration of proton pump inhibitors. While PPI-related fractures through “basic multicellular unit” dysfunction are unlikely, the underlying pathomechanism remains unknown.

1. Introduction

Pantoprazole is one of the most frequently used proton pump inhibitors (PPIs) in the clinical setting. By irreversibly inhibiting H⁺/K⁺ ATPase, or proton pump, in gastric cells, it strongly reduces the proton influx into the gastric lumen and therefore the effective stomach acidity. It is therefore used in the treatment of gastroesophageal reflux disease, in eradication treatment of *Helicobacter pylori* infection, and in combination with nonsteroidal anti-inflammatory drugs (NSAIDs) for prophylaxis of stress ulcers [1–3]. Although the administration of PPIs in a wide range of medical disciplines is necessary, they are often wrongly administered chronically and without a clear indication [4].

The administration of various PPIs has been shown to be significantly associated with an increased fracture risk, while a direct causal link between the two is not yet proven [5–7]. While short-term administration of PPIs already poses a moderately increased risk, long-term administration over a year or more seems to further increase the fracture risk [8, 9].

Insogna hypothesized a cell-independent mechanism why bone density should decrease under PPI therapy. According to his study, altered gastric acidity may lead to reduced calcium absorption, which would ultimately increase the rate of bone loss [10]. Similarly, Vestergaard et al. hypothesized a secondary hyperparathyroidism and subsequent osteoporosis as a possible cause for the increased fracture risk [11].

Our study group has previously investigated the influence of pantoprazole on osteoblasts *in vitro*. Osteoblasts represent a subpopulation of bone cells that synthesize the bone matrix. Pantoprazole seems to have beneficial effects on the viability of osteoblasts and their ability to synthesize new bone matrix [12]. The functional counterpart to osteoblasts within the basic multicellular units is osteoclasts, which actively degrade and resorb bone matrix. Together, these cellular units form a functional entity that secures dynamic bone homeostasis in order to meet the requirements of constant load transmission. It has been described that, additionally to their inhibitory effects on gastric proton pumps, PPIs may inhibit a proton

pump in osteoclasts also known as the vacuolar ATPase [13]. It is mainly responsible for creating an acidic environment between the ruffled border of osteoclasts and bone tissue. In this acidic environment at the bone-apposed plasma membrane of the osteoclast, lytic enzymes are activated and bone is resorbed within the process of remodeling [14]. V-ATPases are related yet not identical to the proton pump that is located in parietal cells. Consequently, effects of PPIs on V-ATPases are less apparent than on gastric proton pumps [15].

Since no pathomechanism has yet been found that adequately explains or indeed proves a causal correlation between the use of PPIs and an increased fracture risk, the aim of this study was to investigate the effect of an important representative of the proton pump inhibitors—pantoprazole—on a cellular level.

2. Materials and Methods

The plastic labware used in our experiments was acquired from Sarstedt (Nümbrecht, Germany). The culture plates were acquired from GE Healthcare (Little Chalfont, UK). The chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA). Dentine chips were kindly provided by Associate Professor Susanne Mayer, MD, Laboratory for Biomechanics and Experimental Orthopaedics, Department of Orthopaedics, Ludwig-Maximilians-University, Munich, Germany.

2.1. Isolation of Peripheral Blood Mononuclear Cells (PBMCs). Buffy coats (i.e., portions of donated blood that contain a concentrated amount of leucocytes) of male and female donors ($N = 4$) ranging from 50 to 67 years in age were obtained from Bayerisches Rotes Kreuz Blutspendedienst (Bavarian Red Cross Blood Donation Centre, Ulm, Germany). We selected donors of higher age since the prescription and therefore potential risks of PPIs are more imminent in older than in younger patients. After resuspension with 250 mL Dulbecco's phosphate buffered saline (DPBS) per buffy coat, 30 mL of suspension was layered on top of 20 mL lymphocyte separation medium (Biowest, Nuaille, France) with a density of 1077 kg/m^3 . After 20 minutes of centrifugation at 1000 g without brakes, the PBMCs were isolated from the formed interphase. Afterwards, the cells were resuspended in DPBS and centrifuged again at 650 g for 10 minutes. This last step was repeated once more for purification. Finally, the PBMCs were resuspended in cultivation medium which consisted of 500 mL alpha modification minimum essential medium (PAA Laboratories GmbH, Cölbe, Germany), 10% fetal calf serum (FCS), 100 U/mL penicillin, and 10 $\mu\text{g/mL}$ streptomycin. The cells were then plated at a concentration of 3 million cells per well on 48-well plates for cell experiments and 6 million cells per well on 24-well plates for gene expression analysis.

