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Effect of magnesium degradation products on mesenchymal stem cell fate and osteoblastogenesis

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Short title: *Mg-degradation, MSC microenvironment, and cell fate*

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ABSTRACT

The unique properties of magnesium (Mg) and its alloys to combine metal related mechanical properties, biocompatibility, and biodegradability (up-to now restricted mainly to polymers) justify its renewal of attention in implantology-field. Some in vivo studies underlined the possible osteoconductive effect of Mg-based-metals and several in vitro studies highlighted positive effect of Mg-enriched biomaterial. However, even if the biological activity of magnesium was observed and is intriguing, it remains largely unexplored. Furthermore, due to increased regulation, introduction of new implants on the market will have to be accompanied with more mechanistic understanding. Therefore, to mimic the in vivo effects of degradation of Mg-based implant on mesenchymal stem cell differentiation during bone remodelling, non-hematopoietic multipotent foetal progenitor cells i.e., HUCPV or human umbilical cord perivascular cells, were cultured for up to three weeks with or without osteoblastic differentiating media and with or without magnesium extract (about 5mM). In an attempt to partially unveil the mechanistic or to select paths to be further investigated; a very broad selection of genes was chosen (e.g., involved in osmolality sensing). Several classical bone markers were also studied on gene and protein levels. The data suggest that Mg extract alone potentiates the cell proliferation or delays the natural fate of maturation/differentiation. While when the cells are driven towards osteoblastic differentiation, Mg extract effects become much more complex influencing positively or negatively the differentiation via various pathways. These preliminary

results confirm the choice of the different parameters followed here and the importance to study them further and deeper.

Keywords: Magnesium; mesenchymal stem cells (MSC); Cell differentiation; Gene expression; Protein expression

1. Introduction

Magnesium (Mg) is the second most abundant intracellular divalent cation and is an essential element in human physiology. It is involved in diverse mechanisms (e.g. acting as a cofactor in over 300 enzymes or in metabolic pathways) via two main modes of interaction: (I) binding the substrate to allow the formation of a substrate/enzyme complex (e.g.in the case of kinases) or by (II) first binding the enzyme to alter its structure or to directly play a catalytic role (e.g. in the case of polymerases). Mg is necessary for proper adenosine triphosphate (ATP) synthesis and enzymatic reactions involving ATP require Mg [1]. Furthermore, the positive effect of Mg on cell proliferation is known already for a long time [1-3]. Therefore, Mg can be seen as a central regulator or coordinator of cell metabolism and proliferation leading to the “membrane, magnesium, mitosis (MMM) model” [4].

The European recommended daily allowance for Mg is about 375 mg [5] and, except in case of advanced chronic kidney disease and laxatives and anti-acids iatrogenesis, in healthy individuals, hypermagnesaemia is a very rare case as the kidneys can rapidly excrete the excess [6]. Mg is mainly found in the bone (67%), 30% of which (present on the crystal surface of the bone) is rapidly exchangeable (complete within 4 h [6]). Due to its bioactivity and resorbability, Mg-based orthopaedic biomaterials belong to the third generation of biomedical materials. Several in vivo studies reported the beneficial effect of Mg-based coatings [7, 8] or of Mg-bulk material [9-12] on implant anchorage in host bone, mineral apposition rates, and bone mass. To study interfacial healing response, histomorphometry, histochemical and immunohistochemical are the major methodologies. Even if quantitation or semi-quantitation is possible, these methods remain mainly qualitative. However, with methods like dual energy X-ray absorptiometry (DXA) and micro-computed tomography (µCT), mineral content, mass and density of the new bone can be quantitatively measured but their resolution (varying from 500 µm –DXA- [13] to 6 µm -µCT- [14]) can limit the analyses. Nevertheless, quantitative or qualitative, these methods give a

phenotypic description of the osseous tissue reparation. Mechanisms why or how Mg-based alloys are influencing this process are not addressed and probably very difficult to assess in vivo. Additionally, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) encourage the mechanistic understanding of clinical pharmacokinetics and drug–drug interactions before the introduction of new compounds to the market. Albeit restricted for clinical drug development, it is reasonable to think that this recommendation will be spread to other medical products such as Mg-based medical devices.

Due to the interwoven functions of Mg, even if restricted to bone metabolism, to elucidate mechanism(s) why Mg-based implants increase osteoinduction or osseointegration is not an easy task. Therefore, in a "shotgun approach", about 70 genes, involved in various biological functions, were selected to study the fate of mesenchymal stem cell cultured for up to three weeks with or without (1) bulk-Mg degradation products (Mg-extract) and (2) osteoblast (OB) differentiation supplements. Moreover, classical markers such as osteocalcin (OC or bone gamma-carboxyglutamate (gla) protein – BGLAP) or collagen 1A1 (COL1A1) were also followed on protein level.

This study has the goal to highlight possible target genes and proteins to clarify the underlying mechanisms of Mg-induced mesenchymal stem cell (MSC) osteoblastogenesis and to reveal noteworthy pathways for future analysis.

2. Material and methods

2.1. Mg-extract solution

Mg extract was prepared according to EN ISO standards 10993:5 [15] and 10993:12 [16] (0.2 g material / mL extraction medium). Magnesium specimens were cut from a cast ingot of pure Mg (99.95%; Helmholtz Centre Geesthacht, Geesthacht, Germany) in cuboid form (1 cm x 1 cm x 0.5 cm). After sterilisation via sonication for 20 min in 100% isopropanol (Merck, Darmstadt, Germany), the samples were incubated in extraction medium (Eagle's minimum essential medium, Alpha modification (α-MEM); Life Technologies GmbH, Karlsruhe, Germany) supplemented with 15% foetal bovine serum for human mesenchymal stem cells (hMSC-FBS; STEMCELL Technologies SARL, Köln, Germany) and 1% penicillin and 100 mg/mL streptomycin (Pen/Strep; Life Sciences, Karlsruhe, Germany) for 72 h under physiological conditions in an incubator (Heraeus BB 6220; Thermo Scientific, Bonn, Germany); under physiological conditions (5%

CO_2, 20% O_2, 95% relative humidity, 37°C). Mg, calcium (Ca), and phosphorous (P) contents were measured via inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700x ICP-MS, Waldbronn, Germany). The Mg extract solutions were further characterised by measuring their osmolality and pH. Osmolality and pH were quantified using a Gonotec 030-D cryoscopic osmometer (Gonotec, Berlin, Germany) and an ArgusX pH Meter (Sentron Europe BV, Roden, the Netherlands), respectively.

2.2. Cell culture

All biological tests were performed with human umbilical cord perivascular cells (HUCPV), derived from Wharton's jelly of umbilical cords. HUCPV isolations were approved by the local ethical committee and performed as previously described [17]. For the experiments cells of the third to fifth passage were used. 50,000 cells per well in 3 mL complete culture media (i.e., α-MEM supplemented with 15% hMSC-FBS and Pen/Strep were seeded in 6-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Cells were further cultured overnight before applying the different cell culture conditions: (1) culture media without Mg extract and without osteoblast differentiation supplements (afterwards marked as "-M-D"), (2) culture media with Mg extract (final Mg concentration of about 5 mM) and without osteoblast differentiation supplements ("+M-D"), (3) culture media without Mg extract and with osteoblastic differentiation factors ("-M+D"), and (4) culture media with Mg extract and with osteoblast differentiation supplements ("+M+D").

For the osteoblastic differentiation, culture medium was supplemented for one week with 10^{-8} M dexamethasone, 5 mg/mL L-ascorbic acid 2 phosphate and 10^{-8} M 1α,25 dihydroxyvitamin D3 (1,25(OH)2D3; Sigma-Aldrich Chemie GmbH, Munich, Germany). Afterwards, 5 mM β-glycerolphosphate (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added and cultures were then maintained for 2 or 3 weeks. Media was refreshed every 2 to 3 days. Supernatant aliquots were removed every week for 3 weeks and stored at -80°C until use. Time points were therefore named 1W, 2W, and 3W. Similarly, ribonucleic acids (RNA) and deoxyribonucleic acids (DNA) extractions were performed.

2.3. DNA extraction

Total DNA content (three independent experiments) was assessed at all-time points to normalise results of further biological tests in order to counteract any cell culture variation. All chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany. Cells were first digested over night at 60°C in a

digestion solution containing 500 µL papain buffer solution (0.1 M NaH_2PO_4 and 5 mM EDTA, pH 6 in double distilled water (ddH2O)), 5 µL β-mercaptoethanol, and 2.5 µL of papain solution (10 µg/mL papain in ddH2O). On the next day samples were diluted 1:5 in DNA dilution buffer (2.5 M NaCl in 19 mM sodium citrate pH 7). Out of the latter solution 100 µL of diluted samples were pipetted in triplicate in 96-well plate, and 50 µL of DNA working buffer (2 M NaCl in 15 mM sodium citrate pH 7) as well as 50 µL of bisbenzimide solution (2 µg/mL bisbenzimide in DNA Working Buffer) were incubated 15 min in the dark. The reactions were fluorometrically measured (excitation: 355 nm, emission: 460 nm) with a VICTOR3 multilabel plate reader (Perkin Elmer, Massachusetts, USA). Unknown DNA concentrations of samples were obtained by plotting measured fluorescent emission to a Human genomic DNA standard curve (ranging from 0 to 500 ng/mL).

2.4. RNA extractions – Reverse transcription real-time polymerase chain reaction (RT-qPCR)

RNA extractions (from three independent experiments) were performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The procedures were followed as outlined in the manufacturer's protocol. RNA concentrations (optical density – OD – at 260 nm) and purity (OD260/280) were measured by a NanoDrop 2000c (Thermo Scientific, Bonn, Germany).Total RNA of each sample was mixed with gDNA Wipeout buffer and RNase-free water and incubated at 42°C for 2 min. Subsequently, complementary deoxyribonucleic acid (cDNA) was synthesised and then stored at -20°C until use. Primers and amplicon designs were carefully designed to ensure specific and efficient amplification (using Primer 3 version 4.0.0) or found in RTPrimerDB database [18] and purchased from Eurofins MWG Operon (Ebersberg, Germany). Details on selected primers employed and amplicons can be found in result part. qPCR was also assessed and validated in terms of optimal primer annealing temperature and specificity (PCR product checked on 1% agarose gel). qPCR were performed with SsoFast EvaGreen supermix (Bio Rad, Munich, Germany) and under the same conditions as described in [16]. PCR and amplification monitoring were run in triplicate for each sample using a CFX96 Touch real-time PCR detection system with CFX Manager software (Bio Rad, Munich, Germany; version 3.1). A melting curve step was inserted to each run to confirm melting temperature (Tm) of PCR products. All Ct values greater than 35 were considered negative calls. Normalised gene expression (ΔC(t) and ΔΔC(t)) [19] methods were chosen. Reference genes (*endoplasmic reticulum membrane protein complex subunit 7* (*EMC7*), *glyceraldehyde-3-*

phosphate dehydrogenase (*GAPDH*), *polymerase* (*RNA*) *II* (*DNA directed*) *polypeptide A* (*POLR2A*), *and ribosomal protein L10* (*RPL10*)) were carefully selected based on the geNorm algorithm method [20] and automatically calculated with the CFX Manager software. Due to inherent biological variability between samples three biological and three technical replicates were performed. Differential expressions between two conditions (-M-D vs +M-D and -M+D vs +M+D) were then statistically analysed with the *t*-test. Regulation threshold (expression fold change) and *p*-values (measure of the evidence against the null hypothesis in a statistical test) were set to 1.2 and 0.05, respectively.

