1 **GPETAFLR, a novel bioactive peptide from *Lupinus***
2 ***angustifolius* L. protein hydrolysate, reduces**
3 **osteoclastogenesis**
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15 Running title: Anti-osteoclastogenic effect of GPETAFLR

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ABSTRACT

The effect of GPETAFLR, a peptide isolated from *Lupinus angustifolius* L. protein hydrolysate (LPH), on osteoclastogenesis was investigated. Human osteoclasts generated from monocytes were used to analyse the effects of GPETAFLR (50-100 µg/mL) on osteoclastogenesis using TRAP reaction, RT-qPCR, and ELISA procedures. LPS enhanced TRAP activity and the expression of osteoclast marker genes (TRAP, OSCAR, RANK, and CATHK) while downregulated the expression of OPG gene in human monocyte-derived osteoclasts. These effects were reduced with GPETAFLR. Moreover, LPS increased the release of osteoclastogenic cytokines (TNF- α , IL-1 β , and IL-6) meanwhile GPETAFLR increased the release of anti-osteoclastogenic cytokines (IL-4 and IL-10) in the medium of human monocyte-derived osteoclasts. For the first time, we show that plant peptides from lupine protein hydrolysates have anti-osteoclastogenic activity. These exciting findings open opportunities for developing nutritional strategies with *Lupinus angustifolius* L. as dietary source of plant proteins, notably GPETAFLR, to prevent development and progression of osteoclast-related diseases.

Keywords: Peptide; protein hydrolysate; Lupine; osteoclast; osteoporosis

1. Introduction

Plant proteins have emerged as an alternative for the production of bioactive peptides (Pihlanto, Mattila, Makinen, & Pajari, 2017), which are small amino acid sequences that are inactive in the native protein but can be released by gastrointestinal enzymes and then reach systemic blood stream as active molecules (Lee & Hur, 2017). In previous studies, our research group has described the presence of a novel peptide isolated from *Lupinus angustifolius* L. protein hydrolysate (Millan-Linares, Millan, Pedroche, & Yust, 2015). The sequence was identified as Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg (GPETAFLR) and its anti-inflammatory activity was evaluated in a human monocytic cell line (Millan-Linares, Millan, Pedroche, & Yust, 2015). Monocyte activation by inflammatory cytokines is known to up-regulate osteoclast (OC) activity via increased expression levels of receptor activator for nuclear factor- κ B (RANK) ligand (RANKL) relative to osteoprotegerin (OPG) (Naranjo et al., 2017). OCs are specialized cells derived from monocyte/macrophage lineage precursor cells that adhere to the bone matrix and secrete acids and lithic enzymes resulting in bone degradation (Kalbasi Anaraki et al., 2015). The differentiation of monocytes into OCs, a multi-complex procedure referred to as osteoclastogenesis, is induced by two crucial cytokines: macrophage colony-stimulating factor (M-CSF) and RANKL. In addition to M-CSF and RANKL as the only factors required for basal osteoclastogenesis, several other cytokines such as TNF- α , IL-1 β , and IL-6 also play an important role in OC biology. These osteoclastogenic cytokines promote bone resorption through multiple mechanisms that include the increase of M-CSF and RANKL production (Zupan, Jeras, & Marc, 2013).

Taken together, the global aim of this study is to assess whether GPETAFLR isolated from *Lupinus angustifolius* L. protein hydrolysate may have benefits in the modulation of osteoclastogenesis.

2. Material and methods

2.1. Isolation, purification, and synthesis of GPETAFLR

Seeds of lupine (*Lupinus angustifolius* L.) were a gift from Koipesol Semillas, S.A. (Sevilla, Spain). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). Lupine protein isolate (LPI) was obtained through the method of Yust et al. (2010) at pilot plant scale (Plant Proteins Group, Instituto de la Grasa). The chemical composition of LPI in dry matter was: proteins 86.83%; dietary fibre 5.97%; oil 5.08%; ash 0.78%, carbohydrates 1.34%. Hydrolysis was carried out according to Millan-Linares et al. (2014). Lupine protein isolate (LPI) was suspended in distilled water (10% w/v), and hydrolysis with Alcalase was performed under the following conditions: pH 8, 50 °C, E/S = 0.3 AU/g protein, and a hydrolysis time of 15 min. The mixture was heated at 85 °C for 15 min to inactivate the enzyme, centrifuged at 6500 g for 15 min, and the supernatant constituted the Lupine protein hydrolysate (LPH). LPH was purified by ultrafiltration and chromatographic techniques according to Millán-Linares et al. 2015. The sequence of the purified peptide was identified through sequence analysis by nanoHPLC coupled to a Polaris Q ion-trap mass spectrometer (Mass Spectrometry Unit, Instituto de la Grasa, Sevilla, Spain). Finally, the peptide was synthesised by Fmoc solid-phase method by the Barcelona Scientific Park Foundation (Barcelona, Spain) at 95% purity, measured by HPLC-UV at 220 nm.

