

1 **Association between periodontal disease and inflammatory arthritis reveals**
2 **modulatory functions by melanocortin receptor type 3**

3 Trinidad Montero-Melendez¹, Mila Fernandes Moreira Madeira², Lucy V Norling¹, Asil
4 Alsam³, Michael A Curtis³, Tarcília Aparecida da Silva², Mauro Perretti¹.

5 ¹The William Harvey Research Institute, Barts and The London School of Medicine,
6 Queen Mary University of London, London EC1M 6BQ, United Kingdom.

7 ²Departamento de Clínica, Patologia e Cirurgia Odontológicas, Faculdade de
8 Odontologia, Universidade Federal de Minas Gerais, 31.270-901 Belo Horizonte,
9 MG, Brazil.

10 ³Blizard Institute, Barts and The London School of Medicine, Queen Mary University
11 of London, 4 Newark Street, London E1 2AT, United Kingdom.

12 **Running head:** MC₃ controls bone loss

13 **Number of pages:** 25

14 **Number of Figures:** 6

15 **Financial support:** This work was funded by MRC (MR/K013068/1), William Harvey
16 Research Foundation and CNPq (Brazil).

17 **Corresponding author and reprint requests:** Mauro Perretti, Centre for
18 Biochemical Pharmacology, The William Harvey Research Institute, Barts and The
19 London Medical School, Queen Mary University of London, Charterhouse Square,
20 London EC1M 6BQ, United Kingdom.

21 Email m.perretti@qmul.ac.uk; Phone no: +44-2078828782; Fax no: +44-207-
22 8826076

23

24 **Abstract**

25 **Background.** Since there is clinical evidence for an association between periodontal
26 disease and rheumatoid arthritis, it is important to develop suitable experimental
27 models to explore pathogenic mechanisms and therapeutic opportunities.

28 **Methodology.** The K/BxN serum model of inflammatory arthritis was applied using
29 distinct protocols, and modulation of joint disruption afforded by dexamethasone and
30 calcitonin, in comparison to the melanocortin (MC) receptor agonist DTrp⁸-γMSH
31 (DTrp), established. Wild type and MC receptor type 3 (MC₃) null mice of different
32 ages were also used.

33 **Results.** There was significant association between severity of joint disease, induced
34 with distinct protocols and volumes of the arthritogenic K/BxN serum, and periodontal
35 bone damage. Therapeutic treatment with dexamethasone (10μg/mouse), elcatonin
36 (30ng/mouse) and DTrp (20μg/mouse) revealed unique and distinctive
37 pharmacological properties, with only DTrp protecting both joint and periodontal
38 tissue. Further analyses in non-arthritic animals revealed higher susceptibility to
39 periodontal bone loss in *Mc3r*^{-/-} compared to wild type mice, with significant
40 exacerbation at 14 weeks of age.

41 **Significance.** These data reveal novel protective properties of endogenous MC₃ on
42 periodontal status in health and disease and indicate that MC₃ activation could lead
43 to the development of a new genus of anti-arthritic bone-sparing therapeutics.

44

45

46 **Keywords:** Dexamethasone, Inflammatory Arthritis, K/BxN arthritis model,
47 Melanocortins, Rheumatoid arthritis,

48

49 1. Introduction

50 Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with
51 progressive disability, early death, increased risk of cardiovascular events and other
52 extra-articular manifestations that have a major impact in the quality of life of
53 sufferers [1,2]. The current clinical approach is to start early, after diagnosis, with
54 aggressive therapy, followed by treatment adjustments according to changes in
55 disease activity. However, despite the important progress in RA therapies during the
56 last decade several needs are still unmet. The introduction of biologics in the early
57 '90s revolutionized the treatment of RA and other chronic diseases such as
58 inflammatory bowel disease. However, although highly effective and generally faster
59 acting than disease-modifying anti-rheumatic drugs (DMARDs) a significant
60 proportion of patients are not responsive, and may also experience an increased risk
61 of opportunistic infections and treatments are costly [3]. Thus there is justification for
62 exploiting novel therapies. Additionally, it is also desirable to produce new
63 therapeutics with efficacy not only on pain and inflammation in the joint, but also able
64 to temper systemic complications of RA affecting the heart, lungs, muscles and bone
65 [1].

66 Targeting the melanocortin (MC) system [4] to treat RA may represent an alternative
67 opportunity to drug discovery [5]. Indeed, one of the melanocortin agonists ACTH
68 (adrenocorticotropin hormone) was shown to be effective in human RA over 60 years
69 ago [6], yet it is truly experiencing a renewed interest [7]. This is prompted by the fact
70 that ACTH may afford biological actions beyond the endogenous production of
71 cortisol [8,9], provoking activation of peripheral MC receptors, including the
72 melanocortin receptor 3 (MC₃). This peripheral mechanism of action of ACTH, hence
73 independent from adrenal release of glucocorticoids, might also underlie efficacy in
74 conditions such as proteinuric nephropathies [10] and multiple sclerosis [11].

75 Surmounting evidence indicates an important counter-regulatory role for the
76 melanocortin pathway during inflammation, including in the osteo-articular system,
77 where melanocortin receptors are expressed by osteoblasts, osteoclasts,
78 chondrocytes, fibroblasts and immune cells. Pharmacological targeting with MC
79 peptides leads to a variety of protective actions including increased matrix deposition,
80 reduced fibroblast activation, osteoblast and chondrocyte proliferation
81 [12,13,14,15,16,17]. *In vivo*, the synthetic peptide DTrp⁸-γMSH (DTrp) reduces
82 clinical signs of disease in models of inflammatory arthritis [18] and urate crystal
83 peritonitis [19] by a mechanism involving MC₃. In addition, the pan-MC agonist
84 peptide AP214 also displays anti-arthritic properties [20]. Recent work by Gomez-
85 SanMiguel *et al*, reported that the MC agonist alpha-melanocyte stimulating hormone
86 (αMSH) can reduce joint inflammation together with an improvement of extra-articular
87 signs associated with systemic arthritis, by increasing body weight and reducing
88 levels of muscle wasting markers [21].

89 An **important** clinical manifestation associated with arthritis is periodontal disease.
90 There is epidemiological evidence associating inflammation of the gum to incidence
91 of RA [22] and, conversely, **there is** higher incidence of periodontitis in RA patients
92 [23]. Intriguingly, recent reports demonstrated presence of alveolar bone loss, an
93 important feature of periodontitis, in rodents during the time course of experimental
94 models of arthritis, namely collagen-induced and adjuvant-induced arthritis
95 [24,25,26]. Herein, we investigated the presence of alveolar bone loss in a different
96 model of **experimental** arthritis; one induced by the arthritogenic K/BxN serum **which**
97 **is much faster in its kinetics, and it is characterized by leukocyte infiltration,**
98 **synoviocyte proliferation, cartilage and bone erosion, thus resembling many features**
99 **of human RA in its active flares [27,28],** In addition, we established the involvement
100 **of the melanocortin system in the development of alveolar bone loss by using a**
101 **combination of genetically engineered mice and pharmacological approaches.**

102 **2. Materials and Methods**

103 *2.1. Animals.*

104 Male mice (7-8 weeks old) were maintained on standard chow pellet diet and had
105 free access to water with a 12-hour light-dark cycle. C57BL/6J wild-type (WT) were
106 purchased from Charles River (Kent, UK). *Mc3r*^{-/-} mice were a generous gift of Dr
107 Chen (Merck Laboratories). All animal studies were approved and performed under
108 the guidelines of the Ethical Committee for the Use of Animals, Barts and The
109 London School of Medicine and Home Office Regulations (Guidance on the
110 Operation of Animals, Scientific Procedures Act, 1986).