2.5. Alkaline phosphatase (ALP)

ALP activity was assessed in the cell culture supernatants (QuantiChromeTM Alkaline Phosphatase Assay Kit; BioAssay Systems, Hayward, CA) (three independent experiments, each with n=5) after 1, 2, and 3 weeks), according to the manufacturer's protocol. This assay is based on the inherent catalytic and kinetic activity of ALP, i.e., removal of the phosphate group from ρ nitrophenyl phosphate, resulting in the formation of a coloured product (ρ nitrophenol; yellow), which is easily measured by a spectrophotometer (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany). The kinetic activity of the ALP enzymes contained in each material cell culture supernatant was normalised to a reference (tartrazine) as well as to sample total DNA content.

2.6. Enzyme-linked immunosorbent assay (ELISA) tests

The following ELISA-kits were used: OC (Human Osteocalcin Instant ELISA, eBioscience, Frankfurt, Germany), osteopontin (OPN; EELISA Kit for OPN, Uscn Life Science Inc., Wuhan, China), osteoprotegerin (OPG or tumour necrosis factor receptor superfamily, member 11b - TNFRSF11B; BOSTER BIOLOGICAL TECHNOLOGY Co.,Ltd., Fremont, United States of America), and procollagen I N-terminal propeptide (PINP; Enzyme-linked Immunosorbent Assay Kit for Procollagen I N-Terminal Propeptide, Uscn Life Science Inc., Wuhan, China)

The release of the respective proteins (three independent experiments, each with n=3) in the cell culture media were measured by the ELISA assay kits. The procedures were followed according to the manufacturer protocols. Absorbance of the formed coloured products was measured at 450 nm (620 nm reference wave length; with a Tecan Sunrise (TECAN Deutschland GmbH, Crailsheim, Germany).

2.7. Statistical analysis

Statistics were performed using the SigmaStat package (Systat software GmbH, Erkrath, Germany; version 11.0). Standard analysis comparing treatments was done by one way repeated measurement (RM) analysis of variance (ANOVA). Statistical significance was accepted if the significance level was $p<0.05$. For RT-PCR, to detect differential expression, t test method ($p<0.05$) was employed and directly calculated by CFX Manager Software (Bio Rad, Munich, Germany; version 3.0).

3. Results

3.1. Mg-extract solution

Characterization of the extract is depicted in Table 1. For the following experiments the extract was diluted 1:10 to regain more physiological conditions. A noteworthy fact is that the amount of Ca and P were highly reduced during the preparation of the extracts.

3.2. DNA content

DNA contents were measured for the different conditions every week (Fig. 1). In order to ease reading, only inter conditions statistic differences per week are presented here. Each week, cells cultured with 5 mM Mg extract and osteoblastic differentiation factors (+M+D) exhibited the lowest DNA content and there is no statistical difference between the time points. Similarly, when the cells are cultured with –M+D, the DNA content is constant over the 3 weeks. When cultured only with Mg extract (+M-D), the DNA content is statistically lower only on the third week (compare to 1W) while when cultured with control conditions (-M-D), the DNA content is statistically already lower on 2W (compare to 1W). If analysed per week, there is no different DNA content between -M-D, +M-D, and -M+D conditions.

3.3. RT-qPCR

In order to identify potential genomic processes involved in MSC fate and osteoblastogenesis influenced by Mg, 65 genes (and 4 reference genes) were selected and studied by RT-qPCR (the full from gene abbreviation can be found in Table 2). To simplify data analyses, statistically significant differential gene expressions between the two different conditions -M-D vs +M-D and -M+D vs +M+D are presented in the Table 3 and 4, respectively. However, the complete gene expression patterns over time can be found in supplementary material / data supplement (Table S1).

-M-D vs +M-D—One remarkable trend to be observed is that on 1W 11 genes are up regulated while 10 are down regulated. However, on the second week, only 1 gene out of the 29 differentially expressed is up regulated. On the 3W, this trend is reversed: 18 genes are up-regulated and only one down regulated. To ease the analyses the studied genes were classified in 12 different classes (see Table 3).

(I)- *Bone morphogenetic protein (BMP) / Transforming growth factor beta (TGFB) superfamily*. Only *BMP4* is commonly up regulated on 1W and 3W. On 1W, *BMP6* and on 2W, *BMP2*, bone morphogenetic protein receptor type-1A (*BMPR1A*), and bone morphogenetic protein receptor type-1A (TGFB1) are down regulated. *BMP1* expression is not affected over the 3 weeks with the presence of Mg extract.

(II)- *Calcineurin (CN)*. Only nuclear factor of activated T-cells, cytoplasmic 1 (*NFATC1*) is up regulated on 3W. Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform (*PPP3CC* or calcineurin gamma) expression is similar between both conditions over the 3 weeks.

(III)- *Calcitonin (CT)*. While neither calcitonin (*CALCA*) nor calcitonin receptor (*CALCR*) are influenced on 1W, they are both down regulated and up regulated on 2W and 3W, respectively.

(IV)- *Cytokine (CK)*. Endothelin-1 (*EDN1*) is up regulated on 1W. Interleukin 1, beta (*IL1B*) is down regulated on 1W and 2W. No differential regulation for epidermal growth factor receptor (*EGFR*) is observable over the 3 weeks.

(V)- *Extracellular matrix (ECM)*. On 1W actinin (*ACTN1*) is down regulated while collagen type 1 alpha 1 (*COL1A1*), intercellular adhesion molecule 1 (*ICAM1*), integrin alpha V (ITGAV), and osteopontin (*OPN*; or *SPP1*, secreted phosphoprotein 1) are up regulated. Osteoprotegerin (*OPG*; or *TNFRSF11B*, Tumor necrosis factor receptor superfamily member 11B) remains downregulated up to 2W. On 2W, cluster of differentiation 36 (*CD36*), also known as *FAT* (fatty acid or thrombospondin receptor), cadherin-11 (*CDH11*), claudin 16 (*CLDN16*), and integrin alpha-1 and -2 (*ITGA1* and *ITGA2*), are also downregulated. On 3W, only *CDH11* is up regulated.

Presence of Mg extract does not influence expressions of fibrillin-1 (*FBN1*), fibronectin (*FN1*), osteocalcin (*OC* or or bone gamma-carboxyglutamate (gla) protein *BGLAP*), and members of the Rho family of GTPases (*RHOA* and *RHOC*).

(VI)- *Mineralisation (Mine)*. Phosphoethanolamine/phosphocholine phosphatase (*PHOSPHO1*) is down regulated on 1W and 2W. Alkaline phosphatase, liver/bone/kidney (*ALPL*), progressive ankylosis protein homolog (*ANKH*), and annexin A1 (*ANXA1*) are down regulated on 2W. Only *ANXA1* is up regulated on 3W. Ectonucleotide pyrophosphatase (*ENPP1*) expression remains not influenced.

(VII)- *Response to stress/osmotic stress*. On 1W, heat shock 70kDa (*HSP70*) protein 4-like (*HSPA4L*) and heat shock 27kDa (*HSP27*) protein 1 (*HSPB1*), are up and down regulated, respectively. On 2W, aquaporin-2 (*AQP2*) and aryl hydrocarbon receptor (*AHR*) are up regulated. Both are then down regulated on 3W as well as nuclear factor of activated T-cells 5 (*NFAT5*), *HSPA4L*, heat shock 70 kDa (*HSP70*) protein 1A/1B (*HSPA1A*), and DnaJ (Hsp40) homolog, subfamily B, member 1 (*DNAJB1*). Endoplasmin (*HSP90B1*) and aldose reductase (*AKR1B1*) expressions are similar between both conditions.

(VIII)- *Transcription factor (TF)*. Activating transcription factor 4 (*ATF4*) is down regulated on 1W and 2W. Proto-oncogene c-Fos (*FOS*) is also down regulated on 1W. Runt-related transcription factor 2 (*RUNX2*) is up (1W) and then down regulated (2W). On 2W, proto-oncogene c-Jun (*JUN*) is down regulated. SRY (sex determining region Y)-box 9 (*SOX9*) is not influenced by Mg extract.

(IX)- *Transporter (TP)*. On 1W, 2 genes are down regulated (adenosine triphosphate (ATP)-sensitive inward rectifier potassium channel 14 (*KCNJ14*) and potassium inwardly-rectifying channel, subfamily J, member 2 (*KCNJ2*)) and 2 genes are up regulated (sodium/myo-inositol cotransporter (*SLC5A3*) and transient receptor potential cation channel subfamily M member 7 (*TRPM7*)). On 2W, *KCNJ14* and *SLC5A3* remains down and up regulated, respectively, while magnesium transporter protein 1 (*MAGT1*) and transient receptor potential cation channel subfamily V member 1 (*TRPV1*) are down regulated. On 3W, metal transporters *CNNM2*, *KCNJ14*, *KCNJ2*, *TRPM7*, and *TRPV1* are up regulated. Sodium/potassium-transporting ATPase subunit alpha-1 (*ATP1A1*), magnesium transporter MRS2 homolog, mitochondrial (*MRS2*) and transient receptor potential cation channel subfamily V member 2 (*TRPV2*) are not influenced.

(X)- *Vitamin D3 signalling (VitD3)*. Vitamin D (1,25- dihydroxyvitamin D3) receptor (*VDR*) is up regulated on 1W. On 2W, protein kinase C iota type (*PRKCI*) and protein kinase C alpha type (*PRKCA*) are down

regulated. On 3W, the latter (*PRKCA*) is then up regulated while mitogen-activated protein kinase 3 (*MAPK3*) is down regulated.

(XI)- *Wingless signalling (Wnt)*. In this category, only catenin beta-1 (*CTNNB1*) and low-density lipoprotein receptor-related protein 5 (*LRP5*) are down regulated on 2W. Frizzled homolog 1 (*FZD1*) expression is not affected over the 3 weeks with the presence of Mg extract.

-M+D vs +M+D—Striking and time dependent observations were obtained analysing the effect of Mg extract on the expression of the genes selected to follow HUCPV osteoblastic differentiation. Out of 65 genes, 63%, 55%, and 34%, on 1W, 2W, and 3W, respectively, are differentially expressed in the presence of Mg extract (see Table 4). On 1W, all the genes are up regulated. On 2W, 14 and 22 genes are up and down regulated, respectively. Finally, on 3W, 17 genes are up regulated and 5 genes down regulated.

(I)- *BMP / TGFB superfamily*. *BMP1* is up regulated only on 2W. *BMP4* and *BMP6* remain up regulated over the 3 weeks. *BMPR1A* and *BMP2* are up regulated on 1W but are then down regulated on 2W, even on 3W for *BMP2*. *TGFB1* expression is not affected over the 3 weeks with the presence of Mg extract.

(II)- *Calcineurin (CN)*. *NFATC1* and *PPP3CC* are up regulated on 1W.

(III)- *Calcitonin (CT)*. *CALCA* and *CALCR* are up regulated on 1 W and 3W. Additionally, *CALCR* is in between down regulated on 2W.

(IV)- *Cytokine (CK)*. *EDN1* is up regulated over the whole differentiation period studied while *EGFR* is not influence at all. IL1B is down regulated on 2W and 3W.

(V)- *Extracellular matrix (ECM)*. *ACTN1*, *ITGAV*, and *RHOC* expressions are not affected. *FBN1*, *FN1*, *ITGA2*, & *OC* and *CDH11*, *COL1A1*, & *ICAM1* are solely up regulated on 1W and 2W, respectively. RHOA is down regulated on 3W. *OPN* is up regulated over the whole analysed period while *OPG* only the last 2 weeks. A cyclic regulation can be observed for *CD36*, *CLDN16*, and *ITGA1*. These genes are alternatively up-, down-, and up regulated on 1W, 2W, and 3W, respectively.

