Azathioprine has a deleterious effect on the bone health of mice with DSS-induced inflammatory bowel disease

Stephanie Morgan[1]., Kirsty M. Hooper[1,2]., Elspeth M. Milne[3]., Colin Farquharson[3]., Craig Stevens[1]. and Katherine A. Staines[1],*

[1]School of Applied Sciences, Edinburgh Napier University, Edinburgh, EH11 4BN, UK

[2]Babraham Institute, Cambridge, CB22 3AT, UK

[3]The Royal (Dick) School of Veterinary Studies and The Roslin Institute, The University of Edinburgh, Edinburgh, EH25 9RG UK

*   Correspondence: Katherine Staines, School of Applied Sciences, Edinburgh Napier University, Edinburgh, UK; K.Staines@napier.ac.uk, Tel: +44 (0)131 455 3369, ORCID ID: http://orcid.org/0000-0002-8492-9778

Abstract: Patients with inflammatory bowel disease (IBD) often present poor bone health and are 40% more at risk of bone fracture. Studies have implicated autophagy in IBD pathology and drugs used to treat IBD stimulate autophagy in varying degrees, however, their effect on the skeleton is currently unknown. Here, we have utilised the dextran sulphate sodium (DSS) model of colitis in mice to examine the effects of the thiopurine drug azathioprine on the skeleton. 10-week-old male mice (n=6/group) received 3.0% DSS in their drinking water for 4 days, followed by a 14-day recovery period. Mice were treated with 10mg/kg/day azathioprine or vehicle control. Histopathological analysis of the colon from DSS mice revealed significant increases in scores for inflammation severity, extent and crypt damage (P<0.05). Azathioprine provided partial protection to the colon, as reflected by a lack of significant difference in crypt damage and tissue regeneration with DSS treatment. MicroCT of vehicle-treated DSS mice revealed azathioprine treatment had a significant detrimental effect on the trabecular bone microarchitecture, independent of DSS treatment. Specifically, significant decreases were observed in BV/TV (P<0.01), and trabecular number (P<0.05), with a concurrent significant increase in trabecular pattern factor (P<0.01). Immunohistochemical labelling for LC3 revealed azathioprine to induce autophagy in the bone marrow. Together these data suggest that azathioprine treatment may have a deleterious effect on IBD patients who may already be at increased risk of osteoporotic bone fractures and thus will inform on future treatment strategies for patient stratification.

Keywords: bone, inflammatory bowel disease, autophagy, colitis, osteoporosis, azathioprine

Introduction

Inflammatory bowel disease (IBD) is the name given to a group of conditions that affect the colon and the small intestine. The two main forms of IBD are ulcerative colitis and Crohn's disease. Crohn's disease can affect any part of the gastrointestinal tract and can cause transmural inflammation. In contrast, ulcerative colitis causes mucosal inflammation and is limited to the colon [1]. A recent review by NHS England revealed that the prevalence of IBD was 400 in every 100,000 people which results in a total cost of £720 million a year in costs to the NHS [2].

Osteoporosis has been associated as secondary to a number of gastrointestinal conditions, including IBD [3, 4]. Osteoporosis is a metabolic disease of the bone characterised by a reduction in bone mineral density and alterations in bone structure, which increases the likelihood of bone fracture. Significantly, patients with IBD often present with low bone mineral density (BMD), and are 40% more at risk of bone fracture than a healthy individual [3].

A number of risk factors exist for osteoporosis in IBD patients including malabsorption, chronic inflammation, low body mass index, use of glucocorticoids, vitamin D deficiency and surgery [5].