111 *2.2. Production of K/BxN serum*

112 K/BxN mice were produced by crossing the C57B1/6 mice (carrying the KRN
113 homozygously) and the NOD/Lt mice (carrying the A^{g7} allele homozygously) [27]. The
114 offspring develop spontaneous arthritis, evident at 6 weeks, with 100% incidence. At
115 9 weeks of age (when the titers of **anti-glucose-6-phosphate isomerase (GPI)**
116 **antibodies are maximal implying potent arthritogenic properties of the serum**) mice
117 were exsanguinated by cardiac puncture under anaesthesia (isoflurane). Blood was
118 allowed to clot overnight at 4°C. Serum was recovered with a Pasteur pipette and
119 centrifuged 10 min at 500xg at 4°C. The serum from different mice obtained on a
120 given day was pooled, aliquoted and stored at -80°C until use.

121 *2.3. K/BxN serum transfer arthritis model.*

122 Arthritis was induced by the i.p. injection of serum from K/BxN arthritic mice. Three
123 different protocols were studied: a) protocol 50+50, where mice received two
124 injections of 50µl of serum on days 0 and 2; b) protocol 100+100, where mice
125 received two injections of 100µl of serum on days 0 and 2; c) protocol 200, consisting
126 of one single injection of 200µl of serum on day 0. The protocol 100+100 was then

127 selected for subsequent experiments. The development of the disease was
128 monitored daily by assessing the paw volume using a plethysmometer (Ugo Basile,
129 Comerio, Italy), body weight, clinical score (score per paw: 0= no signs of
130 inflammation, 1=subtle inflammation, localized, 2=easily identified inflammation but
131 localized, 3=evident inflammation, not localized; max score=12 per mouse) and
132 disease incidence (mice showing overt signs of **joint inflammation, i.e. a clinical score**
133 **of 1 or above**) [29]. **Severe arthritis (number of paws per mouse that reached a**
134 **maximum score of 3) was also recorded.**

135 *2.4. Pharmacological treatments.*

136 Mice (n=5) were treated i.p. once daily, starting from day 2 (**1h after the second**
137 **K/BxN injection**), with 10µg/mouse dexamethasone (SIGMA, Poole, UK),
138 20µg/mouse DTrp⁸-γMSH (DTrp; American Peptide, Sunnyvale, CA, USA),
139 30ng/mouse elcatonin (Bachem, Bubendorf, Switzerland) or vehicle (PBS). Doses
140 were selected from previous studies in this or similar rodent models [18,30,31].

141 *2.5. Measurement of alveolar bone loss.*

142 Alveolar bone loss was evaluated as previously described [32]. Mice were
143 euthanized and maxillae were hemisected, exposed overnight in 3% hydrogen
144 peroxide, mechanically defleshed, and stained with 0.3% methylene blue. The palatal
145 faces of the molars were photographed using a stereomicroscope and a digital
146 camera (Kodak EasyShare C743; Rochester, USA). Quantitative analyses included
147 the measurement of the area between the cement enamel junction and the alveolar
148 bone crest in the first molar, using Image J software (Maryland, USA).

149 *2.6. Myeloperoxidase activity assay.*

150 MPO activity was measured as an index of granulocyte infiltration. Briefly, maxillae
151 tissue samples were homogenized in 0.5% hexadecyltrimethylammonium bromide

152 dissolved in phosphate buffer solution (pH = 6) using Precellys®24 homogeniser in
153 Precellys lysing CK14 tubes (Bertin Technologies). The homogenized tissues were
154 centrifuged at 13 000xg for 5 minutes (at 4°C) and the supernatants were placed on
155 96 well plates. Buffer, supplemented with 1% hydrogen peroxide/O-dianisidine
156 dihydrochloride, was added to each well. Optical density readings were taken for 3
157 minutes at 30 seconds intervals at 450 nm using a microplate reader NOVOstar™
158 (BMG Labtech, Aylesbury, UK). Activity was normalized to the sample protein
159 concentration determined with a BCA kit® (Pierce, Cramlington, UK) and expressed
160 as mU/mg protein.

161 *2.7. Histological analyses.*

162 Maxillae tissues were fixed in 10% buffered formalin (pH 7.4) for 24 h at room
163 temperature. The specimens were demineralized in 14% ethylenediamine tetracetic
164 acid (EDTA) for 2 weeks, dehydrated in graded ethanol and embedded in paraffin.
165 Serial sections (5 µm) were stained for tartrate-resistant acid phosphatase (TRAP,
166 Sigma–Aldrich, Saint Louis, MO, USA). Histological osteoclast counting was
167 performed in the coronal two thirds of the distal alveolar bone adjacent to the first
168 molar in five consecutive microscopic fields (40x)/section. Samples were analyzed
169 using an Axioskop 40 microscope (Carl Zeiss, Gottingen, Germany), attached to a
170 digital camera (PowerShot A620; Canon, Tokyo, Japan). For each animal (n=4),
171 three maxillae sections were analyzed. All the slides were counted in a blinded
172 manner by a single examiner. For neutrophil staining tissue specimens were blocked
173 by incubation in 1.5% H₂O₂ in methanol solution for 30 min. Primary antibody against
174 neutrophil elastase (Santa Cruz Biotechnology, Heidelberg, Germany) was used with
175 Vectastain ABC kit anti-rabbit (Vector Laboratories, Burlingame, USA). The sections
176 were counterstained using hematoxylin. Slides were developed using Peroxidase
177 substrate kit DAB Kit (Vector Laboratories, Burlingame, USA).

178 *2.8 Statistical analysis*

179 Data were analyzed by Student's *t*-test, one or two-way ANOVA followed by
180 Bonferroni or multiple comparison test, two-way ANOVA followed Newman-Keuls
181 multiple comparison test or Pearson correlation test, as appropriate. In all cases data
182 are presented as mean \pm SEM of *n* independent observations and were considered
183 statistically significant when $p < 0.05$.

184

185 **3. Results**

186 *3.1. Arthritis severity in the K/BxN serum transfer model correlates with alveolar bone* 187 *loss.*

188 Recent reports have indicated that experimental models of collagen and antigen-
189 induced arthritis are associated with development of alveolar bone loss. We
190 evaluated whether development of aggressive joint inflammation using the K/BxN
191 serum transfer model of arthritis was also associated with this extra-articular
192 manifestation. We therefore utilized three dosing strategies to manipulate the severity
193 of arthritis: mice received two injections of either 50 μ l or 100 μ l of serum on days 0
194 and 2 (*protocol 50+50* or *protocol 100+100* respectively) or a single injection of 200 μ l
195 of serum on day 0; *protocol 200*. All three regimens induced overt signs of arthritis.
196 As expected, the arthritic response produced with protocol 50+50 was milder,
197 evidenced by the low clinical score (Figure 1A), inconsistent increase in paw volume
198 (Figure 1B) and reduced severity (Figure 1C) compared with the other two protocols.
199 Comparatively, although mice received the same total amount of arthritogenic serum
200 using protocols 100+100 and 200, administration in two separate injections (day 0
201 and 2) resulted in higher clinical scores, more gradual and consistent increase in paw
202 volume and, importantly, 100% disease incidence (Figure 1D). We next analysed
203 alveolar bone loss in the maxillae of these mice subjected to serum transfer-induced
204 arthritis. Interestingly, bone loss in the maxillae was highly correlated with the
205 severity of localized inflammation in the joints (Figure 2A), suggesting that extra-
206 articular manifestations of relevance for RA also occur in this model. An overall
207 comparison between non-arthritic and arthritic mice indicated a significant (21%)
208 increase in alveolar bone loss (Figure 2B). This could also be seen macroscopically
209 by an increased corono-apical area between the cement enamel junction (CEJ) and
210 the alveolar bone crest (ABC) on the palatal side of the first molar (Figure 2C).