(VI)- *Mineralisation (Mine)*. *ALPL* and *PHOSPHO1* are also up, down, up cyclically regulated. *ANXA1* is up regulated on 1W while *ENPP1* is down regulated on 2W and 3W.

(VII)- *Response to stress*. *DNAJB1* and *HSP90B1* expressions are similar between both conditions. *AKR1B1* is up regulated over the whole period. *HSPA1A*, *HSPA4L*, *HSPB1*, *NFAT5*, and *AQP2* are up

regulated on 1W and then down regulated (except *NFAT5*) on 2W. Again the same cyclic regulation as previously described (up, down, up) is observed for *AHR*.

(VIII)- *Transcription factor (TF)*. *FOS* is not influenced by Mg extract. *ATF4* and *SOX9* are up regulated on 1W. RUNX2 is down regulated on 2W. *JUN* is up- and then down regulated on 1W and 2W, respectively.

(IX)- *Transporter (TP)*. *MRS2* and *TRPV2* expressions are not influenced. *KCNJ2* and *TRPV1* are again following the cyclic regulation. *ATP1A1* is first up regulated on 1W and then down regulated on 3W. *KCNJ14* is up regulated on 1W while *CNNM2*, *SLC5A3*, and *TRPM7* remain up regulated on the first two weeks. *MAGT1* is up regulated on 3W.

(X)- *Vitamin D3 signalling (VitD3)*. *MAPK3*, *PRKCA*, and *PRKCI* are up regulated on 1W and *VDR* on 2W.

(XI)- *Wingless signalling (Wnt)*. *LRP5* is up- and then down regulated on 1W and 2W, respectively. *CTNNB1* and *LRP5* are down regulated on 2W.

3.4. ALP

ALP is a validated biochemical marker of early bone formation. Therefore, ALP activity was measured in culture supernatants of cells cultured under different conditions (with or without osteoblastic differentiation factors and with or without Mg extract to see if Mg extract has an effect on mineralisation. The measured activities are presented in Fig.2. Under native culture conditions ALP activity is increased on 2W and then remains about the same level on 3W (the differences between ALP activity on 1W -M-D and the ones on 2W -M-D and on 3W -M-D are statistically significant). With Mg extract the activity also increases but more slowly (especially on 2W) to reach the same level on 3W. Similar trend is observable for –M+D but on 3W, its ALP activity is higher than the one of –D+M. With +M+D, the ALP activity reaches its maximum on 2W and is also each week the highest of the ones of the other conditions.

3.3. ELISA tests

OC protein expression was measured every week under the different culture conditions. Each week, only the OC expression on native condition is significantly higher than the expression of cells cultured with Mg extract. However, on 2W, the OC protein expression of +M-D is also significantly lower than the one of –M+D (Figure 3A). On the first week, OPN expression with cell cultured only with differentiating media is significantly higher than the one of cells cultured under native conditions. However, on 2W, the protein expression of cells cultured with full media (differentiation and Mg extract, +M+D) is higher than the one

with native conditions (Figure 3B). Over the analysed weeks, no different PINP expression was measured under the different conditions (Figure 3C). On the first week, +M+D exhibits the lowest OPG:RANKL ratio (Figure 3D). However, on 2W, +M+D ratio is the highest one (significant differences to –M-D and –M+D). –M+D is the lowest (significant differences to –M-D and +M-D). 2W +M-D is also significantly higher than –M+D. On 3W, +M+D ratio remains the highest one (significant differences to all the other culture conditions).

4. Discussion

Due to their mechanical properties closer to the ones of bone than e.g. titanium or stainless steel and their biodegradability, Mg-based orthopaedic materials are promising candidates for orthopaedic applications. Body response after the implantation procedure is really complex. This injury prompts a cascade of events described as “tissue response continuum” [21] starting from inflammation and wound healing and finishing by tissue homeostasis restoration. The healing tissue is therefore a scene full of various factors (e.g., hormones, cytokines, and growth factors) and cell types cooperating to restore the homeostasis. Furthermore, unlike permanent implant material, the degradation of Mg locally causes an increase of extracellular Mg concentration and therefore probably a higher osmolality, gas formation, and an alkaline environment. The media supplemented with Mg extract (5mM) exhibits a slightly higher osmolality and ph. However, the DNA content analyses show that these elevated values do not drastically interfere with the cell viability. Under the native conditions, the DNA content decreases between 1W and 2W, while when cultured with Mg extract, the DNA content decreases between 1W and 3W. Only in the presence of differentiation supplements (-M+D and +M+D), the DNA contents are constant but are higher under (+M-D) conditions. The data suggest that Mg extract alone potentiates the cell proliferation or delays the natural fate of maturation/differentiation. On gene level, much more genes are regulated in the presence of Mg extract when the cells are driven toward osteoblastic differentiation than in the presence of Mg extract alone. BMP/TGFB and Wnt interwoven signalling pathways are quite important for bone homeostasis. Members of the BMP/TGFB superfamily are important key signals of osteogenesis. TGFB promotes osteoprogenitor proliferation, early differentiation, and commitment to the osteoblastic lineage

[22]. BMP2, 4, and 6 have strong osteogenic capacity by (for example) increasing ALP activity, COL1A1 synthesis, and OC expression [23]. BMP1 is involved in ECM organisation by cleaving propeptides of procollagen and promoting fibril formation [24] and can be seen as a marker of a rather advanced OB differentiation. BMP2, BMP4, BMP6 are agonists of BMPR1A. The Wnt pathway is central for all bone cells and promotes the commitment and differentiation along the osteoblastic lineage of the MSC [25]. The canonical Wnt pathway relies on the binding of Wnt ligand to LRP5/6 and FZD1 receptors which will inhibit CTNNB1 (beta catenin) proteosomal destruction leading to its accumulation. CTNNB1 will in turn act as transcriptional coactivator of transcription factors. Here, cells cultured with Mg extract and osteoblastic factors seem more influenced by the BMP/TGFB signalling pathway than by the Wnt one. Moreover, a synergistic effect of Mg extract and osteoblastic factors can be observed on gene and protein levels of classical bone markers. Shortly, osteogenesis can be divided in subsequent sequence of events. First, MSC commit to preosteoblasts and after condensation (which increases the number of committed cells), preosteoblasts start to transform in osteoblasts. Afterwards, the ECM deposition begins followed by terminal differentiation and mineralisation.

RUNX2, activator protein-1 (AP-1; formed by dimers of Jun and Fos protein family members), and ATF4 are multifunctional required transcription factors involved in OB lineage determination, in bone formation and remodelling [26, 27], and in the regulation of several gene expressions (e.g., bone matrix genes, OC, COL1A1 [28]). SOX9 is the primary transcription factor of chondrogenesis [29]. First the organic component of the ECM (90% composed of COL1A1 [30]) is synthesised and then other proteins such as OPN and OC which will colonise the collagenous structure [31]. Even if some differential gene expression is observed for COL1A1, no significant difference is observed on protein level between the different culture conditions. OC (about 20% of the non-collagenous protein) seems to have a role in mineralisation as OC is generally closely associated with calcified area. OC is a rather late marker of osteoblast maturation [32]. On protein level, the production of OC is inhibited by the presence of Mg extract but is restored when the cells are driven toward osteoblastic differentiation. OPN protein expression is also influenced by the culture conditions. Together with ALPL, OPN has a role in controlling hydroxyapatite deposition and is considered as an inhibitor of mineralisation [33]. Only under +M+D conditions (1W and 3W), ALPL expression is upregulated. Similar observations are seen on protein level: Mg extract alone

tends to decrease ALP activity, while a synergistic effect is observed under +M+D conditions. Therefore, it could be postulated that, Mg extract alone seems to inhibit the non-collagenous protein colonisation of the ECM as well as the mineralisation itself. However, these effects are reversed for MSC driven toward osteogenic differentiation. The OPG:RANKL ratio is a useful marker of bone turnover and mineralisation [34]. OPG is a RANKL decoy receptor, inhibiting it to bind RANK and therefore negatively regulating osteoclastic differentiation/resorption. Here, Mg extract seems to decrease bone turnover on 2W. Under +M+D condition, an antagonistic effect is observed after 1W favouring bone resorption. However, on 2 and 3W, bone formation seems preferred.

Calcium (Ca^{2+}) homeostasis is tightly regulated: parathyroid hormone (PTH) acts to increase the concentration of Ca^{2+} in the blood, whereas calcitonin acts to decrease it. As bone is a large reservoir of Ca^{2+} both hormones influence the bone turnover by increasing bone resorption by osteoclasts or inhibiting it via osteoblast OPG production. It was postulated that hypermagnesaemia may decrease PTH secretion and/or synthesis [35]. Similarly, Rodríguez-Ortiz et al. demonstrated that 5 mM Mg decreases PTH expression at various Ca^{2+} concentrations. However, for lower Mg concentrations, an inhibitory effect of Mg was observed only when lower basal calcium concentration was present. Mg was also modulating parathyroid glands function via up regulation of (e.g.) calcium sensing receptor and VDR expressions [36]. In addition to its role in calcium homeostasis, VDR and its high affinity ligand (1α,25(OH)2D3 – vitamin D3) stimulates osteoblastogenesis in MSC also in a potential autocrine/paracrine manner [37]. In this study, Mg extracts with and without osteoblastic factors influences VDR signalling member expressions.

It was reported that CALCA induces osteoblast proliferation, production of monocyte chemoattractants [38] and inhibits OPG production in human osteoblast-like cells via cAMP/PKA-dependent pathway [39]. Furthermore, Mg was measured to enhance serum calcitonin in mice [40]. PPP3CC is one of the three known isoforms of mammalian calcineurin A (isoform gamma). This serine-threonine protein phosphatase is sensitive to Ca^{2+} and calmodulin and plays a critical role in coupling Ca^{2+} signals to cellular responses. Calcium signalling and the Ca^{2+}-Calcineurin-NFATc1 signalling pathway play a significant role in the process of osteoclastogenesis [41]. However, it has also been demonstrated that this signalling pathway exists in osteoblast [42]. It regulates bone formation and resorption by enhancing osteoblast

differentiation and by regulating osteoclast precursor chemoattractants expression [43], respectively. High extracellular Ca^{2+} has been shown to induce RANKL expression via NFATc1/NFATc3 in OB [44]. Furthermore increased extracellular zinc was measured to inhibit osteoclast differentiation by acting on the Ca^{2+}-Calcineurin-NFATc1 signalling pathway [45]. Therefore, high extracellular Mg, also a divalent cation, may influence these processes. Indeed, on gene level, PPP3CC and NFATc1 are both up regulated with Mg extract and osteoblastic factors only on 1W while Mg extract alone seems to have a delayed effect on NFATc1 (3W).

Several class of TRSPT (e.g., TRP) are suspected to be able to transduce physical signal such as osmolality or stretch to intracellular Ca^{2+} signalling ("osmotransduction"), thus playing important roles in growth and differentiation of various stem cells [46]. Furthermore, it has been demonstrated that high extracellular Mg influences intracellular Ca signalling [47]. This study showed on gene level, that genes involved in stress response and transporter genes are differentially expressed under the studied culture conditions. Further analyses, on protein levels, should be performed to further investigate these aspects.