2.2. Monocyte isolation

Peripheral blood mononuclear cells (MNCs) from buffy coat were isolated by centrifugation over a Ficoll-Histopaque (Sigma, Madrid, Spain) gradient (Montserrat-de la Paz et al., 2017). Monocytes were isolated from peripheral blood MNCs using CD14 microbeads and LS columns on a midiMACS system (MiltenyiBiotec, Madrid, Spain). Monocyte (CD14⁺) purity was routinely >90% by flow cytometry analysis (FACScanto II flow cytometer and FACSDiva software, BD) and cell viability >95% by trypan blue exclusion (Sigma). The monocytes were seeded in 24-well culture plates at a density of 1×10^6 cells/mL and cultured in ultra low attachment flasks in RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (complete culture medium).

2.3. Osteoclast differentiation

Monocytes were induced to differentiate for 6 days in the presence of human recombinant M-CSF (50 ng/mL) and RANKL (50 ng/mL) to obtain OCs (Naranjo et al., 2017). Complete culture medium was replaced every 2 days with fresh medium and the cytokines. To study the effect of GPETAFLR in osteoclastogenesis, monocytes were treated for 6 days with GPETAFLR at 50 and 100 μ g/mL in presence of M-CSF and RANKL. LPS (1 μ g/mL) was used as positive control.

2.4. Osteoclast viability

For cell viability, monocytes were seeded in 96-well plates (1×10^5 cells/well) and differentiated into OCs as indicated above. At day 1, 2, 4, and 6, MTT solution (Sigma) was added to cells for 2 hours until a purple precipitate was visible. MTT-formazan crystals were then solubilized with DMSO (Sigma) and measured with a microplate reader at 570 nm corrected to 650 nm. Cell survival was expressed as the percentage of absorbance compared with that of control, non-treated cells.

2.5. TRAP staining and activity assay

TRAP staining was performed using an acid phosphatase leukocyte kit (Sigma) in OCs fixed with 4% paraformaldehyde. TRAP-positive multinuclear cells containing more than 3 nuclei were scored as OCs and examined using an Olympus microscope (40 $\times$) (Model IX71, Olympus Corp, Center Valley, PA, USA). TRAP activity was determined using a TRAP assay kit (Takara Bio, Shiga, Japan). Briefly, 50 μ L of cell extract and 50 μ L of p-nitro-phenyl phosphate (pNPP) were mixed with a sodium tartrate solution and incubated at 37 $^{\circ}$ C for 25 min. The reaction was stopped with 0.5 N NaOH and the absorbance was measured at 405 nm. A solution containing acid phosphatase and pNPP, but not the sodium tartrate solution, was used as a control.

2.6. RNA isolation and RT-qPCR

Total RNA was extracted by using Trisure Reagent (Bioline). RNA quality was assessed by A_{260}/A_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Briefly, RNA (250 ng) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain). An amount of 20 ng of the

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150 resulting cDNA was used as a template for real-time PCR amplifications. The
151 mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad).
152 For each PCR reaction, cDNA template was added to Brilliant SYBR green
153 QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for
154 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene
155 (**Supplemental Table 1**). All amplification reactions were performed in triplicate
156 and average threshold cycle (Ct) numbers of the triplicates were used to
157 calculate the relative mRNA expression of candidate genes. The magnitude of
158 change of mRNA expression for candidate genes was calculated by using the
159 standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference
160 (GAPDH) gene content and expressed as relative fold-change of control.

161 162 **2.7. Cytokine quantification**

163 The pro-osteoclastogenic cytokines TNF- α , IL-1 β , and IL-6, and the anti-
164 osteoclastogenic cytokines IL-4 and IL-10 were determined by enzyme-linked
165 immunosorbent assay (ELISA), following the indications of the manufacturer
166 (Diacclone, Besançon, France).

167 168 **2.8. Statistical analysis**

169 All values are expressed as mean $\pm$ standard deviation (SD). Data were
170 evaluated with Graph Pad Prism Version 5.01 software (San Diego, USA). The
171 statistical significance of any difference in each parameter among the groups
172 was evaluated by one-way analysis of variance (ANOVA), following Tukey's or
173 Dunnett's test for multiple comparisons. *P* values less than 0.05 were
174 considered statistically significant.