2.2. Osteoclast Differentiation. 24 hours after isolation, the isolation medium was exchanged for cultivation medium that contained 25 ng/mL macrophage colony-stimulating factor (M-CSF). All following media exchanges were performed on Mondays, Wednesdays, and Fridays. After

a week of M-CSF stimulation, cultivation medium containing 12.5 ng/mL M-CSF and 10 ng/mL receptor activator of nuclear factor κB (RANKL) was administered. For the following two weeks, the monocyte cultures were stimulated with medium containing 20 ng/mL RANKL.

2.3. Experimental Setup. After 24 days of differentiation, the osteoclasts were stimulated every other day with freshly prepared pantoprazole (PP) at the following concentrations: 0 $\mu\text{g/mL}$ (negative control), 0.1 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 3 $\mu\text{g/mL}$, and 10 $\mu\text{g/mL}$. These include the concentrations that correspond to the *in vivo* serum concentrations (1 $\mu\text{g/mL}$ and 3 $\mu\text{g/mL}$) of the two most common prescription doses of pantoprazole (20 mg and 40 mg). This was done to reveal effects that are most relevant to clinical application. The effects of pantoprazole on osteoclast function, metabolism, and gene expression were analysed on days 1, 3, and 7 after the initial stimulation.

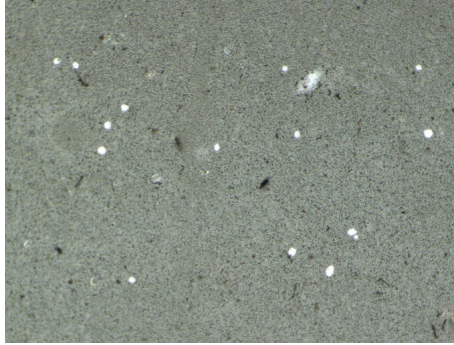
2.4. Osteoclast Phenotype: von Kossa Staining. In order to demonstrate and prove the osteoclastic phenotype and the ability to absorb mineralised matrix, PBMCs were plated on a culture plate coated with a thin layer of calcium phosphate (Corning, Tewksbury, MA, USA). After differentiation and stimulation, the medium was removed and the osteoclasts were incubated with NaClO for 5 minutes at room temperature. Afterwards, the wells were washed twice with dH_2O and subsequently incubated with a 3% solution of silver nitrate in H_2O for 30 minutes at room temperature. The silver nitrate solution was removed and the wells were washed repeatedly in dH_2O over 10 minutes. Then, a 1% solution of pyrogallol in H_2O was added and incubated for 3 minutes. After two additional washing steps with dH_2O , the samples were fixated with a 5% solution of sodium thiosulfate in H_2O for 5 minutes. After multiple washing steps with dH_2O , pictures were taken using the BZ9000 fluorescence microscope (Keyence, Osaka, OSK, Japan). Through the von Kossa staining, the remaining calcium phosphate appears as a dark grey and the resorption pits appear as a light grey to white shade.

2.5. Osteoclast Phenotype: Toluidine Blue Staining. Toluidine blue staining was performed on osteoclasts that were cultivated on dentine chips in suspension culture plates to show the formation of resorption lacunae. The cells were lysed with sodium hypochlorite solution and wiped off the dentine chips with a paper towel. After short immersion in 1% toluidine blue solution, pictures were taken with the BZ9000 fluorescence microscope (Keyence, Osaka, OSK, Japan).

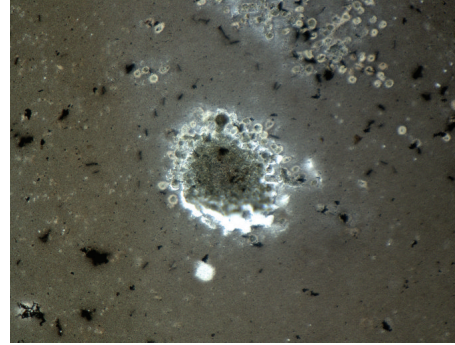
2.6. Osteoclast Phenotype: TRAP Staining. To visualise the presence and resorptive activity of osteoclasts and thus prove the successful differentiation, TRAP staining was performed on control samples after 1 day, 3 days, and 7 days. The cells were fixated with a solution of 10% DPBS, 4% formaldehyde, and 0.2% Triton X-100 in dH_2O for 5 minutes. The staining buffer consisted of 40 mM sodium acetate and 10 mM disodium tartrate dihydrate in ddH_2O (pH = 5). 0.01% naphthol