5. Conclusion

A very broad selection of genes, from classical bone markers, transporters, to genes involved in stress response, was chosen to study the effect of Mg extract on MSC cultured with native medium supplemented with or without osteoblastic factors. The results obtained in this study emphasize the complexity of the effect of Mg extract on cellular mechanisms involved when cells are driven toward osteogenesis. As stated in Aubin et al. [48] "not all mature osteoblasts develop via the same regulatory mechanisms nor are they identical molecularly or functionally" therefore, the pathways mentioned here have to be further studied. Furthermore, in vivo, the environment is much more complex as several cells are involved and other complex biological responses are interplaying. Inflammation, for example, is crucial after implantation and should be also implemented in an in vitro model (e.g., via monocyte/macrophage and MSC coculture).

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Figure legends

Fig.1: DNA content (µg/mL). DNA contents were measured up to 3W after induced or non-induced osteoblastic differentiation (+/- D) with or without presence of Mg extract (+/- M). Bars represent mean ±standard deviation (SD) of three independent experiments, each with n=9. Stars indicate statistically significant difference between two groups (one way RM ANOVA; * $p \leq 0.05$).

Fig.2: ALP activity measurements. ALP activity was assessed in cell culture supernatants for up to 3W after induced or non-induced osteoblastic differentiation (+/- D) with or without presence of Mg extract (+/- M). ALP activities were further normalised by corresponding sample DNA content to prevent inter-sample variation. Bars represent mean ±SD of three independent experiments, each with n=6. Stars indicate statistically significant difference between two groups (one way RM ANOVA; * $p \leq 0.05$).

Fig.3: ELISA assays. OC (A), OPN (B), and PINP (C) protein expressions measured in supernatants of cell cultured with different conditions (induced or non-induced osteoblastic differentiation (+/- D) with or without presence of Mg extract (+/- M)) from 1 to 3W and further normalised by corresponding sample. Similarly, for OPG:RANKL ratio (D), OPG and RANKL protein expressions were subjected to the same data treatment but OPG expression was further normalised by RANKL respective ones. Bars represent mean ±SD of three independent experiments, each with n=6. Stars indicate statistically significant difference between two groups (one way RM ANOVA; * $p \leq 0.05$).

Abbreviations list:

Actinin (ACTN1)

Activating transcription factor 4 (ATF4)

Activator protein-1 (AP-1)

Adenosine triphosphate (ATP)

Adenosine triphosphate (ATP)-sensitive inward rectifier potassium channel 14 (KCNJ14)

Aldose reductase (AKR1B1)

Alkaline phosphatase (ALP)

Alkaline phosphatase, liver/bone/kidney (ALPL)

Ankylosis protein homolog (ANKH)

Annexin A1 (ANXA1)

Aquaporin-2 (AQP2)

Aryl hydrocarbon receptor (AHR)

Bone morphogenetic protein (BMP)

Bone morphogenetic protein receptor type-1A (BMPR1A)

Cadherin-11 (CDH11)

Calcineurin (CN)

Calcitonin (CALCA)

Calcitonin (CT)

Calcitonin receptor (CALCR)

Calcium (Ca)

Catenin (cadherin-associated protein), beta 1, 88kDa (CTNNB1)

Catenin beta-1 (CTNNB1)

c-Fos (FOS)

c-Jun (JUN)

Claudin 16 (CLDN16)

Cluster of differentiation 36 (CD36; or FAT (fatty acid or thrombospondin receptor))

Collagen type 1 alpha 1 (COL1A1)

Cytokine (CK)

Deoxyribonucleic acids (DNA)

DnaJ (Hsp40) homolog, subfamily B, member 1 (DNAJB1)

Dual energy X-ray absorptiometry (DXA)

Eagle's minimum essential medium, Alpha modification (α-MEM)

Ectonucleotide pyrophosphatase (ENPP1)

Endoplasmic reticulum membrane protein complex subunit 7 (EMC7)

Endoplasmin (HSP90B1)

Endothelin-1 (EDN1)

Enzyme-linked immunosorbent assay (ELISA)

Epidermal growth factor receptor (EGFR)

Ethylenediaminetetraacetic acid (EDTA)

European Medicines Agency (EMA)

Extracellular matrix (ECM)

Fibrillin-1 (FBN1)

Fibronectin (FN1),

Foetal bovine serum for human mesenchymal stem cells (hMSC-FBS)

Food and Drug Administration (FDA)

Frizzled homolog 1 (FZD1)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Heat shock 27kDa (HSP27) protein 1 (HSPB1)

Heat shock 70 kDa (HSP70) protein 1A/1B (HSPA1A)

Heat shock 70kDa (HSP70) protein 4-like (HSPA4L)

Human umbilical cord perivascular cells (HUCPV)

Inductively coupled plasma mass spectrometry (ICP-MS)

Integrin alpha V (ITGAV)

Integrin alpha-1 (ITGA1)

Integrin alpha-2 (ITGA2)

Intercellular adhesion molecule 1 (ICAM1)

Interleukin 1, beta (IL1B)

Low-density lipoprotein receptor-related protein 5 (LRP5)

Magnesium (Mg)

Magnesium transporter MRS2 homolog, mitochondrial (MRS2)

Magnesium transporter protein 1 (MAGT1)

membrane, magnesium, mitosis model (MMM)

Mesenchymal stem cells (MSC)

Micro-computed tomography (μCT)

Mitogen-activated protein kinase 3 (MAPK3)

Nuclear factor of activated T-cells 5 (NFAT5)

Nuclear factor of activated T-cells, cytoplasmic 1 (NFATC1)

Optical density (OD)

Osteocalcin (OC or bone gamma-carboxyglutamate (gla) protein – BGLAP)

Osteopontin (OPN; or SPP1, secreted phosphoprotein 1)

Osteoprotegerin (OPG or tumour necrosis factor receptor superfamily, member 11b - TNFRSF11B)

Osteoprotegerin (OPG; or TNFRSF11B, Tumor necrosis factor receptor superfamily member 11B)

Parathyroid hormone (PTH)

Phosphoethanolamine/phosphocholine phosphatase (PHOSPHO1)

Phosphorous (P)

Polymerase (RNA) II (DNA directed) polypeptide A (POLR2A)

Potassium inwardly-rectifying channel, subfamily J, member 2 (KCNJ2)

Potential cation channel subfamily M member 7 (TRPM7)

Protein kinase C alpha type (PRKCA)

Protein kinase C iota type (PRKCI)

Repeated measurement (RM) analysis of variance (ANOVA)

Reverse transcription real-time polymerase chain reaction (RT-qPCR)

Rho family of GTPases (RHOA and RHOC).

Ribonucleic acids (RNA)

Ribosomal protein L10 (RPL10)

Runt-related transcription factor 2 (RUNX2)

Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform (PPP3CC or calcineurin gamma)

Sodium/myo-inositol cotransporter (SLC5A3)

Sodium/potassium-transporting ATPase subunit alpha-1 (ATP1A1)

SRY (sex determining region Y)-box 9 (SOX9)

Standard deviation (SD)

Transcription factor (TF)

Transforming growth factor beta (TGFB)

Transforming growth factor, beta 1 (TGFB1)

Transient receptor potential cation channel subfamily V member 1 (TRPV1)

Transient receptor potential cation channel subfamily V member 2 (TRPV2)

Transporter (TP)

Vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR)

Vitamin D3 signalling (VitD3)

Wingless signalling (Wnt)

With or without Mg extract (+M and –M)

With or without osteoblast differentiation supplements (+D and –D)

Table(s)

Table 1: Mg, Ca, and P contents (mM), osmolality (Osmol/kg) and pH of the different solutions.

	Mg (mM)	Ca (mM)	P (mM)	Osmolality (Osmol/L)	pH
Complete culture media	0.74	1.74	1.25	0.29	7.36
Mg extract	53.07	0.73	0.31	0.41	8.39
Complete culture media + diluted Mg extract	5.60	1.64	1.16	0.31	8.00

Table 2: Real-time PCR primers. For each gene official gene name and abbreviation (human genome organisation, HUGO) and corresponding trivial synonym (in italic), accession number (from national centre for biotechnology information (NCBI) reference sequence collection database), primers, and amplicon size are indicated. The 69 selected genes are regrouped in 12 different groups. CN: calcineurin, CC: calcitonin, CK: cytokine ECM: extracellular matrix, Mine: Mineralisation, Ref. Gene: reference genes, Response to stress/ Osmotic stress, TF: transcription factors, TP: transporters, VitD3: vitamin D3, and Wnt: Wingless family.

Group	Target name / NCBI RefSeq			
	Abbreviation	**Forward**	**Reverse**	**Amplicon length (bp)**
BMP / TGF-β superfamily	Bone morphogenetic protein 1 / **NM_006129**			
	BMP1	GGGTCATCCCCTTTGTCATTG	GCAAGGTCGATAGGTGAACACA	152 bp
	Bone morphogenetic protein 2 / **NM_001200**			
	BMP2	ACCCGCTGTCTTCTAGCGT	CTCAGGACCTCGTCAGAGGG	140 bp
	Bone morphogenetic protein 4 / **NM_001202**			
	BMP4	ATGATTCCTGGTAACCGAATGC	CCCCGTCTCAGGTATCAAACT	93 bp
	Bone morphogenetic protein 6 / **NM_001718**			
	BMP6	AGCGACACCACAAAGAGTTCA	GCTGATGCTCCTGTAAGACTTGA	159 bp
	Bone morphogenetic protein receptor type-1A / **NM_004329**			
	BMPR1A	ACTGCCCCCTGTTGTCATAG	AATGAGCAAAACCAGCCATC	64 bp
	Transforming growth factor beta-1 / **NM_000660**			
	TGFB1	CAAGCAGAGTACACACAGCAT	TGCTCCACTTTTAACTTGAGCC	129 bp
CN	Nuclear factor of activated T-cells, cytoplasmic 1 / **NM_172387**			
	NFATC1	CACCAAAGTCCTGGAGATCCCA	TTCTTCCTCCCGATGTCCGTCT	132 bp
	Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform (*calcineurin gamma*) / **NM_005605**			
	PPP3CC	CTCCATCCTGAGGCAAGAGAAG	AGTCACCCAGAAAGAGGTAGCG	149 bp
CT	Calcitonin / **NM_001741**			
	CALCA	TCTAAGCGGTGCGGTAATCT	CCCAGCATGCAAGTACTCAG	94 bp
	Calcitonin receptor / **NM_001164738**			
	CALCR	CTAAATCACCCAACCCCAATTCT	CTGCATTCGGTCATAGCATTTG	135 bp
CK	Endothelin-1 / **NM_001955**			
	EDN1	TCCTCTGCTGGTTCCTGACT	ATGGAAGCCAGTGAAGATGG	99 bp
	Epidermal growth factor recepto / **NM_005228**			
	EGFR	AACACCCTGGTCTGGAAGTACG	TCGTTGGACAGCCTTCAAGACC	106 bp
	Interleukin 1, beta / **NM_000576**			
	IL1B	CAGCTACGAATCTCCGACCAC	GGCAGGGAACCAGCATCTTC	100 bp
ECM	Actinin / **NM_001130004**			
	ACTN1	GTGACATTCCAGGCCTTCAT	TATCTGTGTCGGCTGTCTCG	55 bp
	Platelet glycoprotein 4 / **NM_000072**			
	CD36	GCCAAGGAAAATGTAACCCAGG	GCCTCTGTTCCAACTGATAGTGA	101 bp
	Cadherin-11 / **NM_001797**			
	CDH11	AACCTGAGTATCAGTACATGCCT	CCTGCCTTCATAACCGTAGATTT	155 bp