211 *3.2. Melanocortin treatment reduces arthritis and prevents alveolar bone loss.*

212 The melanocortin system is widely associated with joint disease and inflammation
213 both mechanistically and therapeutically [5,8,18,19]. We studied if pharmacological
214 intervention with melanocortin-based compounds could have an impact, not only in
215 joint inflammation or other arthritis-related manifestations like cachexia, as recently
216 described [21], but also in alveolar bone loss. We therefore assessed whether the
217 peptide DTrp could prevent the development of alveolar bone loss we observed
218 using this passive transfer model of arthritis. Dexamethasone (Dex), very potent as
219 an anti-inflammatory drug but with detrimental bone effects, and elcatonin (ECT), a
220 bone protective molecule but with mild anti-inflammatory effects, were used for
221 comparison. As expected Dex afforded a potent anti-inflammatory effect, reducing
222 clinical score (-40% at day 8; Figure 3A), paw volume (-59%; Figure 3B), and
223 incidence of severe arthritis (-93%; Figure 3C). The effect of ECT was less
224 pronounced with a significant statistical difference only in reducing paw volume. DTrp
225 presented a moderate and significant attenuation in all parameters measured (clinical
226 score -23%, paw volume -44%, severity -57%).

227 Determination of myeloperoxidase (MPO) activity in maxillae tissues (analyzed at day
228 8) showed a significant reduction in the groups treated with either Dex or DTrp, but
229 not with ECT (Figure 3E). Although the degree of anti-inflammatory activity attained
230 by treatment of animals with Dex and DTrp in the periodontal tissue appears to be
231 similar, assessment of alveolar bone loss revealed interesting differences: the anti-
232 arthritic effect of DTrp is associated with bone protection, as evident from the positive
233 correlation ($R=0.87$) between bone loss and clinical score (Figure 3F). In contrast,
234 the anti-arthritic effect of Dex was inversely correlated with alveolar bone loss ($R=-$
235 0.87), in accordance with the well-known effects of Dex on bone metabolism. These
236 findings suggest that melanocortin therapy, in addition to potent modulation of

237 inflammation and arthritis, could also have the advantage over corticoids therapy in
238 preserving bone integrity.

239

240 *3.4. MC₃ deficient mice display increased alveolar bone loss.*

241 Our next approach consisted of the study of the role of the melanocortin receptor 3
242 (MC₃) using genetically modified mice lacking this receptor, as it has been reported
243 that MC₃ is **as pivotal target for the** anti-inflammatory and anti-arthritic actions of
244 melanocortin drugs, including ACTH [8,9]. Arthritis was induced using the 100+100
245 protocol, as in previous experiments, although in this case 12-week old mice were
246 used (due to stock availability). As shown in Figure 4, arthritis developed similarly to
247 previous experiments with younger mice. The clinical score and disease severity was
248 identical in WT and *Mc3r*^{-/-} mice although some differences were found in the paw
249 volume (Figure 4). As expected, when alveolar bone loss was assessed in maxillae
250 (day 8), WT arthritic mice presented an increase in bone loss corroborating our
251 previous findings. However, the basal values **of alveolar bone loss** obtained in *Mc3r*^{-/-}
252 *-/-* mice were **elevated compared with WT mice**, and no further alveolar bone loss was
253 obtained **following** arthritis induction (Figure 4D). The fact that the mice used in this
254 experiment were older made us **hypothesise** that there might be an association
255 between MC₃ deficiency and **physiological** bone metabolism.

256

257 *3.5. Aging-associated alveolar bone loss is accelerated in MC₃ deficient mice.*

258 The evidence obtained in our previous experiments **suggests** that activation of MC₃
259 (the main receptor that mediates DTrp actions [19]) might protect from alveolar bone
260 loss. **Since there is** evidence **in the literature** **furthering** an association between the
261 melanocortin system and bone metabolism [12], **as well as linking** normal bone loss

262 associated with aging [33,34], next we sought to investigate if MC₃ played any role in
263 these phenomena.

264 Maxillae from WT and *Mc3r*^{-/-} mice harvested at different ages (1.5, 3.5 and 4.5
265 months old) were analyzed for alveolar bone loss. Interestingly, periodontal bone loss
266 was accelerated in *Mc3r*^{-/-} mice, showing a 39% increase at 3.5 months of age
267 compared to younger mice (1.5 months), while only 15% increase was observed in
268 same age WT mice (Figure 5).

269 There is some indication for a functional association between MC₃ and
270 osteoclastogenesis, as the number of osteoclasts is increased in *Mc3r*^{-/-} arthritic
271 mice, compared to WT [18]. We then studied if osteoclasts numbers were also
272 different in the maxillae of the two genotypes by quantifying the Trap⁺ cells in the
273 cervical area of the first molar of the left lower maxillae (n=4 mice). Although basal
274 levels (1.5 months/old) were different, we quantified a 40% increase in Trap⁺ cells in
275 older mice in *Mc3r*^{-/-} but not in WT mice, where values remained stable (Figure 6A).
276 We also observed a significant increase in the number of neutrophils in the junctional
277 epithelium of *Mc3r*^{-/-} mice compared to WT (Figure 6B). However, no differences
278 were found in the rest of epithelial and connective tissue suggesting that neutrophils
279 might not be playing a crucial role in the alveolar bone loss as measured in our
280 experimental conditions.

281

282 4. Discussion

283 There is clinical evidence that periodontal disease is associated with RA, **although it**
284 **is unclear whether the link is causal or casual.** Bone resorption in the maxillae
285 (associated with **a** high degree of immune cell infiltration) is reminiscent of the RA
286 joint where large numbers of blood-borne cells can be found in the exudate during
287 the active phases of the disease. To gain information on pathogenesis **to**
288 **subsequently inform on** therapeutic opportunities, it is important to develop **animal**
289 models where disease development could be monitored **at** the two sites in parallel.

290

291 The recent appreciation that mice subjected to the gold standard model of RA,
292 collagen induced arthritis, develop alveolar bone loss that parallel ankle joint damage
293 [25] represents an important **conceptual and experimental** advance. Park *et al*
294 reported severe periodontal bone damage 16 weeks after induction of arthritis using
295 type-II collagen: this was associated with increased osteoclastic activity and impaired
296 repair ability due to reduced bone formation by osteoblasts [25]. Here we used the
297 K/BxN serum model of inflammatory arthritis, an aggressive model that mimics the
298 active phases of RA **and it is much faster in its onset.** Injection of **the** serum rich in
299 anti-glucose-6-phosphate isomerase immunoglobulins fixes complement onto
300 cartilage with initiation of **an** inflammatory reaction, highly reliant on cytokines and
301 eicosanoids [27,29]. Herein we first **determined** whether this rapid model (~7-8 days
302 against >30 days for the collagen-induced arthritis) also led to periodontal disease.
303 Thus, administration of the arthritogenic serum along three different protocols
304 induced evident joint inflammation, **with more consistent and reproducible data** with
305 the 100+100 µl protocol and truly mild when using the 50+50 µl protocol. A good
306 association was observed between serum dosage and protocol of administration,
307 which resulted in distinct severity of arthritis, and the corresponding degree of

308 alveolar bone loss measured in maxillae. Collectively these data, coupled to the two
309 recent studies [24,25] indicate that experimental polyarthritis in rodents is indeed
310 associated with periodontal disease features.

311

312 The melanocortin **receptor agonist** ACTH is an anti-arthritic drug indicated for the
313 treatment of acute inflammatory episodes of gout [35] as well as RA, as **shown by** the
314 seminal work of Hench and colleagues of the Mayo Clinic taught us [36,37]. The
315 equally old observations of Gutman [6] have been repeated in more rigorous clinical
316 studies confirming ACTH efficacy in human gouty arthritis [38,39,40]. **All these**
317 studies indicate that ACTH is effective and safe for the treatment of acute gout and
318 presents as a good alternative in patients with comorbidities in which steroids and
319 colchicine are not recommended. Why is ACTH so effective in arthritides? Ritter and
320 colleagues indicated the possible existence of mechanisms aside adrenal stimulation
321 and glucocorticoid release [40], inciting us to identify peripheral modulation of MC₃ as
322 a very important contributor of the anti-inflammatory actions of the peptide [8].
323 However, ACTH is a pan-MC receptor agonist. Herein we used peptide DTrp⁸-γMSH
324 (abbreviated DTrp), which although not totally selective in *in vitro* **expression** cell
325 systems, retains functional selectivity in the mouse as demonstrated by its lack of
326 efficacy in *Mc3r*^{-/-} animals. Importantly, DTrp displays anti-arthritic effects in the
327 K/BxN animal model of arthritis [18,19,41].