TABLE 1: Primers used for RT-PCR analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>ACTB</i>	ACAGAGCCTCGCCTTTGCCGAT	GCGAAGCCGGCCTTGCACAT
<i>NFκB</i>	GCGCCGCTTAGGAGGGAG	GAAGGTATGGCCATCTGCTGTT
<i>TRAP</i>	CCCTCGGAGAAACTGCATCAT	CATGTCCATCCAGGGGGAGA
<i>VATP</i>	GGGCTGCTCATGTTCCCTCTT	CACGTGGTTGAAGACTCCGA
<i>NFATC1</i>	GCTTTAAAAAGGCAGGAGGCA	GAGGAAAGTCATCGAGGGGC



(a)



(b)

FIGURE 1: Depicted are two exemplary fields of view of the von Kossa-stained calcium phosphate plate. The pictures were taken of samples that were not stimulated with pantoprazole, 7 days after differentiation. Multiple resorption zones (white spots) where osteoclasts were located prior to lysis are visible at 20x magnification (a). A large cluster of osteoclasts is visible in the middle of the picture with several smaller clusters in the periphery of the picture at 10x magnification (b).

AS-MX phosphate, 0.06% Fast Red Violet LB salt, and 1% N-N dimethylformamide were added to get the final staining solution. After the fixation, the cells were air-dried at room temperature. Finally, the staining solution was added and incubated for 20 minutes at 37°C. After a washing step with DPBS, pictures were taken using the BZ-9000 fluorescence microscope (Keyence, Osaka, OSK, Japan).

2.7. TRAP Activity. In order to determine the level of osteoclast resorption activity, the TRAP activity of the osteoclasts was assessed. The assay buffer consisted of 100 mM sodium acetate and 50 mM disodium tartrate dihydrate in ddH₂O (pH 5.5). The substrate buffer solution consisted of 5 mM 4-nitrophenyl phosphate disodium salt hexahydrate in assay buffer. For TRAP activity analysis, 50 μL of cell supernatant of each sample was transferred to a 96-well plate. The aliquots were incubated at 37°C with 150 μg/mL assay buffer per well for 1 hour. A standard curve was prepared by stepwise dilution of 1 mM 4-nitrophenol in a 1 : 4 solution of osteoclast culture medium and assay buffer. After the total incubation time, the reaction was stopped using 50 μL of 3 M NaOH solution. Finally, the absorbance was detected at λ = 405 nm. TRAP activity was calculated according to the standard curve.

2.8. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. In order to quantify cell viability, the MTT assay was performed and set in relation to the negative control. The samples were incubated with 1.2 mM thiazolyl blue solution for 120 minutes at 37°C and 5% CO₂. Afterwards, the supernatant was removed and the remaining formazan crystals were resolved in a dimethyl sulfoxide

solution containing 10% sodium dodecyl sulfate and 0.6% acetic acid. The formazan content was determined through photometrical measurements at λ = 570 nm and λ = 690 nm.

2.9. Gene Expression. In order to evaluate gene expression activity, semiquantitative analyses of reverse transcription polymerase chain reaction (RT-PCR) products were performed. The total RNA was isolated from the cells using TriFast (Sigma-Aldrich, St. Louis, MO, United States), phenol chloroform extraction, and subsequent ethanol precipitation. The RNA was transcribed into complementary DNA (cDNA) using the First-Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). RT-PCRs were performed after a standard protocol. The respective primers of the investigated genes are listed in Table 1. Ethidium bromide was used for visualisation of the PCR products that were separated in 1.8% w/v agarose gels. The densitometric analysis of the generated signals was performed with the ImageJ software (NIH, Bethesda, MD, USA). The housekeeping gene β-actin (*ACTB*) was used for normalisation.

3. Statistics

The experiments were performed independently on four individual specimens ($N = 4$). The measurements were performed as duplicates or more ($n \geq 2$). The gathered results are depicted as mean ± SEM. Kruskal-Wallis test and Dunn's multiple comparisons test (GraphPad Prism Software, El Camino Real, USA) were used for data comparison. The minimal value of significance was $P < 0.05$.