	Claudin-16 / **NM_006580**			
	CLDN16	TGGTAACTCGAGCGTTGATG	TTCACGCAGTCAAGACCAAG	85 bp
	Collagen alpha-1(I) chain / **NM_000088**			
	COL1A1	AAGACATCCCACCAATCACC	GCAGTTCTTGGTCTCGTCAC	159 bp
	Fibrillin-1 / **NM_000138**			
	FBN1	AAAGGGGGTTTGTACCTTGG	AGGTGCAGCGTTAGAAGGAA	67 bp
	Fibronectin / **NM_212474**			
	FN1	AGTGGACACCACCCAATGTT	TTCATTGGTCCGGTCTTCTC	76 bp
	Intercellular adhesion molecule 1 / **NM_000201**			
	ICAM1	TCTGTGTCCCCCTCAAAAGTC	GGGGTCTCTATGCCCAACAA	101 bp
	Integrin alpha-1 / **NM_181501**			
	ITGA1	GTGCTTATTGGTTCTCCGTTAGT	GCCCACAAGCCAGAAATCCT	211 bp
	Integrin alpha-2 / **NM_002203**			
	ITGA2	TGTTGTTTGGCCTACAATGTTGG	CAACCAGTAACCAGTTGCCTT	121 bp
	Integrin alpha-V / **NM_002210**			
	ITGAV	ATCTGTGAGGTCGAAACAGGA	TCCAATGGTACAATGGGGCAC	56 bp
	Osteocalcin (or BGLAP, bone gamma-carboxyglutamate (gla) protein) / **NM_199173**			
	OC	GGCAGCGAGGTAGTGAAGAG	CTGGAGAGGAGCAGAACTGG	95 bp
	Osteoprotegerin (or TNFRSF11B, Tumor necrosis factor receptor superfamily member 11B) / **NM_002546**			
	OPG	CGCTCGTGTTTCTGGACAT	GGACATTTGTCACACAACAGC	112 bp
	Osteopontin (or SPP1, secreted phosphoprotein 1) / **NM_000582**			
	OPN	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT	230 bp
	Transforming protein RhoA / **NM_001664**			
	RHOA	AAGGACCAGTTCCCAGAGGT	GCTTTCCATCCACCTCGATA	76 bp
	Rho-related GTP-binding protein RhoC / **NM_001042679**			
	RHOC	ACAGCAGGGCAGGAAGACTA	GTCGATGGAGAAGCACATGA	84 bp
Mine	Alkaline phosphatase, liver/bone/kidney / **NM_000478**			
	ALPL	CACCCACGTCGATTGCATCT	TAGCCACGTTGGTGTTGAGC	211 bp
	Progressive ankylosis protein homolog / **NM_054027**			
	ANKH	GTTCACCTTCGTCTGCATGGCT	GGCAAAGTCCACTCCGATGATG	109 bp
	Annexin A1 / **NM_000700**			
	ANXA1	AAGGTGTGGATGAAGCAACC	GCTGTGCATTGTTTCGCTTA	57 bp
	Ectonucleotide pyrophosphatase / **NM_006208**			
	ENPP1	TCCTCTGCTGGTTCCTGACT	ATGGAAGCCAGTGAAGATGG	99 bp
	Phosphoethanolamine/phosphocholine phosphatase / **NM_001143804**			
	PHOSPHO1	ATCGTGGACGAAAACAGCGACG	GGTACTTGAAGACGCGCTGCAT	127 bp
Ref. Gene	ER membrane protein complex subunit 7 **/ NM_020154**			
	EMC7	TGCCCTATCCTCTCCAAATG	CCCCACGATTCCCTTTTAAT	105 bp
	Glyceraldehyde-3-phosphate dehydrogenase / **NM_002046**			
	GAPDH	GTCGGAGTCAACGGATTTG	TGGGTGGAATCATATTGGAA	143 bp
	Polymerase (RNA) II (DNA directed) polypeptide A, 220kDa / **NM_000937**			
	POLR2A	GAGAGCGTTGAGTTCCAGAACC	TGGATGTGTGCGTTGCTCAGCA	152 bp
	Ribosomal protein L10 / **NM_006013**			
	RPL10	AGTGGATGAGTTTCCGCTTT	ATATGGAAGCCATCTTTGCC	135 bp
Response to stress/ Osmotic stress	Aldose reductase / **NM_001628**			
	AKR1B1	AGCAGCCAGGATATGACCAC	GGGTAATCCTTGTGGGAGGT	89 bp
	DnaJ homolog subfamily B member 1 (HSP40) / **NM_006145**			
	DNAJB1	CCAGTGAGTGACTCCAGCAA	CTTCACTGGAGCCAGAGGTC	97 bp
	Endoplasmin / **NM_003299**			
	HSP90B1	TATGTGCGCCGTGTATTCAT	GGGGAGATCATCTGAGTCCA	96 bp
	Heat shock 70 kDa protein 1A/1B (*HSP70*) / **NM_005345**			
	HSPA1A	CCGAGAAGGACGAGTTTGAG	CTGGTACAGTCCGCTGATGA	77 bp
	Heat shock 70 kDa protein 4L (*HSP70*) / **NM_014278**			
	HSPA4L	CAGCAATAGCCCAGAAGAGG	TCAATGCCAACCACAGACAT	96 bp

	Heat shock 27kDa protein 1 (*HSP27*) / **NM_001540**			
	HSPB1	ACGAGATCACCATCCCAGTC	CTTTACTTGGCGGCAGTCTC	91 bp
	Nuclear factor of activated T-cells 5 / **NM_138713**			
	NFAT5	AACCATATGACCCTGCTTGC	ACCAGGCAAATGACTTCTGG	83 bp
	Aquaporin-2 / **NM_000486**			
	AQP2	TCTCCTGCAGTGGATGAGTG	CTTCAGCCCTCTCAAAGTGG	81 bp
	Aryl hydrocarbon receptor / **NM_001621**			
	AHR	AGCCACTGCATTCAGCTCTT	TGGGATTCCATCAGATTTTCA	95
TF	Activating transcription factor 4 / **NM_182810**			
	ATF4	GCTCCTATTTGGAGAGCCCCT	AGTGGCATCTGTATGAGCCCA	77 bp
	Proto-oncogene c-Fos / **NM_005252**			
	FOS	CTCCGGTGGTCACCTGTACT	GGTGAAGACGAAGGAAGACG	59 bp
	Proto-oncogene c-Jun, Transcription factor AP-1 / **NM_002228**			
	JUN	CAGGTGGCACAGCTTAAACA	TGAGTTGGCACCCACTGTTA	58 bp
	Runt-related transcription factor 2 / **NM_001024630**			
	RUNX2	CAGTAGATGGACCTCGGGAA	ATACTGGGATGAGGAATGCG	112 bp
	SRY (sex determining region Y)-box 9 / **NM_000346**			
	SOX9	AGACCTTTGGGCTGCCTTAT	TAGCCTCCCTCACTCCAAGA	121 bp
TP	Sodium/potassium-transporting ATPase subunit alpha-1 / **NM_000701**			
	ATP1A1	GGCAGTGTTTCAGGCTAACCAG	TCTCCTTCACGGAACCACAGCA	119 bp
	Metal transporter CNNM2 / **NM_017649**			
	CNNM2	CACCAAGTTGGACGCTATGCTG	CATCTTCTAAGGTGACGATTCCC	131 bp
	ATP-sensitive inward rectifier potassium channel 14 / **NM_013348**			
	KCNJ14	CTGTGCTCCCTCCTGAGAAC	TTTCCTCTTGGGTGATCCAG	102 bp
	Potassium inwardly-rectifying channel, subfamily J, member 2 / **NM_000891**			
	KCNJ2	CATCAATGTGGGTGAGAAGG	CAGGCAGAAGATAACCAGCA	97 bp
	Magnesium transporter protein 1 / **NM_032121**			
	MAGT1	CGTCATGTTCACTGCTCTCCAAC	CCTGTTGGTGAATGCACTGGAG	115 bp
	Magnesium transporter MRS2 homolog, mitochondrial / **NM_020662**			
	MRS2	ACCAAGAGTTAGGTCTTCAAGCC	GGAACAGCCATTGCTCTAAGTT	171 bp
	Sodium/myo-inositol cotransporter / **NM_006933**			
	SLC5A3	TTGCTTTCCCCCACTGTTAC	AACGGGTAAATCCCTGCTCT	77 bp
	Transient receptor potential cation channel subfamily M member 7 / **NM_017672**			
	TRPM7	CCCTACCGACCAAAGATTGA	CTTCATAAGGCAAGCCCAAA	144 bp
	Transient receptor potential cation channel subfamily V member 1 / **NM_080705**			
	TRPV1	GCCTGGAGCTGTTCAAGTTC	GCAGCAGGATGATGAAGACA	90 bp
	Transient receptor potential cation channel subfamily V member 2 / **NM_016113**			
	TRPV2	CCAGCTCTCCAGTTTTCAGG	CCTCAGAGCCATCTTCTTGG	60 bp
VitD3	Mitogen-activated protein kinase 3 / **NM_002746**			
	MAPK3	CTACACGCAGTTGCAGTACAT	CTACACGCAGTTGCAGTACAT	157 bp
	Protein kinase C alpha type / **NM_002737**			
	PRKCA	GTGGCAAAGGAGCAGAGAAC	GTGGTGTTAAGACGGGCTGT	63 bp
	Protein kinase C iota type / **NM_002740**			
	PRKCI	CAACGAACAGCTCTTCACCA	ACGGGTCTCCTTCCTCATCT	50 bp
	Vitamin D (1,25- dihydroxyvitamin D3) receptor / **NM_001017536**			
	VDR	CTGACCCTGGAGACTTTGAC	TTCCTCTGCACTTCCTCATC	277 bp
Wnt	Catenin beta-1 / **NM_001904**			
	CTNNB1	GAAACGGCTTTCAGTTGAGC	CCTGGGCACCAATATCAAGT	98 bp
	Frizzled homolog 1 / **NM_003505**			
	FZD1	GTGAGCCGACCAAGGTGTAT	AGCACAGCACTGACCAAATG	93 bp
	Low-density lipoprotein receptor-related protein 5 / **NM_002335**			
	LRP5	ATGTTGATGGGACGAAGAGG	GTAGATGAAGTCCCCCAGCA	86 bp

Table 3: Gene expressions analyses -M-D (control samples) vs +M-D (experimental samples). Regulation: fold expression values are compared to regulation threshold (⇧ up regulation and ⇩down regulation). Non-regulated gene are represented by emboldening their symbol names. *p*-value given are absolute values and significance levels where ✳ ≤ 0.05, ✳✳ ≤ 0.01, and ✳✳✳ ≤ 0.001.