3. Results

3.1. GPETAFLR modulates phenotype markers in human monocyte-derived osteoclasts

We investigated the role of GPETAFLR on the TRAP activity and OC marker gene expression during human monocyte differentiation into monocyte-derived OCs. GPETAFLR at 100 µg/mL added to the differentiation medium including M-CSF and RANKL for 6 days did not induce cytotoxicity (data not shown). As depicted in **Figure 1A**, TRAP activity was increased in monocyte-derived OCs treated with LPS, whereas a reduction was observed with GPETAFLR when compared to LPS. In addition, only LPS induced an increase in TRAP (**Figure 1B**), RANK (**Figure 1D**) CATHK (**Figure 1F**) gene expression. The transcriptional activity of RANK gene was also increased by GPETAFLR at 50 µg/mL when compared to untreated cells. Therefore, GPETAFLR induced a decrease in OC marker gene expression.

3.2. GPETAFLR modulates cytokine gene expression and secretion in human monocyte-derived osteoclasts

To further explore the possible osteoclastogenicity of GPETAFLR in human monocyte-derived OCs, gene expression and secretion of TNF-α, IL-1β, IL-6, IL-4, and IL-10 were analysed. LPS promoted the transcriptional activity of TNF-α, IL-1β, and IL-6 genes (**Figures 2A, 2C and 2G**) and the release of these cytokines into the culture media (**Figures 2B, 2D and 2H**). All doses of GPETAFLR diminished the gene expression and release of these pro-

osteoclastogenic cytokines. IL-4 and IL-10 gene expression (**Figure 2I**) and release (**Figure 2J**) were increased by GPETAFLR when compared to LPS.

4. Discussion

Lupin is a popular pulse worldwide. From more than 450 species of the *Lupinus* family, *Lupinus angustifolius* are being used in food production. *L. angustifolius* is used in bakery products and other food to improve the nutritional value, with additional health related benefits of a high percentage of protein, low fat, and starch content, reasonable levels of dietary fibre and the absence of gluten (Villarino et al., 2016).

Currently, a clear trend for the utilization of enzyme technology in food sciences can be observed. There is an interest in obtaining biologically and functionally active peptides, which can be obtained by hydrolysis of animal proteins, as well as those of plant origin (Czubinski, Montowska, Pospiech, & Lampart-Szczapa, 2017). In the literature, there is a previous research that study plant peptides and its anti-inflammatory effects (Malaguti et al., 2014). Inflammation is one of the factors that contributes to the appearance of osteoporosis and others bone-related diseases (Polat et al., 2013). The pro-inflammatory cytokines play a pivotal role in the osteoclast activation but if it is exit a balance between pro- and anti-inflammatory cytokines the bone loss can be balanced (Montalcini et al., 2013). Health care in these diseases includes calcium, vitamin D, and antiresorptive agents among others (Polat et al., 2013). At present, there is a line in food chemistry researches that explore plant compounds with anti-inflammatory properties in osteoporosis disease. Although

the research about anti-inflammatory effects of plant peptides and osteoporosis is not very extensive, there is a sufficient research that talks about compounds in plants with anti-osteoclastogenic activity. For example, *Cistanches herba* aqueous extract has demonstrated a decreased in TRAP levels (Liang et al., 2013). *Equisetum arvense* hydromethalonic extract has shown that can negatively modulate osteoclast development (Costa-Rodrigues, Carmo, Silva & Fernandes, 2012). Saikosaponins, a bioactive compound derivate from *Bupleurum scorzonerifolium* Willd, and *Glechoma hederacea*, an aromatic creeper of the mint family Lamiaceae, can suppress RANKL which is necessary in the differentiation of osteoclast precursors into osteoclast (Zhou, et al., 2014; Hwan et al., 2014).

GPETAFLR (Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg) is a octapeptide isolated and purified from a LPH and its anti-inflammatory activity was evaluated in a human monocytic cell line (Millan-Linares, Millan, Pedroche, & Yust, 2015) and in THP-1-derived macrophages (Millan-Linares et al., 2014). In both articles the results showed an attenuated expression of pro-inflammatory cytokines as IL-1 β and TNF- α , and an increased expression of anti-inflammatory cytoquines as IL-10 and IL-6.