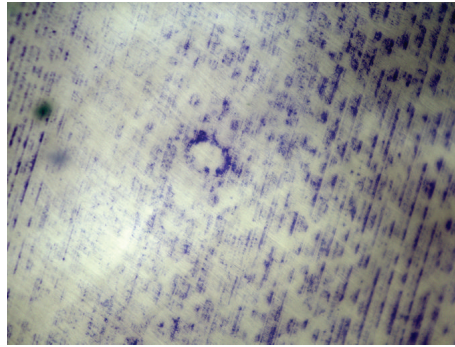


FIGURE 2: A resorption lacuna is made visible on the dentine chip through toluidine blue staining. The picture was taken under light microscopy at 10x magnification, 7 days after differentiation. The sample in this picture consisted of osteoclasts that were not stimulated with pantoprazole.

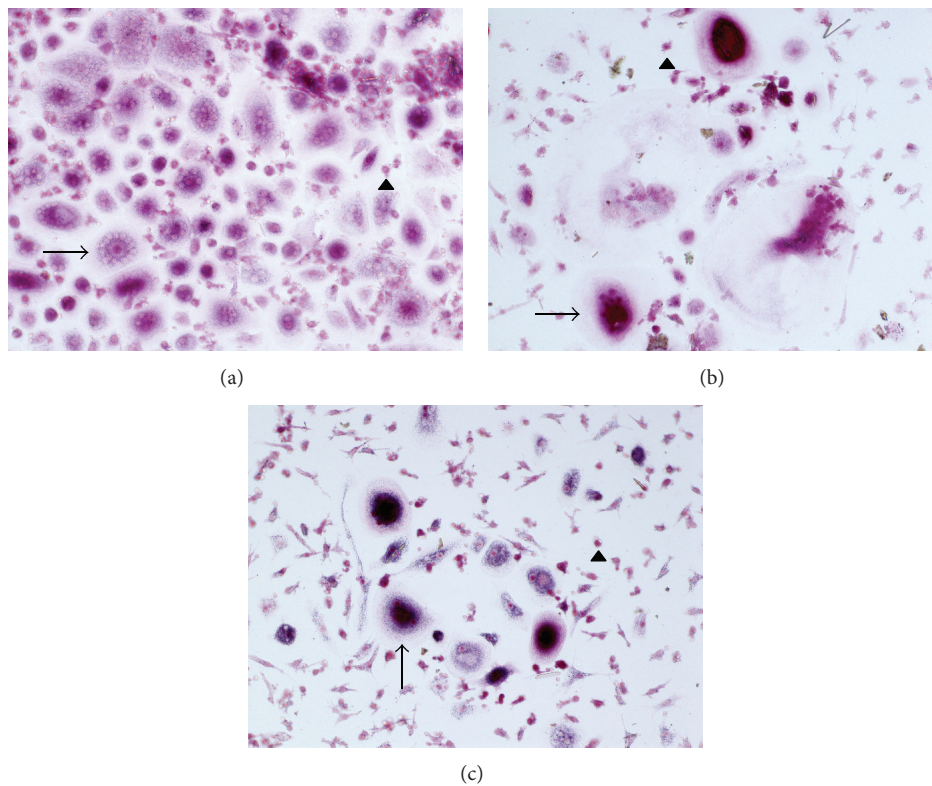


FIGURE 3: Depicted are three representative fields of view of TRAP-stained samples, 1 day (a), 3 days (b), and 7 (c) days after differentiation and without pantoprazole stimulation. Visible are multinucleated osteoclasts (arrows) as well as mononuclear cells that have not yet merged (arrowheads).

4. Results

4.1. von Kossa Staining/Toluidine Blue Staining/TRAP Staining. The stainings were performed on negative control samples of each donor 7 days after completed differentiation. The von Kossa-stained samples show that the differentiated cells actively resorbed calcium phosphate (Figures 1(a) and 1(b)) while the exemplary toluidine blue-stained dentine chip shows the formation of Howship's lacuna (Figure 2). Furthermore, the strong accumulation of TRAP stain in the depicted cells suggests successful differentiation of the PBMCs into active osteoclasts after days 1, 3, and 7 after differentiation (Figures 3(a), 3(b), and 3(c)).

4.2. TRAP Activity. TRAP activity assessed on days 1 and 3 after initial differentiation showed no significant differences between the control and samples stimulated with PP (Figures 4(a) and 4(b)). On day 7, however, the TRAP activity of the 0.1 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{g}/\text{mL}$ PP groups showed a decrease of 6.12% ($P < 0.05$) and 8.56% ($P < 0.01$), respectively (Figure 4(c)).