		1W				2W				3W			
Group	**Gene**	**Regulation**		***p*-Value**		**Regulation**		***p*-Value**		**Regulation**		***p*-Value**	
BMP / TGFB superfamily	*BMP1*												
	BMP2					-2.53	⇩	8.63E-04	✳✳✳				
	BMP4	1.71	⇧	6.81E-05	✳✳✳					1.82	⇧	1.87E-02	✳
	BMP6	-2.43	⇩	5.95E-04	✳✳✳								
	BMPR1A					-2.03	⇩	1.15E-04	✳✳✳				
	TGFB1					-1.92	⇩	1.14E-02	✳				
CN	NFATC1									2.50	⇧	2.12E-03	✳✳
	PPP3CC												
CT	CALCA					-1.26	⇩	3.79E-02	✳	3.27	⇧	1.45E-04	✳✳✳
	CALCR					-2.91	⇩	1.40E-06	✳✳✳	2.52	⇧	1.96E-05	✳✳✳
CK	EDN1	1.82	⇧	2.48E-06	✳✳✳								
	EGFR												
	IL1B	-1.50	⇩	6.24E-06	✳✳✳	-3.33	⇩	6.63E-05	✳✳✳				
ECM	ACTN1	-1.37	⇩	3.11E-03	✳✳								
	CD36					-3.37	⇩	3.50E-06	✳✳✳				
	CDH11					-1.54	⇩	3.56E-02	✳	1.85	⇧	7.04E-03	✳✳
	CLDN16					-3.79	⇩	5.62E-05	✳✳✳				
	COL1A1	1.72	⇧	8.94E-05	✳✳✳								
	FBN1												
	FN1												
	ICAM1	1.59	⇧	2.51E-03	✳✳								
	ITGA1					-1.83	⇩	2.60E-05	✳✳✳				
	ITGA2					-1.65	⇩	2.05E-02	✳				
	ITGAV	1.71	⇧	5.87E-06	✳✳✳								
	OC												
	OPG	-1.72	⇩	3.58E-05	✳✳✳	-1.82	⇩	1.32E-02	✳				
	OPN	1.46	⇧	1.78E-04	✳✳✳								
	RHOA												
	RHOC												
Mine	ALPL					-1.51	⇩	2.71E-04	✳✳✳				
	ANKH					-2.07	⇩	2.72E-03	✳✳				
	ANXA1					-1.45	⇩	4.94E-02	✳	1.66	⇧	1.68E-02	✳
	ENPP1												
	PHOSPHO1	-3.22	⇩	1.24E-04	✳✳✳	-5.23	⇩	6.82E-03	✳✳				
Response to stress	*AKR1B1*												
	DNAJB1									1.52	⇧	4.03E-02	✳
	HSP90B1												
	HSPA1A									1.88	⇧	1.51E-02	✳
	HSPA4L	1.31	⇧	1.77E-03	✳✳					2.28	⇧	1.25E-02	✳
	HSPB1	-1.43	⇩	1.63E-05	✳✳✳								
	NFAT5									2.22	⇧	1.39E-02	✳
	AQP2					-3.50	⇩	5.98E-05	✳✳✳	3.53	⇧	3.62E-02	✳
	AHR					-2.01	⇩	3.61E-02	✳	1.81	⇧	1.61E-02	✳
TF	ATF4	-1.25	⇩	4.69E-02	✳	-1.79	⇩	3.66E-03	✳✳				
	FOS	-1.83	⇩	9.16E-05	✳✳✳								
	JUN					-2.64	⇩	1.91E-05	✳✳✳				
	RUNX2	1.40	⇧	3.01E-02	✳	-2.07	⇩	8.67E-03	✳✳				
	SOX9												
TP	*ATP1A1*												
	CNNM2									1.90	⇧	3.17E-03	✳✳
	KCNJ14	-1.44	⇩	1.32E-04	✳✳✳	-1.81	⇩	5.79E-04	✳✳✳	2.39	⇧	5.62E-04	✳✳✳
	KCNJ2	-1.21	⇩	3.09E-02	✳					3.47	⇧	7.66E-03	✳✳
	MAGT1					-2.86	⇩	3.88E-02	✳				
	MRS2												
	SLC5A3	1.37	⇧	4.16E-05	✳✳✳	1.57	⇧	2.81E-02	✳				
	TRPM7	1.63	⇧	5.55E-05	✳✳✳					1.77	⇧	1.76E-02	✳
	TRPV1					-2.39	⇩	1.80E-04	✳✳✳	2.70	⇧	4.89E-05	✳✳✳
	TRPV2												
VitD3	MAPK3									-2.52	⇩	1.28E-02	✳
	PRKCA					-2.70	⇩	2.78E-02	✳	1.75	⇧	1.77E-02	✳
	PRKCI					-2.66	⇩	1.13E-06	✳✳✳				
	VDR	7.88	⇧	1.71E-11	✳✳✳								

Wnt	CTNNB1					-2.51	⇩	6.33E-06	✳✳✳				
	FZD1												
	LRP5					-2.06	⇩	5.43E-03	✳✳				

Table 4: Gene expressions analyses: -M+D (control samples) vs +M+D (experimental samples). Regulation: fold expression values are compared to regulation threshold (⇧ up regulation and ⇩down regulation). Non-regulated genes are represented by emboldening their symbol names. *p*-value given are absolute values and significance levels where ✳ ≤ 0.05, ✳✳ ≤ 0.01, and ✳✳✳ ≤ 0.001.

		1W				2W				3W			
Group	**Gene**	**Regulation**		***p*-Value**		**Regulation**		***p*-Value**		**Regulation**		***p*-Value**	
BMP / TGFB superfamily	BMP1					2.40	⇧	3.73E-03	✳✳				
	BMP2	1.50	⇧	1.04E-03	✳✳	-1.41	⇩	3.09E-03	✳✳	-1.73	⇩	3.45E-02	✳
	BMP4	4.83	⇧	1.11E-03	✳✳	2.25	⇧	1.75E-04	✳✳✳	2.77	⇧	2.51E-03	✳✳
	BMP6	2.18	⇧	1.94E-02	✳	2.27	⇧	1.97E-04	✳✳✳	2.59	⇧	7.54E-03	✳✳
	BMPR1A	4.42	⇧	2.64E-04	✳✳✳	-2.80	⇩	3.10E-09	✳✳✳				
	TGFB1												
CN	NFATC1	2.60	⇧	5.04E-04	✳✳✳								
	PPP3CC	3.27	⇧	3.11E-07	✳✳✳								
CT	CALCA	4.56	⇧	1.24E-08	✳✳✳					1.45	⇧	2.84E-04	✳✳✳
	CALCR	7.33	⇧	1.79E-11	✳✳✳	-5.50	⇩	3.40E-08	✳✳✳	1.51	⇧	4.61E-03	✳✳
CK	EDN1	6.34	⇧	1.79E-03	✳✳	1.92	⇧	1.74E-03	✳✳	3.65	⇧	5.98E-03	✳✳
	EGFR												
	IL1B					-1.87	⇩	4.27E-04	✳✳✳	-2.18	⇩	3.15E-02	✳
ECM	*ACTN1*												
	CD36	7.43	⇧	1.03E-06	✳✳✳	-3.11	⇩	2.32E-05	✳✳✳	1.43	⇧	4.61E-02	✳
	CDH11					2.11	⇧	1.13E-02	✳				
	CLDN16	5.65	⇧	9.07E-03	✳✳	-4.25	⇩	9.45E-07	✳✳✳	2.06	⇧	1.57E-02	✳
	COL1A1					3.16	⇧	2.53E-04	✳✳✳				
	FBN1	1.88	⇧	4.69E-02	✳								
	FN1	1.66	⇧	3.47E-02	✳								
	ICAM1					2.11	⇧	9.23E-06	✳✳✳				
	ITGA1	3.86	⇧	1.38E-12	✳✳✳	-1.55	⇩	5.72E-06	✳✳✳	1.31	⇧	2.29E-02	✳
	ITGA2	1.48	⇧	3.71E-02	✳								
	ITGAV												
	OC	2.41	⇧	8.74E-06	✳✳✳								
	OPG					2.01	⇧	4.61E-04	✳✳✳	2.18	⇧	2.29E-02	✳
	OPN	1.64	⇧	4.92E-02	✳	2.76	⇧	1.00E-06	✳✳✳	1.80	⇧	3.27E-02	✳
	RHOA									-2.17	⇩	2.16E-02	✳
	RHOC												
Mine	ALPL	3.73	⇧	4.68E-09	✳✳✳	-1.85	⇩	9.90E-06	✳✳✳	1.56	⇧	4.75E-04	✳✳✳
	ANKH												
	ANXA1	1.84	⇧	1.47E-02	✳								
	ENPP1					-2.04	⇩	1.12E-02	✳	-2.56	⇩	8.20E-04	✳✳✳
	PHOSPHO1	10.63	⇧	8.42E-11	✳✳✳	-3.96	⇩	1.31E-04	✳✳✳	1.96	⇧	6.75E-04	✳✳✳
Response to stress	AKR1B1	2.11	⇧	4.00E-03	✳✳	2.57	⇧	1.58E-04	✳✳✳	1.64	⇧	2.52E-02	✳
	DNAJB1												
	HSP90B1												
	HSPA1A	1.72	⇧	9.53E-03	✳✳	-1.59	⇩	3.15E-03	✳✳				
	HSPA4L	2.36	⇧	1.18E-02	✳	-1.60	⇩	3.07E-02	✳				
	HSPB1	1.62	⇧	2.48E-02	✳	-1.96	⇩	1.66E-05	✳✳✳				
	NFAT5	1.99	⇧	2.87E-02	✳								
	AQP2	9.40	⇧	3.83E-04	✳✳✳	-7.80	⇩	6.31E-10	✳✳✳				
	AHR	3.83	⇧	1.75E-09	✳✳✳	-1.51	⇩	5.02E-03	✳✳	1.53	⇧	4.77E-02	✳
TF	ATF4	1.48	⇧	4.20E-02	✳								
	FOS												
	JUN	2.19	⇧	3.33E-03	✳✳	-1.89	⇩	2.78E-08	✳✳✳				
	RUNX2					-1.92	⇩	3.36E-02	✳				
	SOX9	4.27	⇧	1.35E-06	✳✳✳								
TP	ATP1A1	2.58	⇧	4.48E-03	✳✳					-2.11	⇩	5.37E-03	✳✳
	CNNM2	3.36	⇧	8.60E-07	✳✳✳	2.39	⇧	3.90E-06	✳✳✳				
	KCNJ14	5.36	⇧	1.83E-08	✳✳✳								
	KCNJ2	10.50	⇧	1.66E-08	✳✳✳	-2.52	⇩	2.13E-05	✳✳✳	1.93	⇧	6.12E-03	✳✳
	MAGT1									2.51	⇧	4.44E-02	✳
	MRS2												

	SLC5A3	2.72	⇧	1.18E-05	✳✳✳	1.49	⇧	1.25E-02	✳				
	TRPM7	2.53	⇧	9.79E-03	✳✳	1.89	⇧	1.97E-02	✳				
	TRPV1	2.15	⇧	2.59E-02	✳	-2.79	⇩	2.86E-05	✳✳✳	2,34	⇧	4,26E-03	✳✳
	TRPV2												
VitD3	MAPK3	2.93	⇧	2.18E-05	✳✳✳								
	PRKCA	2.85	⇧	6.09E-08	✳✳✳								
	PRKCI	3.21	⇧	9.80E-03	✳✳								
	VDR					6.33	⇧	5.71E-03	✳✳				
Wnt	CTNNB1					-8.45	⇩	9.10E-04	✳✳✳				
	FZD1					-4.48	⇩	3.27E-05	✳✳✳				
	LRP5	4.32	⇧	6.02E-07	✳✳✳	-1.96	⇩	1.94E-04	✳✳✳				