328

329 The pharmacological experiments suggested that MC-based therapy may yield a
330 unique opportunity. Whilst the glucocorticoid dexamethasone afforded the expected
331 therapeutic effect on the arthritic joint [18], measured in terms of score, swelling,
332 disease severity and MPO activity, it did not affect – and rather worsened – alveolar
333 bone loss. This effect can be chiefly due to the **osteoclast** activating property of

334 glucocorticoids [42]. Calcitonin, on the other hand, was selected because
335 representing an opposite therapeutic, with **very little** modulation of inflammatory
336 arthritis [43] yet its daily delivery to mice from day 2 significantly **protected from**
337 alveolar bone loss associated with this model of experimental arthritis. This was
338 predicted in view of the potent action of calcitonin in stopping bone resorption [44],
339 being able for instance to override the activating effect of glucocorticoids [43].

340

341 DTrp revealed unique properties since was able to inhibit arthritis, albeit to an
342 intermediate level between dexamethasone and calcitonin, and also attenuate bone
343 loss associated with periodontal disease. **In** more detail, the DTrp group showed a
344 positive high correlation between clinical score and bone loss (i.e. reduced bone loss
345 associated with the anti-arthritic effect, $R=0.87$), and this was the exact opposite of
346 **that** calculated **for** Dex-treated mice ($R=-0.87$). This finding is of relevance as
347 **prolonged steroid therapy** is associated with bone density loss, osteoporosis and
348 fractures. These results indicate that MC receptor agonists, possibly better if
349 selective for MC_3 , represent a novel class of anti-arthritic therapeutics able to target
350 joint disease without aggravating unwanted effects on distant organs and tissues.
351 This notion is further substantiated by a recent study **wh**ere the beneficial effect of
352 melanocortin treatment on **joint** inflammation and against systemic muscle **wasting**
353 (cachexia) was demonstrated [21].

354

355 The bone-protective effect of DTrp is likely due a direct **osteoclast** effect that it is
356 additive to modulation of local inflammation, as shown by the MPO activity
357 measurements in maxillae samples. In agreement **with these pharmacological data**,
358 we have reported a higher degree of osteoclastogenesis in *Mc3r*^{-/-} mice, measured
359 both *in vivo* in arthritic joints and *in vitro*, using bone marrow derived osteoclasts [18].

360 Furthermore, in wild type osteoclasts, application of DTrp reduced cell activation and
361 resorptive activity. In these experiments, *Mc3r*^{-/-} mice did not presented more
362 pronounced arthritis, at variance from what we reported previously [18], likely due to
363 differences in protocol and animal age. On the same token though, this new result
364 allows to separate periodontal bone loss and joint arthritis, indicating that the former
365 occurs independently from modulation of the latter. Collectively, these data prompt us
366 to identify MC₃ as a modulatory receptor on osteoclast differentiation and activation.

367

368 The use of genetically engineered mice can shed new insights into the biological
369 functions of genes of interest. The involvement of MC₃ in bone metabolism emerges
370 from pharmacological evidence or from the use of *Mc3r*^{-/-} in settings of experimental
371 pathology. But if MC₃ plays important non-redundant role in bone physiology, then
372 its absence might produce a phenotype in 'healthy' mice. Indeed, these mice present
373 decreased linear growth and femur length as well as reduced bone mineral density
374 [45,46]. To this end we monitored the degree of bone erosion in the maxillae of mice
375 at different ages, comparing wild type and *Mc3r*^{-/-} animals. These experiments
376 confirmed a higher susceptibility to alveolar bone loss in the transgenic lacking the
377 MC₃ receptor, with presence of significant bone loss as early as 14 weeks of age,
378 whilst wild type mice displayed similar degrees of damage at 18 weeks. Thus,
379 endogenous MC₃, possibly activated by circulating ACTH or α MSH, exerts a tonic
380 inhibitory role on bone metabolism in the maxillae hence in its absence there is a
381 higher susceptibility to bone loss hence disease. Though congruent with the data
382 presented above, this hypothesis requires corroboration by future studies. With
383 ageing *Mc3r*^{-/-} mice become obese, an effect evident at 12 weeks of age [47]. Since
384 it is reported that obese mice have a different microbiota compared to lean animals
385 [48], one could not exclude that a different microbiota predisposes to higher
386 susceptibility to periodontal disease. Again, focused and systematic analyses on the

387 periodontal compartment of MC₃ deficient mice can shed light onto this novel biology
388 of the MC system we have unveiled here.

389

390 In summary, we describe a novel experimental association between periodontal
391 disease and inflammatory arthritis with two distinct outcomes: first, the modulatory
392 function of MC₃ on periodontal status in **health and disease**; second, the distinct
393 pharmacology of DTrp as compared to other anti-arthritic or bone-protective
394 compounds, suggesting the potential development of a new genus of anti-arthritic
395 therapeutics, centred on MC₃ activation and able to spare or correct alveolar bone
396 damage.

397

398 **5. References**

- 399 1. McInnes IB, Schett G: The pathogenesis of rheumatoid arthritis. *N Engl J Med*
400 2011, 365: 2205-2219.
- 401 2. Boissier MC, Semerano L, Challal S, Saldenber-Kermanac'h N, Falgarone G:
402 Rheumatoid arthritis: from autoimmunity to synovitis and joint
403 destruction. *J Autoimmun* 2012, 39: 222-228.
- 404 3. Taylor PC: Developing anti-TNF and biologic agents. *Rheumatology (Oxford)*
405 2011, 50: 1351-1353.
- 406 4. Gantz I, Fong TM: The melanocortin system. *Am J Physiol Endocrinol Metab*
407 2003, 284: E468-474.
- 408 5. Catania A, Lonati C, Sordi A, Carlin A, Leonardi P, Gatti S: The melanocortin
409 system in control of inflammation. *ScientificWorldJournal* 2010, 10: 1840-
410 1853.
- 411 6. Gutman AB, Yu TF: Effects of adrenocorticotrophic hormone (ACTH) in gout. *Am*
412 *J Med* 1950, 9: 24-30.
- 413 7. Cronstein BN, Terkeltaub R: The inflammatory process of gout and its
414 treatment. *Arthritis Res Ther* 2006, 8 Suppl 1: S3.
- 415 8. Getting SJ, Christian HC, Flower RJ, Perretti M: Activation of melanocortin type
416 3 receptor as a molecular mechanism for adrenocorticotrophic hormone
417 efficacy in gouty arthritis. *Arthritis Rheum* 2002, 46: 2765-2775.
- 418 9. Getting SJ, Gibbs L, Clark AJ, Flower RJ, Perretti M: POMC gene-derived
419 peptides activate melanocortin type 3 receptor on murine macrophages,
420 suppress cytokine release, and inhibit neutrophil migration in acute
421 experimental inflammation. *J Immunol* 1999, 162: 7446-7453.
- 422 10. Gong R: The renaissance of corticotropin therapy in proteinuric
423 nephropathies. *Nat Rev Nephrol* 2012, 8: 122-128.
- 424 11. Berkovich R: Treatment of acute relapses in multiple sclerosis.
425 *Neurotherapeutics* 2013, 10: 97-105.
- 426 12. Dumont LM, Wu CS, Tatnell MA, Cornish J, Mountjoy KG: Evidence for direct
427 actions of melanocortin peptides on bone metabolism. *Peptides* 2005, 26:
428 1929-1935.
- 429 13. Cornish J, Callon KE, Mountjoy KG, Bava U, Lin JM, Myers DE, Naot D, Reid IR:
430 alpha -melanocyte-stimulating hormone is a novel regulator of bone. *Am J*
431 *Physiol Endocrinol Metab* 2003, 284: E1181-1190.
- 432 14. Grassel S, Opolka A, Anders S, Straub RH, Grifka J, Luger TA, Bohm M: The
433 melanocortin system in articular chondrocytes: melanocortin receptors,
434 pro-opiomelanocortin, precursor proteases, and a regulatory effect of
435 alpha-melanocyte-stimulating hormone on proinflammatory cytokines
436 and extracellular matrix components. *Arthritis Rheum* 2009, 60: 3017-
437 3027.
- 438 15. Evans JF, Niu QT, Canas JA, Shen CL, Aloia JF, Yeh JK: ACTH enhances
439 chondrogenesis in multipotential progenitor cells and matrix production
440 in chondrocytes. *Bone* 2004, 35: 96-107.
- 441 16. Bohm M, Raghunath M, Sunderkotter C, Schiller M, Stander S, Brzoska T,
442 Cauvet T, Schioth HB, Schwarz T, Luger TA: Collagen metabolism is a
443 novel target of the neuropeptide alpha-melanocyte-stimulating hormone.
444 *J Biol Chem* 2004, 279: 6959-6966.