4.3. MTT Assay. The performed MTT assay revealed a decreased viability in osteoclast cultures stimulated with PP. On day 1, the viability of the samples exposed to 3 $\mu\text{g}/\text{mL}$ PP was decreased by 13.04% ($P < 0.05$). The viability of the other sample groups decreased as well, yet without

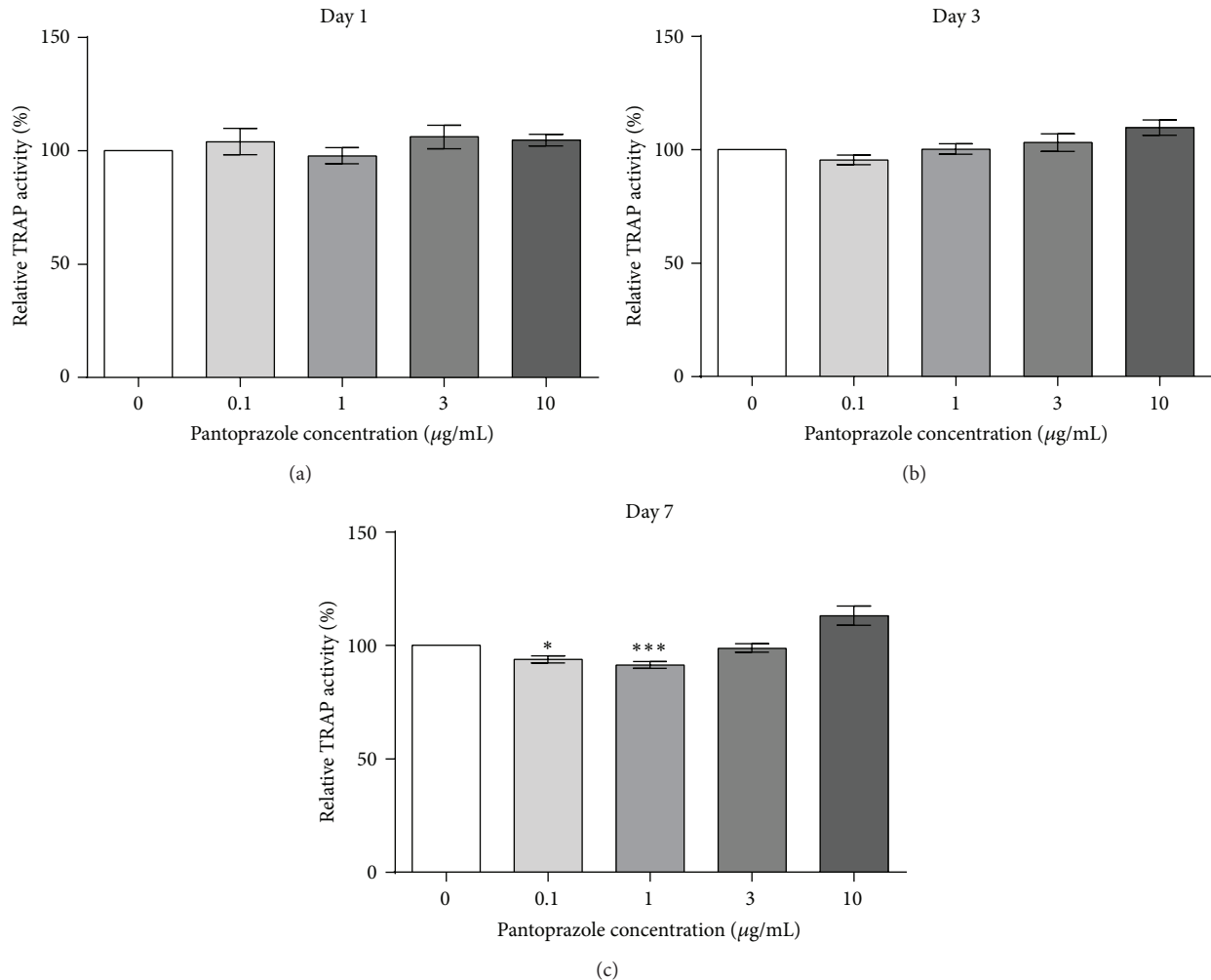


FIGURE 4: The relative TRAP activity of osteoclast samples ($N = 4$, $n = 6$), that is, the activity of samples stimulated with pantoprazole normalized to untreated samples, was assessed on days 1, 3, and 7 of stimulation. The results were accordingly expressed as a percentage of the negative control. Shown are column bar graphs of means \pm SEM. * $P < 0.05$ and *** $P < 0.001$ versus the untreated controls.

statistical significance (Figure 5(a)). On day 3, stimulation with 1 µg/mL PP or more led to a decreased viability and was also statistically insignificant (Figure 5(b)). On day 7, the viability of samples that were stimulated with 10 µg/mL PP was decreased by 15.33% ($P < 0.01$) (Figure 5(c)).