FIGURE 1

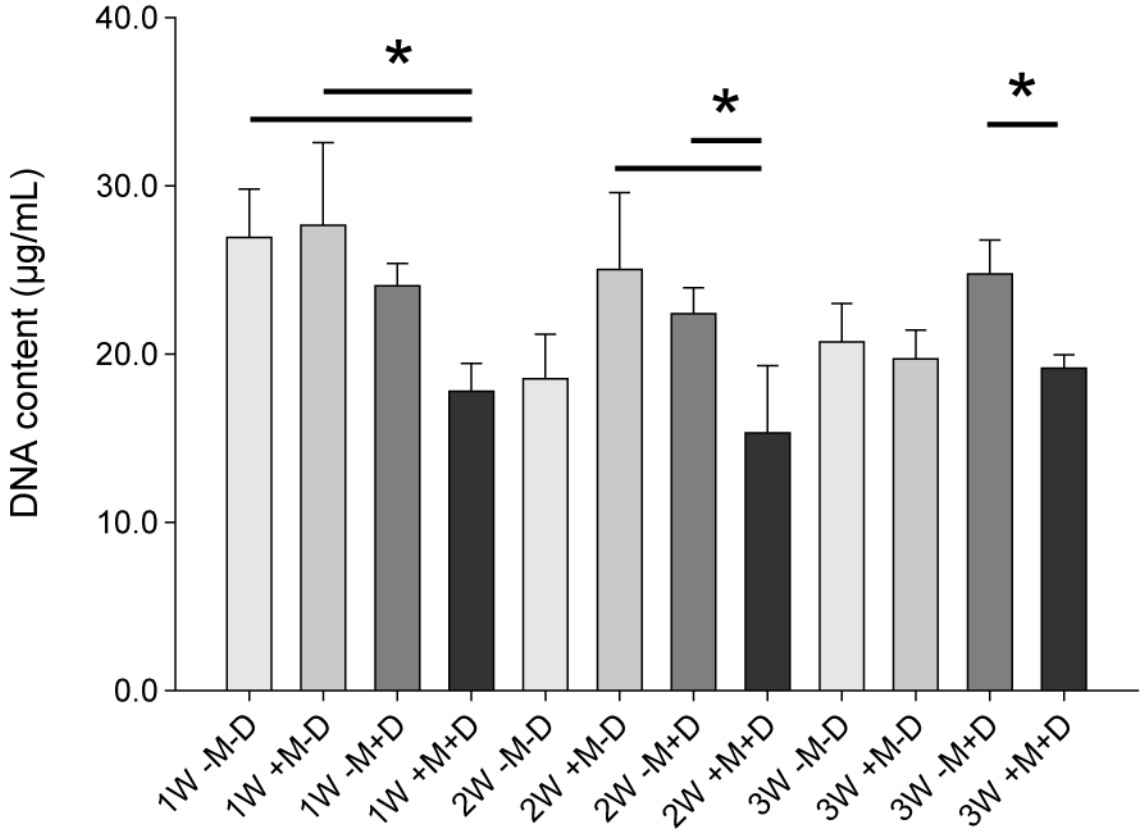
DNA content (µg/mL)
40.0
30.0
20.0
10.0
0.0
*
*
*
1W -M-D
1W +M-D
1W -M+D
1W +M+D
2W -M-D
2W +M-D
2W -M+D
2W +M+D
3W -M-D
3W +M-D
3W -M+D
3W +M+D

FIGURE 2

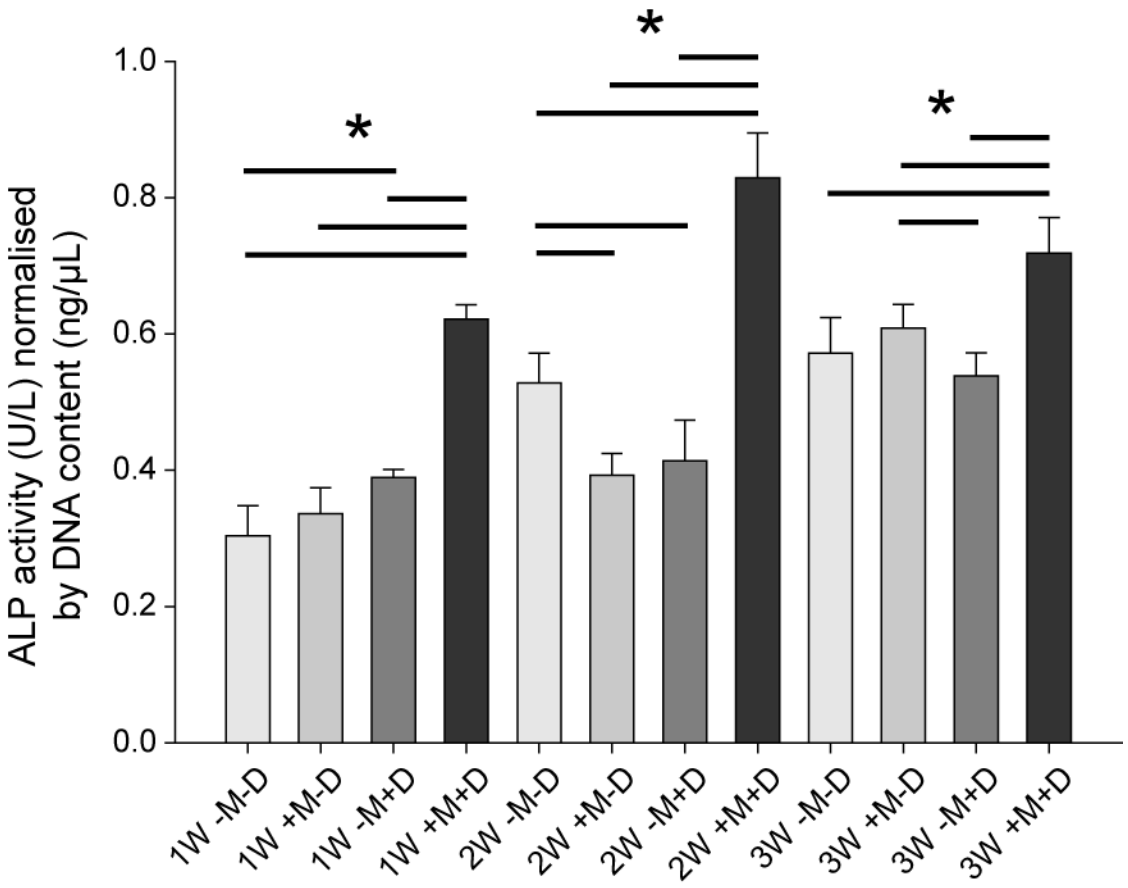
ALP activity (U/L) normalised by DNA content (ng/µL)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
1W -M-D
1W +M-D
1W -M+D
1W +M+D
2W -M-D
2W +M-D
2W -M+D
2W +M+D
3W -M-D
3W +M-D
3W -M+D
3W +M+D

FIGURE 3

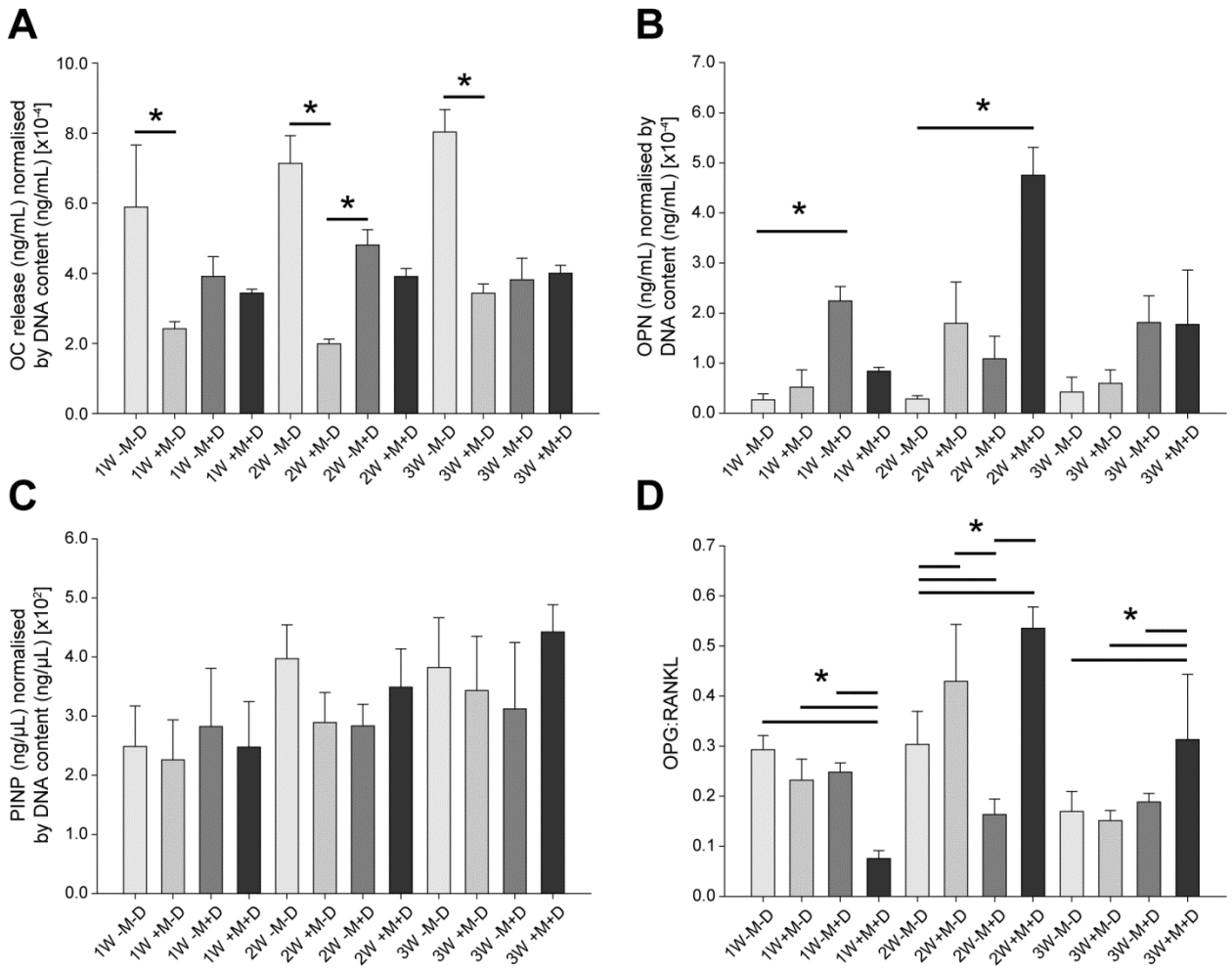
A
OC release (ng/mL) normalised by DNA content (ng/mL) [x10⁻⁴]
10.0
8.0
6.0
4.0
2.0
0.0
*
*
*
*
1W -M-D
1W +M-D
1W -M+D
1W +M+D
2W -M-D
2W +M-D
2W -M+D
2W +M+D
3W -M-D
3W +M-D
3W -M+D
3W +M+D
B
OPN (ng/mL) normalised by DNA content (ng/mL) [x10⁻⁴]
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
1W -M-D
1W +M-D
1W -M+D
1W +M+D
2W -M-D
2W +M-D
2W -M+D
2W +M+D
3W -M-D
3W +M-D
3W -M+D
3W +M+D
C
PINP (ng/µL) normalised by DNA content (ng/µL) [x10²]
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1W -M-D
1W +M-D
1W -M+D
1W +M+D
2W -M-D
2W +M-D
2W -M+D
2W +M+D
3W -M-D
3W +M-D
3W -M+D
3W +M+D
D
OPG:RANKL
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
1W-M-D
1W+M-D
1W-M+D
1W+M+D
2W-M-D
2W+M-D
2W-M+D
2W+M+D
3W-M-D
3W+M-D
3W-M+D
3W+M+D