The present study suggests that GPETAFLR peptide decreased TRAP (Tartrate-resistant acid phosphatase) activity. TRAP is an enzyme necessary to characterize the osteoclastic lineage in mature osteoclast (Yoshimatsu et al., 2015). In line with the previous research with GPETAFLR, this plant peptide reduced the gene expression of TNF- α , IL-1 β and IL-6, meanwhile IL-4 and IL-10 gene expression were raised. The possible mechanism that could explain that is the RANKL/OPG ratio. OPG is secreted by osteoblast and protects the

bone from excessive resorption by binding to RANKL, avoiding the RANK/RANKL union (Martin& Sims, 2015; Naranjo et al., 2017). In our research, the cells that received GPETAFLR show a smaller RANK mRNA expression than those that were provided with LPS. At the same time, the OPG expression was increased in the cell treatment with GPETAFLER and reduced with LPS treatment. This point is very interesting because RANK/RANKL/OPG system could be a target for new treatments in bone diseases (Fernandes et al., 2014; Kushilinskii et al., 2014; Pitari et al., 2015; Ndip et al., 2014). The concentrations of pro-inflammatory and anti-inflammatory cytokines are quite related with OPG and RANKL concentrations (Ndip et al., 2014).

In conclusion, our results suggest that GPETAFLR is a bioactive peptide with anti-inflammatory effects that can largely suppress osteoclastogenesis and could be used as adjuvant in osteoclast-related diseases.

5. Conclusion

In conclusion, an anti-osteoclastogenic octapeptide GPETAFLR (Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg) was purified from a *L. angustifolius* protein hydrolysate by ultrafiltration and reversed-phase and gel filtration chromatographies. Anti-osteoclastogenic activity of the synthetic peptide was corroborated by analyzing. The TRAP activity, the expression of markers and cytokines by RT-qPCR, and the levels of these proteins by ELISA, in monocyte-derived OCs. To the best of our knowledge, this is the first report of an anti-osteoclastogenic peptide purified from a *L. angustifolius* protein hydrolysate. Further investigations on in vivo studies of these activities would be necessary

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to establish strategies for specific pathophysiological conditions that could mark
the possible use of these compounds in specialised nutrition.

Conflicts of interest

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. (A) TRAP activity, (B) TRAP, (C) OSCAR, (D) RANK, (E) OPG, and (F) CATHK relative gene expression after the treatment of human monocyte-derived OCs with GPETAFLR at 50 and 100 $\mu\text{g/mL}$ in presence of M-CSF and RANKL. Control means non-treated cells and LPS (1 $\mu\text{g/mL}$) was used as positive control. Values are presented as means $\pm$ SD ($n = 6$) and those marked with different letters are significantly different ($P < 0.05$).

Figure 2. (A,B) $\text{TNF-}\alpha$, (C,D) $\text{IL-1}\beta$, (E,F) IL-4, (G,H) IL-6, and (I,J) IL-10 release (left panels) and relative gene expression (right panels) after the treatment of human monocyte-derived OCs with GPETAFLR at 50 and 100 $\mu\text{g/mL}$ in presence of M-CSF and RANKL. Control means non-treated cells and LPS (1 $\mu\text{g/mL}$) was used as positive control. Values are presented as means $\pm$ SD ($n = 6$) and those marked with different letters are significantly different ($P < 0.05$).

Supplemental Table 1. Detailed information about primers' sequences used in this study.

Target	GenBank accession Number	Direction	Sequence (5'→3')
CATHK	NM_000396	Forward	TTCTGCTGCTACCTGTGGTG
		Reverse	CCAGGTGGTTCATAGCCAGT
GAPDH	NM_001289746	Forward	CACATGGCCTCCAAGGAGTAAG
		Reverse	CCAGCAGTGAGGGGTCTCTCT
IL-10	NM_000572	Forward	CTGACATCAAGGAGCACGTG
		Reverse	GGCTTTGTAGACACCCCTCT
IL-1 β	NM_000576	Forward	AAGGAGAGCTCTTTCCCACC
		Reverse	GCCTCTGGTCTCCTTGGATT
IL-4	NM_000589	Forward	CAACAAGGAACACCACGGAG
		Reverse	TTTCAGTGTTGTGAGCGTGG
IL-6	NM_000600_	Forward	TAACCATCCCTGTCCCAACC
		Reverse	AGTTACATGCCCAGTGGACA
OPG	NM_002546	Forward	GGCAACACAGCTCACAAGAA
		Reverse	CTGGGTTTGCATGCCTTTAT
OSCAR	NM_001282349	Forward	CCCAGCTTCATACCACCCTA
		Reverse	GAAGAGAAGGGGAGCGATCT
RANK	NM_001270949	Forward	GGTGCAGCCTCTAACTCCTG
		Reverse	TTGAGACCAGGCTGGGTAAC
TNF- α	NM_000594	Forward	TCCTTCAGACACCCTCCACC
		Reverse	AGGCCCCAGTTTGAATTCTT
TRAP	NM_001111034	Forward	CTTTCTACCGCCTGCACTTC
		Reverse	GTTTCTTGAGCCAGGACAGC

Figure 1
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Figure 2
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