47 Intestinal absorption of key determinants of bone health – *e.g.* calcium and vitamin D - is often
48 compromised in IBD due to the reduction of intestinal mucosa [4]. Indeed, patients with IBD present
49 with vitamin D [6, 7] and calcium deficiencies [8]. IBD is also characterised by the chronic release of
50 pro-inflammatory cytokines such as IL-6 and tumour necrosis factor-α (TNF-alpha) - the increased
51 production of these cytokines can stimulate the RANK/RANKL/OPG axis and thus drive osteoclastic
52 bone resorption [9–11]. Low body mass index is a well established risk factor for low BMD and
53 fracture, and studies have shown similar associations in patients with IBD [12]. However, other
54 mechanisms may exist and the development of targeted therapies for bone loss in IBD requires a
55 better understanding of the underlying cellular mechanisms.
56   Drugs used to treat IBD include glucocorticoids, immunosuppressants, aminosalicylates (5-
57 ASAs) and biologic agents. Risk of osteoporosis has been shown to be twice as high in IBD patients
58 on corticosteroids [13], thought to be due to their effects on the RANK/RANKL/OPG axis, sex
59 hormones and calcium absorption [14]. Conversely, IBD patients on the monoclonal antibody
60 infliximab have shown increases in their BMD and markers of bone turnover [15–17]. Similarly,
61 patients on dual anti-TNF-α (infliximab or adalimumab) and azathioprine saw a significant positive
62 effect on BMD at the lumbar spine [18]. There is an increasing demand to optimise existing medical
63 therapies through patient stratification and personalised medicine [19]. We, and others, have
64 previously shown that drugs currently used for treating IBD can affect the autophagy pathway [20].
65 Autophagy is an essential self-eating process that can degrade intracellular components such as
66 organelles, foreign bodies and insoluble protein aggregates through the formation and maturation of
67 double membrane vesicles, known as autophagosomes [21]. Specifically, we have shown that the
68 thiopurine drug azathioprine is a potent inducer of autophagy in the colon, however its effects on
69 bone health have yet to be established [22].
70   To directly examine this we have studied bones from a mouse model of IBD using dextran
71 sulphate sodium (DSS)-induced colitis, which has been extensively used and validated by others to
72 induce acute and chronic forms of inflammatory bowel disease. This model of colitis in mice is
73 thought to involve the deterioration of the intestinal epithelial barrier through increased cell
74 apoptosis, therefore allowing the influx of antigens and micro-organisms and the subsequent
75 increased expression of inflammatory markers [23, 24]. Histologically, the colitis induced in this
76 murine model exhibits a wide range of features similar to that seen in man [23]. Further, we and
77 others have previously shown that DSS treatment causes detrimental effects on bone trabecular
78 microarchitecture [25, 26]. Here we have used the DSS model to examine the effects of azathioprine
79 treatment on the skeleton in the context of IBD. The dose and duration of the DSS treatment, and the
80 age, sex and strain of the mice used were based on our previous study [26]. Male mice were also used
81 to avoid any confounding actions of oestrogen on the skeleton. Data from this approach will help
82 inform on personalised therapies for patients with poor bone health in IBD.

83 **Results**

84 *Effect of azathioprine on body weight in DSS treated mice*

85   To assess the effects of azathioprine on body weight of mice, colitis was induced with 3% DSS
86 for 4 days in the presence of azathioprine (DSS/azathioprine) or vehicle control (DSS/vehicle). During
87 the DSS treatment period (0-4 days), no significant weight loss was observed in any mouse treatment
88 groups (Fig. 1). Independent of azathioprine treatment, mice exhibited a rapid and significant weight
89 loss from day 4 following DSS treatment (up to 7% in comparison to non-DSS treated mice, P<0.05).
90 Following this period of rapid weight loss, DSS/vehicle treated mice proceeded to gain weight until
91 the end of the study.   Weight gain was observed throughout the study period in the non-DSS/vehicle
92 treated mice (Fig. 1). In contrast, DSS/azathioprine treated mice exhibited a rapid and significant
93 weight loss, followed by a brief period of weight gain, which plateaued from day 10 onwards (Fig.
94 1). Non-DSS/azathioprine treated mice showed no significant weight gain throughout the experiment
95 (Fig. 1). Full details of the weight measurements and statistical significance over the 18-day treatment
96 period is detailed in Suppl. Table 1.