- 445 17. Bohm M, Luger TA: Melanocortins in fibroblast biology--current update and
446 future perspective for dermatology. *Exp Dermatol* 2004, 13 Suppl 4: 16-
447 21.
- 448 18. Patel HB, Bombardieri M, Sampaio AL, D'Acquisto F, Gray M, Grieco P, Getting
449 SJ, Pitzalis C, Perretti M: Anti-inflammatory and antiosteoclastogenesis
450 properties of endogenous melanocortin receptor type 3 in experimental
451 arthritis. *FASEB J* 2010, 24: 4835-4843.
- 452 19. Getting SJ, Lam CW, Chen AS, Grieco P, Perretti M: Melanocortin 3 receptors
453 control crystal-induced inflammation. *FASEB J* 2006, 20: 2234-2241.
- 454 20. Montero-Melendez T, Patel HB, Seed M, Nielsen S, Jonassen TE, Perretti M:
455 The melanocortin agonist AP214 exerts anti-inflammatory and
456 proresolving properties. *Am J Pathol* 2011, 179: 259-269.
- 457 21. Gomez-Sanmiguel AB, Martin AI, Nieto-Bona MP, Fernandez-Galaz C, Lopez-
458 Menduina M, Villanua MA, Lopez-Calderon A: Systemic alpha melanocyte
459 stimulating hormone administration decreases arthritis-induced anorexia
460 and muscle wasting. *Am J Physiol Regul Integr Comp Physiol* 2013.
- 461 22. Scher JU, Abramson SB: Periodontal disease, *Porphyromonas gingivalis*, and
462 rheumatoid arthritis: what triggers autoimmunity and clinical disease?
463 *Arthritis Res Ther* 2013, 15: 122.
- 464 23. Scher JU, Ubeda C, Equinda M, Khanin R, Buischi Y, Viale A, Lipuma L, Attur M,
465 Pillinger MH, Weissmann G, Littman DR, Pamer EG, Bretz WA, Abramson
466 SB: Periodontal disease and the oral microbiota in new-onset rheumatoid
467 arthritis. *Arthritis Rheum* 2012, 64: 3083-3094.
- 468 24. Queiroz-Junior CM, Madeira MF, Coelho FM, Costa VV, Bessoni RL, Sousa LF,
469 Garlet GP, Souza Dda G, Teixeira MM, Silva TA: Experimental arthritis
470 triggers periodontal disease in mice: involvement of TNF-alpha and the
471 oral Microbiota. *J Immunol* 2011, 187: 3821-3830.
- 472 25. Park JC, Su C, Jung IH, Choi SH, Cho KS, Kim CK, Park YB, Lee SK, Kim CS:
473 Mechanism of alveolar bone loss in a collagen-induced arthritis model in
474 mice. *J Clin Periodontol* 2011, 38: 122-130.
- 475 26. Queiroz-Junior CM, Madeira MF, Coelho FM, de Oliveira CR, Candido LC,
476 Garlet GP, Teixeira MM, de Souza Dda G, Silva TA: Experimental arthritis
477 exacerbates *Aggregatibacter actinomycetemcomitans*-induced
478 periodontitis in mice. *J Clin Periodontol* 2012, 39: 608-616.
- 479 27. Monach PA, Mathis D, Benoist C: The K/BxN arthritis model. *Curr Protoc*
480 *Immunol* 2008, Chapter 15: Unit 15 22.
- 481 28. Ditzel HJ: The K/BxN mouse: a model of human inflammatory arthritis.
482 *Trends Mol Med* 2004, 10: 40-45.
- 483 29. Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, Brenner MB: Mast cells: a
484 cellular link between autoantibodies and inflammatory arthritis. *Science*
485 2002, 297: 1689-1692.
- 486 30. Patel HB, Kornerup KN, Sampaio AL, D'Acquisto F, Seed MP, Girol AP, Gray M,
487 Pitzalis C, Oliani SM, Perretti M: The impact of endogenous annexin A1 on
488 glucocorticoid control of inflammatory arthritis. *Ann Rheum Dis* 2012, 71:
489 1872-1880.
- 490 31. Al-Kashi A, Montero-Melendez T, Moradi-Bidhendi N, Gilligan JP, Mehta N,
491 Perretti M: The calcitonin and glucocorticoids combination: mechanistic
492 insights into their class-effect synergy in experimental arthritis. *PLoS One*
493 2013, 8: e54299.

- 494 32. Madeira MF, Queiroz-Junior CM, Costa GM, Santos PC, Silveira EM, Garlet GP,
495 Cisalpino PS, Teixeira MM, Silva TA, Souza Dda G: MIF induces osteoclast
496 differentiation and contributes to progression of periodontal disease in
497 mice. *Microbes Infect* 2012, 14: 198-206.
- 498 33. Liang S, Hosur KB, Domon H, Hajishengallis G: Periodontal inflammation and
499 bone loss in aged mice. *J Periodontal Res* 2010, 45: 574-578.
- 500 34. Rivaldo EG, Padilha DP, Hugo FN: Alveolar bone loss and aging: a model for
501 the study in mice. *J Periodontol* 2005, 76: 1966-1971.
- 502 35. Khanna D, Khanna PP, Fitzgerald JD, Singh MK, Bae S, Neogi T, Pillinger MH,
503 Merrill J, Lee S, Prakash S, Kaldas M, Gogia M, Perez-Ruiz F, Taylor W, Liote
504 F, Choi H, Singh JA, Dalbeth N, Kaplan S, Niyyar V, Jones D, Yarows SA,
505 Roessler B, Kerr G, King C, Levy G, Furst DE, Edwards NL, Mandell B,
506 Schumacher HR, Robbins M, Wenger N, Terkeltaub R: 2012 American
507 College of Rheumatology guidelines for management of gout. Part 2:
508 therapy and antiinflammatory prophylaxis of acute gouty arthritis.
509 *Arthritis Care Res (Hoboken)* 2012, 64: 1447-1461.
- 510 36. Hench PS: Introduction: cortisone and ACTH in clinical medicine. *Proc Staff*
511 *Meet Mayo Clin* 1950, 25: 474-476.
- 512 37. Slocumb CH, Polley HF, Hench PS, Kendall EC: Effects of cortisone and ACTH
513 on patients with rheumatoid arthritis. *Proc Staff Meet Mayo Clin* 1950, 25:
514 476-478.
- 515 38. Axelrod D, Preston S: Comparison of parenteral adrenocorticotrophic hormone
516 with oral indomethacin in the treatment of acute gout. *Arthritis Rheum*
517 1988, 31: 803-805.
- 518 39. Daoussis D, Antonopoulos I, Yiannopoulos G, Andonopoulos AP: ACTH as first
519 line treatment for acute gout in 181 hospitalized patients. *Joint Bone*
520 *Spine* 2013, 80: 291-294.
- 521 40. Ritter J, Kerr LD, Valeriano-Marcet J, Spiera H: ACTH revisited: effective
522 treatment for acute crystal induced synovitis in patients with multiple
523 medical problems. *J Rheumatol* 1994, 21: 696-699.
- 524 41. Leoni G, Patel HB, Sampaio AL, Gavins FN, Murray JF, Grieco P, Getting SJ,
525 Perretti M: Inflamed phenotype of the mesenteric microcirculation of
526 melanocortin type 3 receptor-null mice after ischemia-reperfusion.
527 *FASEB J* 2008, 22: 4228-4238.
- 528 42. Reid IR: Steroid-induced osteoporosis. *Osteoporos Int* 1997, 7 Suppl 3: S213-
529 216.
- 530 43. Mancini L, Paul-Clark MJ, Rosignoli G, Hannon R, Martin JE, Macintyre I,
531 Perretti M: Calcitonin and prednisolone display antagonistic actions on
532 bone and have synergistic effects in experimental arthritis. *Am J Pathol*
533 2007, 170: 1018-1027.
- 534 44. Karsdal MA, Henriksen K, Arnold M, Christiansen C: Calcitonin: a drug of the
535 past or for the future? Physiologic inhibition of bone resorption while
536 sustaining osteoclast numbers improves bone quality. *BioDrugs* 2008, 22:
537 137-144.
- 538 45. Renquist BJ, Murphy JG, Larson EA, Olsen D, Klein RF, Ellacott KL, Cone RD:
539 Melanocortin-3 receptor regulates the normal fasting response. *Proc Natl*
540 *Acad Sci U S A* 2012, 109: E1489-1498.
- 541 46. Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI,
542 Vongs A, Feng Y, Cao L, Metzger JM, Strack AM, Camacho RE, Mellin TN,