Figure 1

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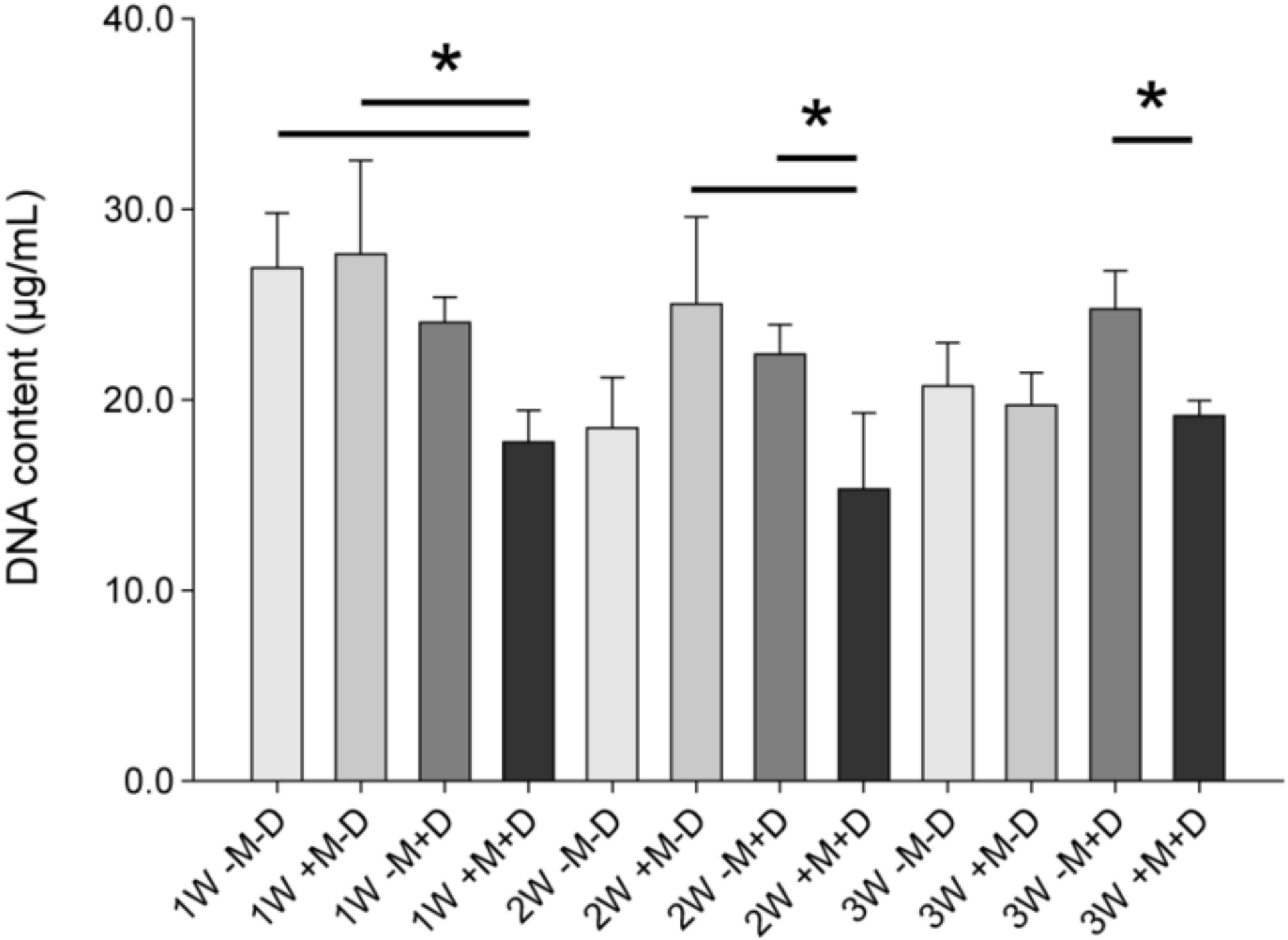

Figure 2

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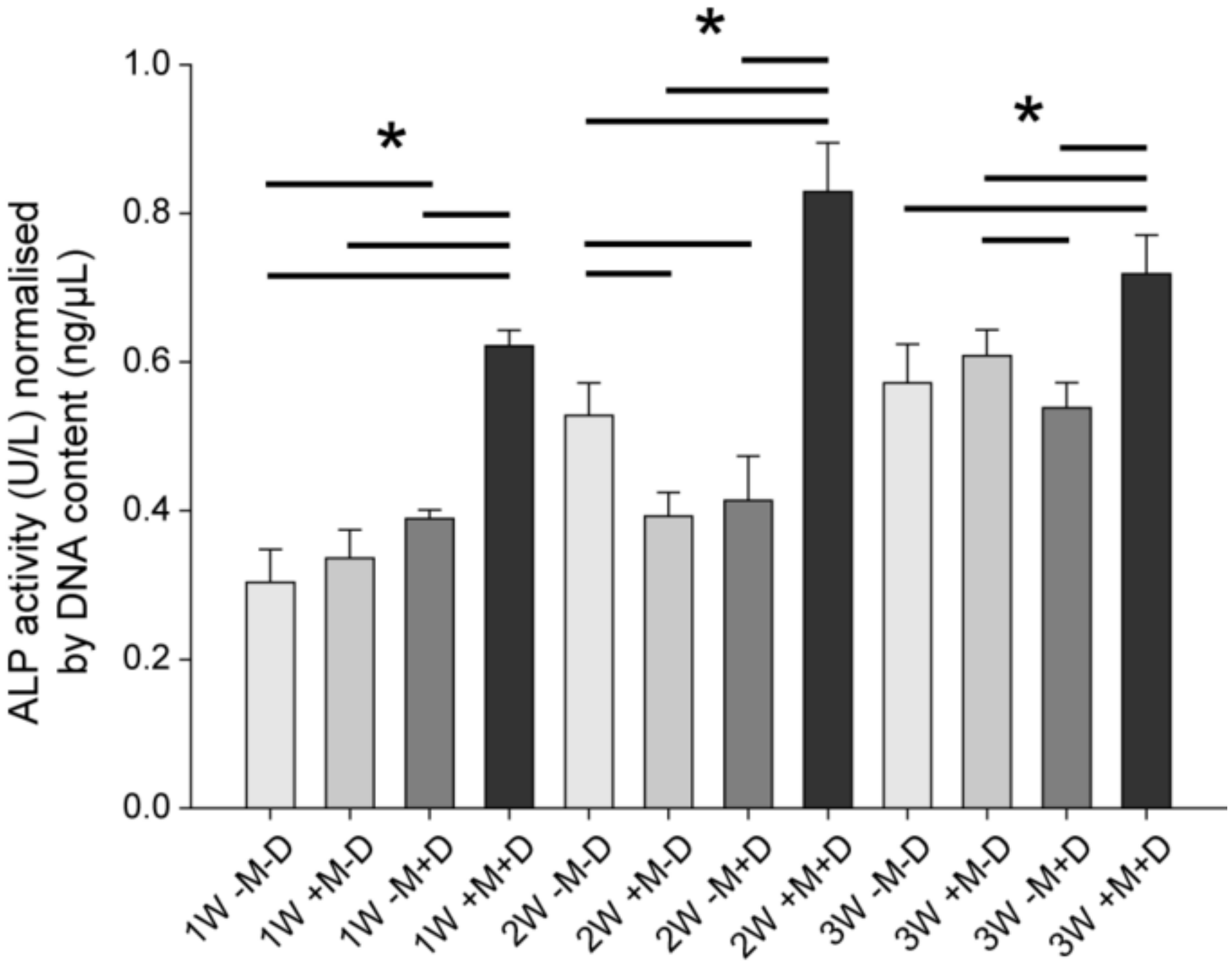

Figure 3

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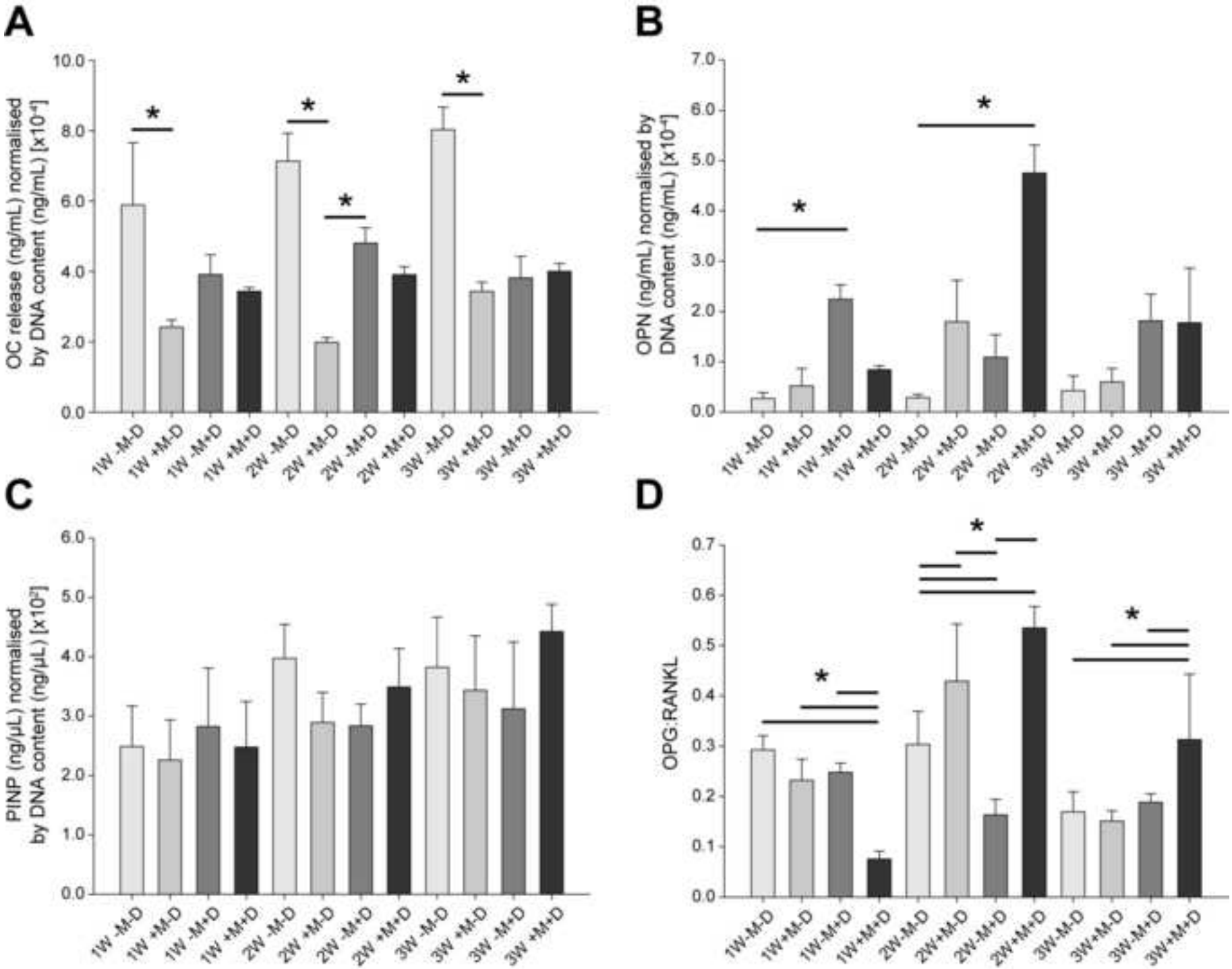

Supplementary material for on-line publication only

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