4.4. Gene Expression. The gene expression analysis of nuclear factor kappa-light-chain-enhancer of activated B cells (*NFκB*), tartrate-resistant acid phosphatase (*TRAP*), a subunit of the V-ATPase (*VATP*), and nuclear factor of activated T-cells, cytoplasmic 1 (*NFATc1*), revealed no significant differences after 7 days of stimulation (Figures 6(a), 6(b), 6(c), and 6(d)).

5. Discussion

The question whether PPIs have detrimental effects on bone metabolism and may in fact cause an increased fracture risk, as well as being a risk factor for other pathologies, such as community-acquired pneumonia, is of great interest in

the clinical setting [5, 6, 8, 16, 17]. The fact that most standard pain management therapies include the concomitant administration of PPIs, in many cases over long periods of time, would make negative effects particularly harmful. In this study, we explored the effects of pantoprazole, one commonly prescribed PPI, on a cellular level.

After successful initial differentiation of the isolated PBMCs into osteoclasts, our stimulation experiments were carried out over a period of 7 days. The performed cell viability assays, as well as the TRAP activity assays, suggest that human osteoclasts are significantly inhibited in their overall ability to degrade and absorb bone matrix. This is in accordance with the findings of Seraly et al., who showed in their *in vivo* experiments that pantoprazole (0.5 mg/mL) and omeprazole (40 mg/mL) decrease the resorptive activity of osteoclasts when instilled into calcium phosphate cement [18]. Additionally, Hyun et al., who investigated the effects of omeprazole on murine osteoclast-like RAW 264.7 cells, revealed that osteoclast function is overall inhibited, while osteoblast function is enhanced, leading to