- 543 Nunes CN, Min W, Fisher J, Gopal-Truter S, MacIntyre DE, Chen HY, Van
544 der Ploeg LH: Inactivation of the mouse melanocortin-3 receptor results
545 in increased fat mass and reduced lean body mass. *Nat Genet* 2000, 26:
546 97-102.
- 547 47. Butler AA, Kesterson RA, Khong K, Cullen MJ, Pellemounter MA, Dekoning J,
548 Baetscher M, Cone RD: A unique metabolic syndrome causes obesity in
549 the melanocortin-3 receptor-deficient mouse. *Endocrinology* 2000, 141:
550 3518-3521.
- 551 48. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI: An
552 obesity-associated gut microbiome with increased capacity for energy
553 harvest. *Nature* 2006, 444: 1027-1031.
- 554
- 555

556 **List of abbreviations**

557 ACTH (adrenocorticotropic hormone), Dex (dexamethasone), DMARDs (disease-
558 modifying anti-rheumatic drugs), DTrp⁸-γMSH (DTrp), ECT (elcatonin), MC
559 (melanocortin), MC₃ (melanocortin receptor 3), myeloperoxidase (MPO), rheumatoid
560 arthritis (RA), Trap (tartrate-resistant acid phosphatase), wild type (WT)

561

562 **Competing interests**

563 Authors declare no competing interests.

564

565 **Authors contributions**

566 TMM designed study, performed experiments, analyzed and interpreted data and
567 wrote manuscript; MFMM performed experiments, analyzed and interpreted data and
568 revised manuscript; LVN performed experiments and revised manuscript; AA
569 performed experiments and revised manuscript; MAC interpreted data and revised
570 manuscript; TAS interpreted data and revised manuscript; MP designed study, wrote
571 manuscript and provided funding.

572

573

574 **Figure Legends**

575 **Figure 1.** *Comparison of three protocols for the K/BxN serum transfer arthritis model.*

576 Arthritis was induced by the i.p. injection of serum from K/BxN arthritic mice using
577 three different protocols: 50+50 (two injections of 50µl on days 0 and 2); 100+100
578 (two injections of 100µl on days 0 and 2); 200 (one single injection on day 0). Clinical
579 score (A), paw volume (B) and disease incidence (C) were recorded for 7 days.
580 Panel (D) shows the number of **paws** per mouse that reached the maximum score
581 (3). Representative images of ankle, wrist and digits swelling are shown in panel (E).
582 Data are the mean±SEM of 4-6 mice per group. * $p < 0.05$ two-way ANOVA followed
583 by Bonferroni multiple comparison test.

584

585 **Figure 2.** *Correlation of arthritis with alveolar bone loss in the K/BxN serum transfer*

586 *model.* Alveolar bone loss was evaluated at day 7 in the palatal aspect of the first
587 upper molar of the right hemi-maxillae. (A) Correlation between alveolar bone loss
588 and clinical score on mice studied in the protocol comparison experiment (see Figure
589 1) analyzed by Pearson correlation test (n=14). (B) Overall increase in alveolar bone
590 loss in arthritic mice (pooled data from all mice, mean±SEM, n=14) compared to
591 control mice, analyzed by t-test (* $p < 0.05$). (C) Representative photographs of the
592 maxillae showing evidence of alveolar bone loss (white arrows).

593

594 **Figure 3.** *Effect of dexametasone, DTrp and elcatonin in arthritis and alveolar bone*

595 *loss.* Arthritis was induced using the 100+100 protocol (100µl of serum on days 0 and
596 2) and monitored by daily recording the clinical score (A), paw volume (B), severity
597 (number of **paws** reaching the maximum score) (C) and disease incidence (D).
598 Myeloperoxidase activity was measured in the left hemi-maxillae at day 8 (E).
599 Alveolar bone loss was analyzed in the right hemi-maxillae at day 8 and correlated

600 with clinical score recorded that day (F). Drugs were administered i.p. once daily:
601 dexamethasone (Dex) 10µg/mouse; DTrp⁸-γMSH (DTrp) 20µg/mouse; elcatonin
602 (ECT) 30ng/mouse; Vehicle PBS (Veh). Non-arthritic mice were included as controls
603 (Ctrl). Data are the mean±SEM of 5-6 mice per group. Data **were** analyzed by two-
604 way ANOVA followed by Bonferroni multiple comparison test (A-C), one-way ANOVA
605 followed by Bonferroni multiple comparison test (E) and Pearson correlation test (F).
606 In all cases * $p < 0.05$.

607

608 **Figure 4.** *Arthritis and alveolar bone loss in MC₃ deficient mice.* Arthritis was induced
609 in C57BL/6J wild type mice (WT) and melanocortin receptor 3 deficient mice (*Mc3r*^{-/-})
610 using the 100+100 protocol (100µl of serum on days 0 and 2). Disease was
611 monitored by daily recording of the clinical score (A), paw volume (B), and disease
612 severity (number of **paws** reaching the maximum score) (C). Alveolar bone loss was
613 analyzed in the right hemi-maxillae on the last day of the experiment (day 8). Data
614 are the mean±SEM of 5 mice per group. Statistical analyses were carried out by two-
615 way ANOVA followed by Bonferroni multiple comparison test (A-C) and one-way
616 ANOVA followed by Newman-Keuls multiple comparison test vs. WT-Ctrl (D). In all
617 cases * $p < 0.05$.

618

619 **Figure 5.** *Impact of aging on alveolar bone loss in MC₃ deficient mice.* (A) Alveolar
620 bone loss was evaluated in the right hemi-maxillae in mice from different ages (1.5,
621 3.5 and 4.5 months old) in both C57BL/6J wild type mice (WT) and melanocortin
622 receptor 3 deficient mice (*Mc3r*^{-/-}). Data are the mean±SEM of 6-17 mice. Data **were**
623 analyzed by two-way ANOVA followed by Bonferroni multiple comparison test,
624 * $p < 0.05$ vs. 1.5months, † $p < 0.05$ WT vs. *Mc3r*^{-/-}.

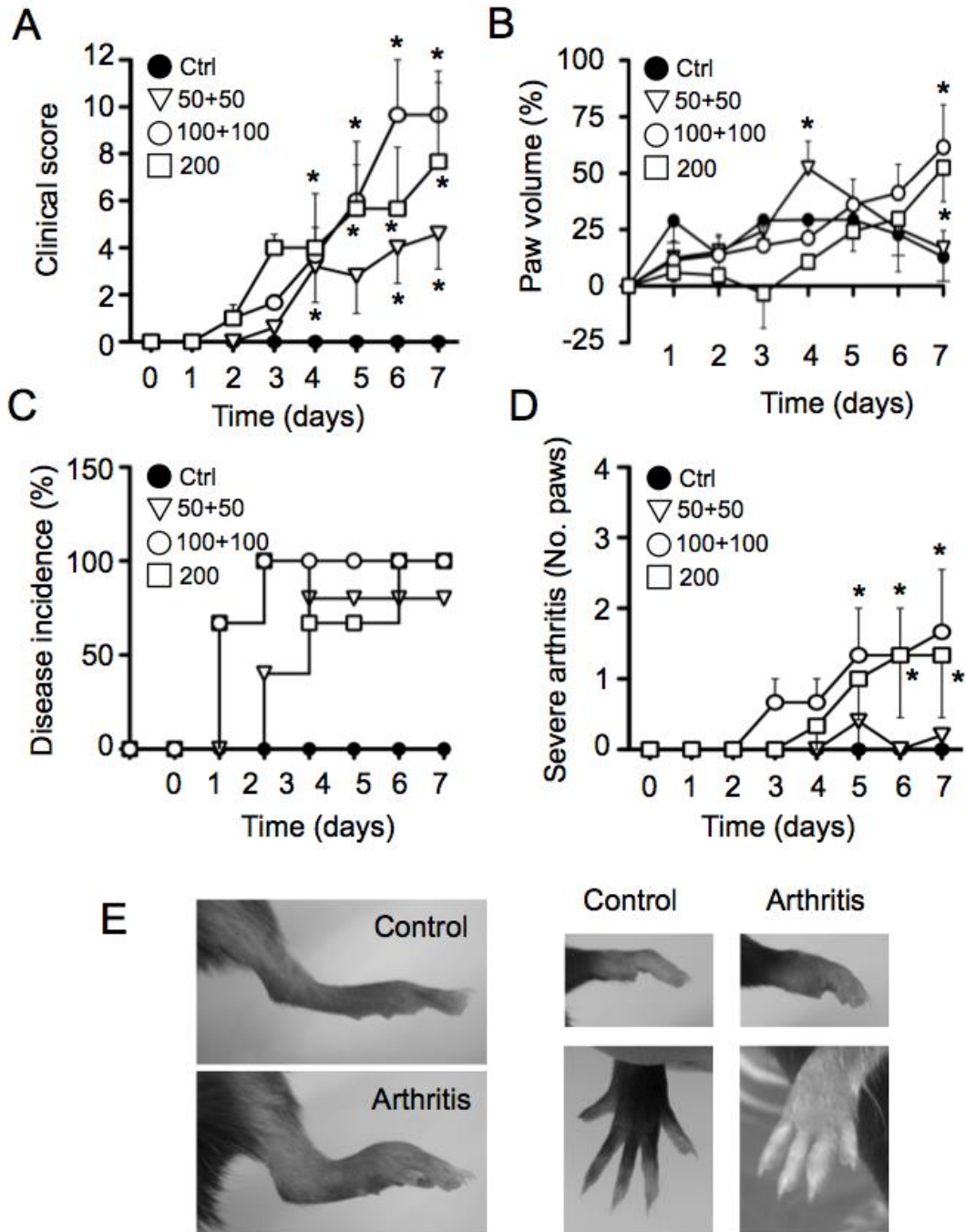
625

626 **Figure 6.** *Analysis of osteoclasts and neutrophils in gingival tissues.* The left hemi-
627 maxillae were used for histological evaluation of osteoclast activity by Trap staining
628 and neutrophil infiltration. (A) The number of osteoclasts on the cervical area of the
629 first molar was counted. Representative images of Trap⁺ cells are shown. (B)
630 Sections were stained for neutrophil elastase as a marker of neutrophils.
631 Representative images of 1.5 months old mice are shown. Data are the mean±SEM
632 of 2-4 mice. Data **were** analyzed by one-way ANOVA followed by Bonferroni multiple
633 comparison test, * $p < 0.05$.