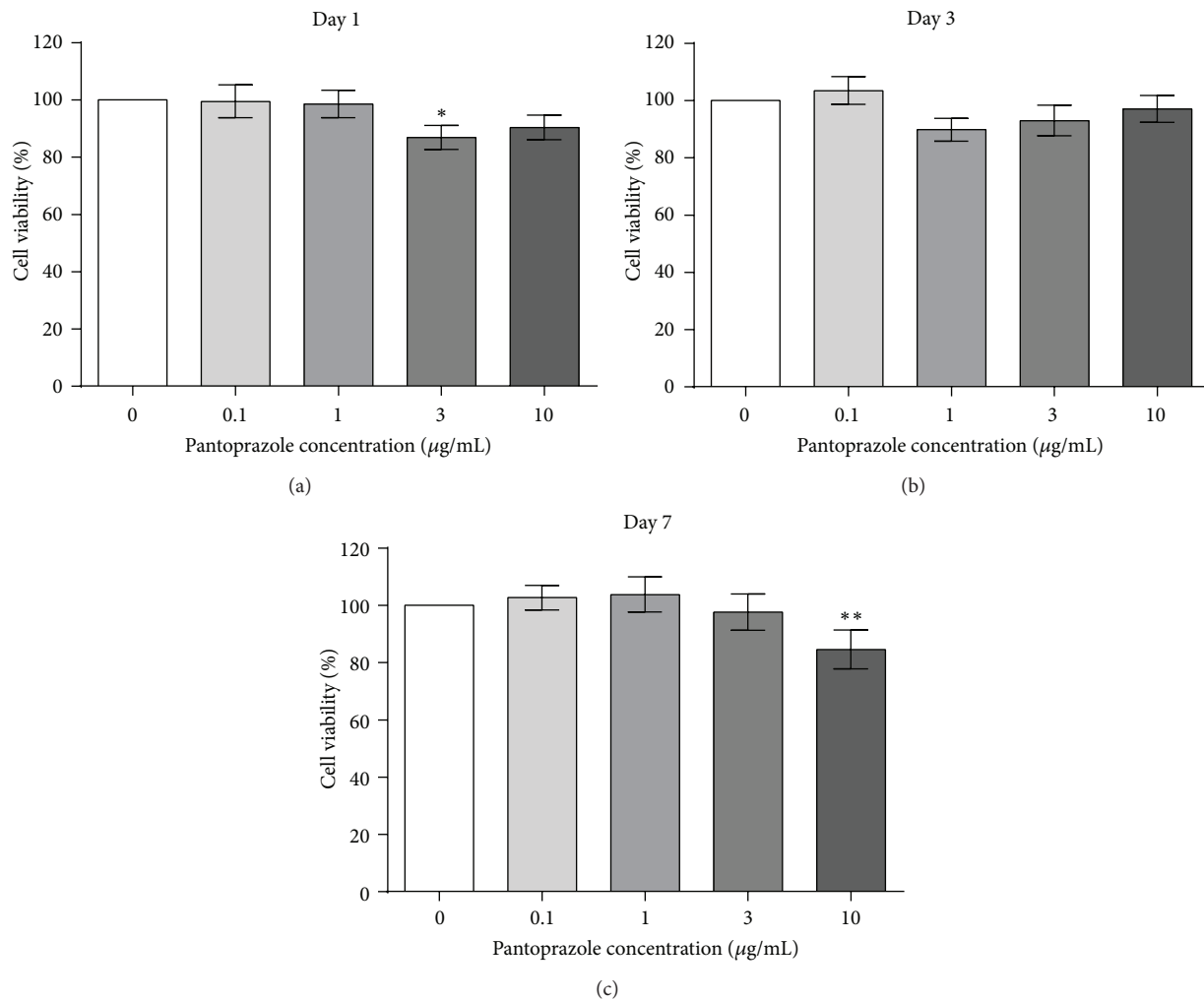


FIGURE 5: The MTT cell viability assays performed on days 1, 3, and 7 of stimulation with pantoprazole ($N = 4, n = 6$). The viability in relation to the respective negative control was assessed. The results were expressed as percentages of the negative control values. Shown are column bar graphs of means \pm SEM. * $P < 0.05$ and ** $P < 0.01$ versus the untreated controls.

an osteopetroricket-like bone matrix [19]. In synopsis of our studies on human osteoblasts and osteoclasts, as well as various studies that confirm the inhibitory effects of PPI on osteoclast function, the development of osteoporosis through effects on a cellular level as a causal link between PPI treatment and an increased fracture risk seems unlikely [6, 12, 20]. Accordingly, Targownik et al., who used a multivariate linear regression model on a set of data from the Canadian Multicentre Osteoporosis Study to determine whether there is a relevant decrease in bone mineral density (BMD) after administration of PPIs, revealed that there was no association between PPI usage and accelerated BMD loss over a ten-year period, although PPI users had a lower average baseline BMD [21].

However, not all studies agree on the effects of PPIs on bone homeostasis. In an early study by Mizunashi et al., for example, the administration of omeprazole led to an increase in serum levels of parathyroid hormone and subsequently TRAP, alkaline phosphatase, and bone gla protein (osteocalcin) while decreasing urinary excretion of calcium. This was thought to suggest overall increased bone turnover

[22]. Costa-Rodrigues et al. on the other hand proposed in their study that there is the possibility of a dose-dependent decrease in bone turnover with the administration of PPIs [23]. Finally, several meta-analyses that were performed on recent observational studies hypothesized that the heterogeneity of results concerning the increased fracture risk could be due to hitherto unknown confounders and called for further investigation [6, 7].

6. Conclusions

PPIs are commonly used in combination with NSAID in pain management regimens for patients with bone-related pathologies. An increased fracture risk implying adverse effects on bone homeostasis would especially limit the safe application of this group of drugs as first line therapy in this context. In synopsis of this and several other *in vitro* studies, we think that the development of osteoporosis through direct cellular impairment is unlikely. Under PPI stimulation, the ability of osteoclasts to resorb mineralized matrix seems to