634

635 Figure 1

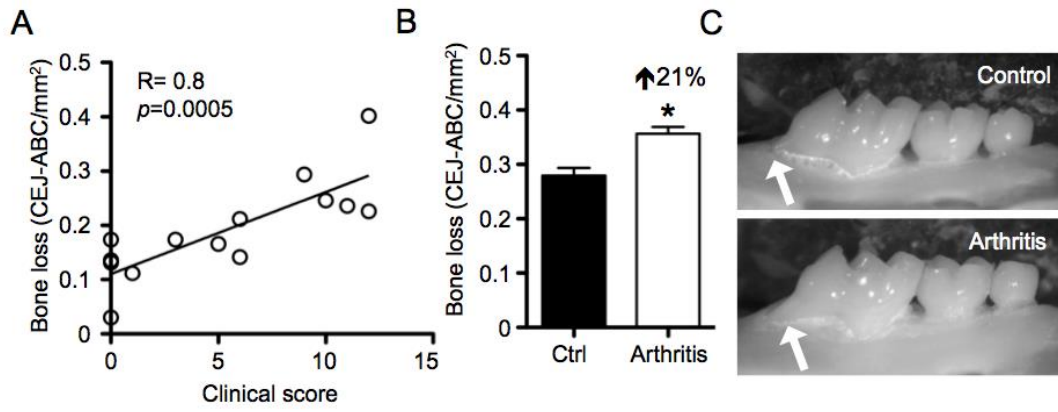
636



637

638 Figure 2

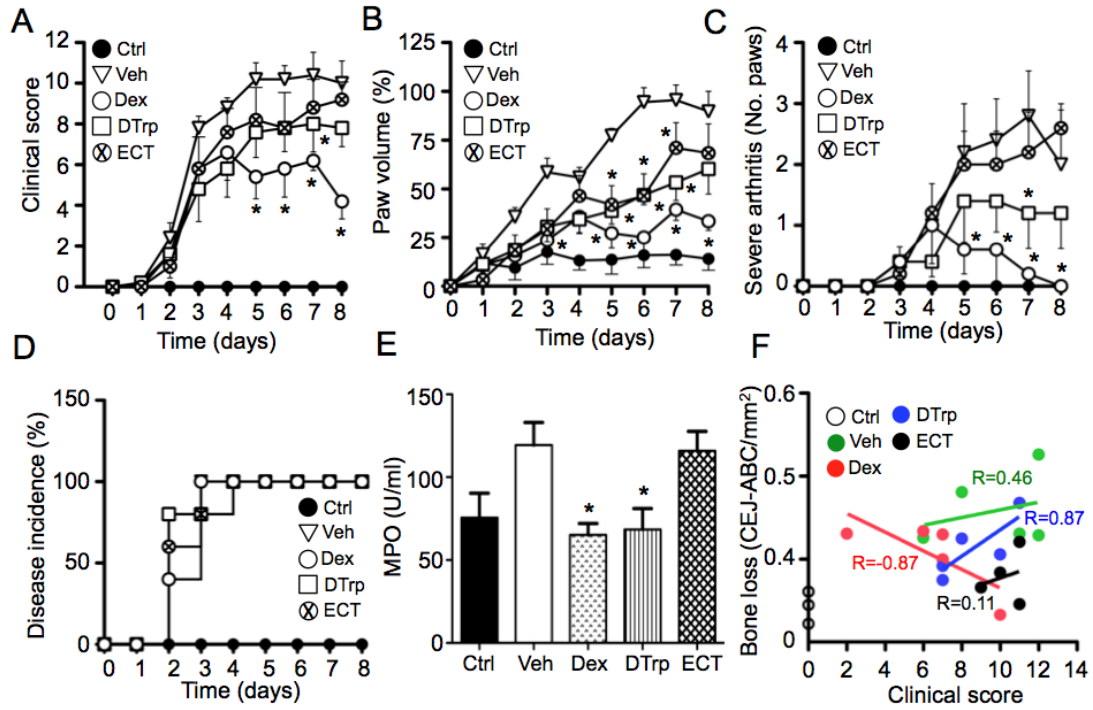
639



640

641 Figure 3

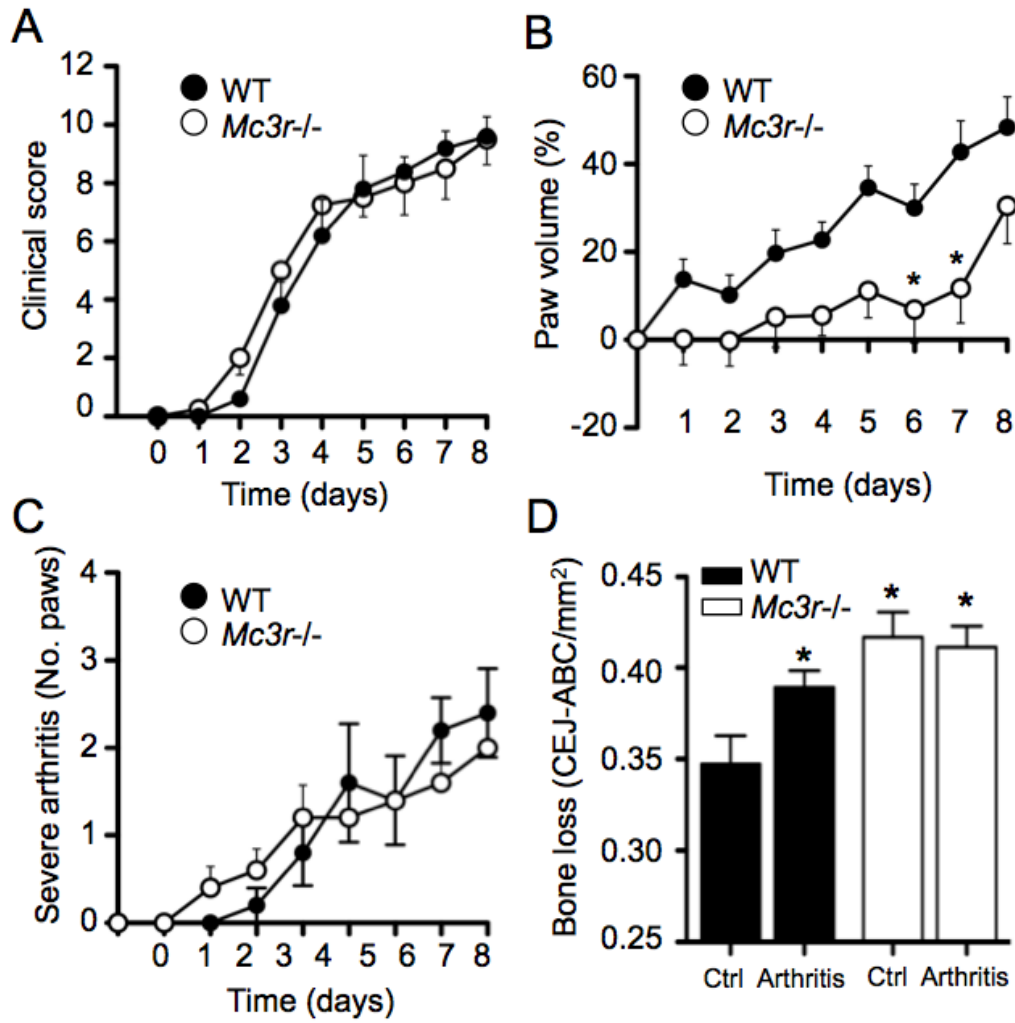
642



643

644 Figure 4

645



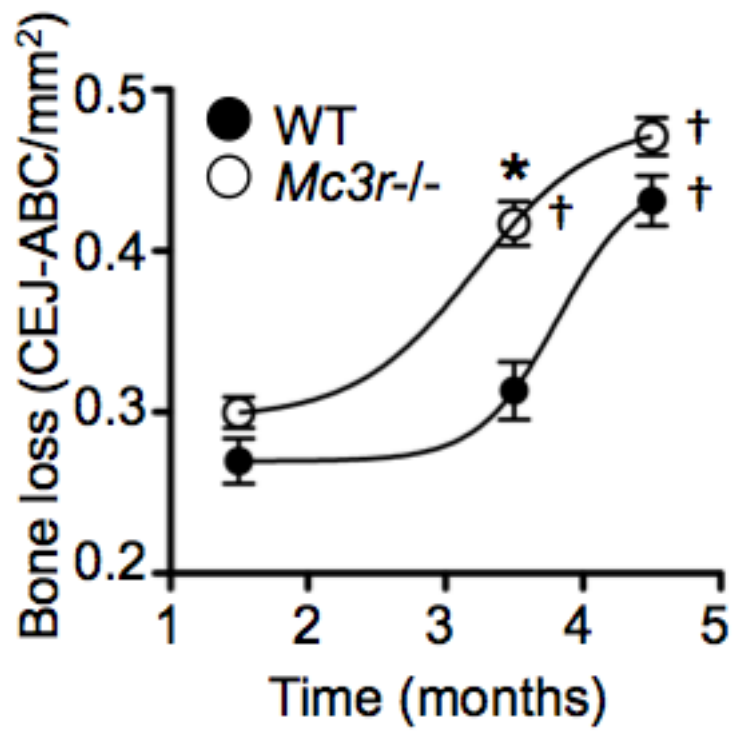
646

647 Figure 5

648

649

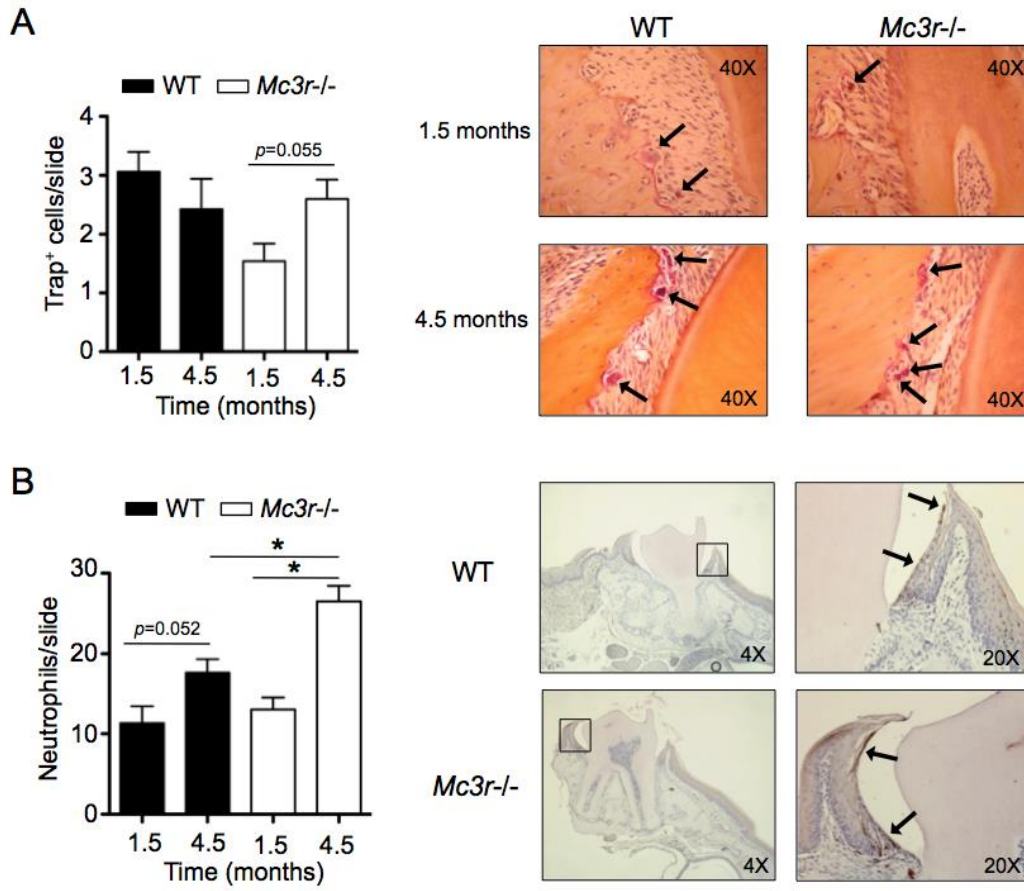
650



651

652 Figure 6

653



654