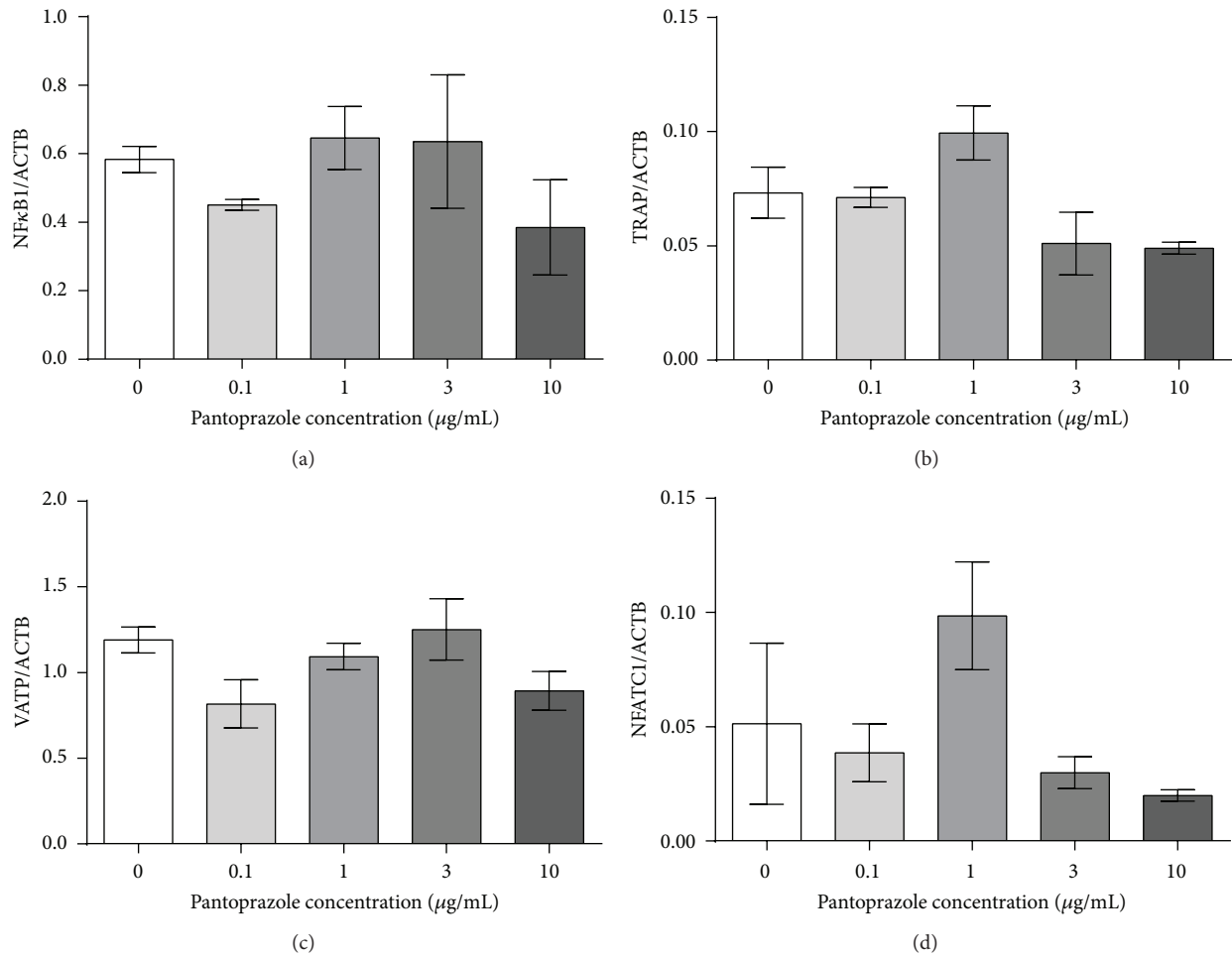


FIGURE 6: Gene expression analysis of *NFκB* (a), *TRAP* (b), *VAP* (c), and *NFATC1* (d) was performed after 7 days of stimulation with pantoprazole ($N = 4$, $n = 2$). The results were normalized to the housekeeping gene β -actin (ACTB). Shown are column bar graphs of means \pm SEM.

be inhibited, while the ability of osteoblasts to synthesize new matrix is increased.

Reduced bone turnover as a result of the decreased resorption rate could potentially contribute to an increased fracture risk, as the bone matrix is not optimally adapted to its constantly changing load. Additionally, cell-independent causes of osteoporosis, for example, decreased calcium absorption or increased parathyroid hormone excretion, remain possible explanations for the association between PPI use and fracture risk. Finally, it must also be considered that the increased fracture risk found in observational studies is not causally related to the administration of PPIs but rather caused by an unknown confounder and therefore factually nonexistent. To prove a causal link between the use of PPIs and an increased fracture risk, further investigations into potential confounding factors as well as *in vivo* studies to further illustrate effects of the drugs on the cells in their natural environment are necessary.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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