97
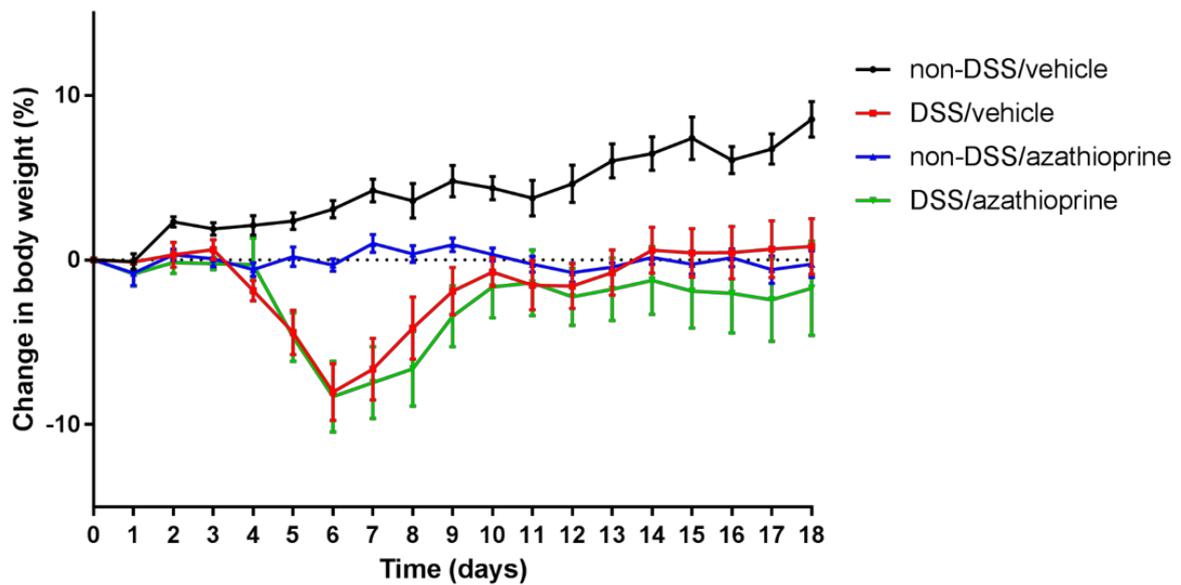
Figure 1. Body weight changes of azathioprine and vehicle treated mice treated with DSS followed
by a recovery period. Percentage change in body weight of azathioprine and vehicle mice treated with
or without 3% DSS for 4 days. Data are presented as mean ± S.E.M (n=6/group).

Effect of azathioprine on colon pathology in DSS treated mice

To assess the effects of DSS on mucosal integrity, detailed histological analysis was performed
on the colon from control and DSS/azathioprine or DSS/vehicle mice. Histological scores for all
parameters were minimal in the non-DSS treated mice, and there were no notable differences
observed with azathioprine treatment in this group (Fig. 2). In contrast, histological analysis of the
colon from DSS mice revealed significant increases in scores for inflammation severity (Fig. 2A,
$P<0.05$) and extent (Fig. 2B, $P<0.01$), consistent with previous studies and indicative of successful
induction of colitis. It was also observed that the colons from DSS/vehicle mice showed decreased
tissue regeneration (as indicated by the higher regeneration score; Suppl. Table 2 & Fig. 2C, $P<0.05$)
and increased crypt damage (Fig. 2D, $P<0.05$) in comparison to the non-DSS/vehicle mice.

No significant differences were observed in tissue regeneration (Fig. 2C) and crypt damage (Fig.
2D) in non-DSS/azathioprine and DSS/azathioprine treated mice, indicative of a partial protection of
azathioprine treatment to the colon. Regional specific changes in the parameters examined were also
observed, with significant pathology localised to the distal aspect of the colon (Suppl. Fig. 1).
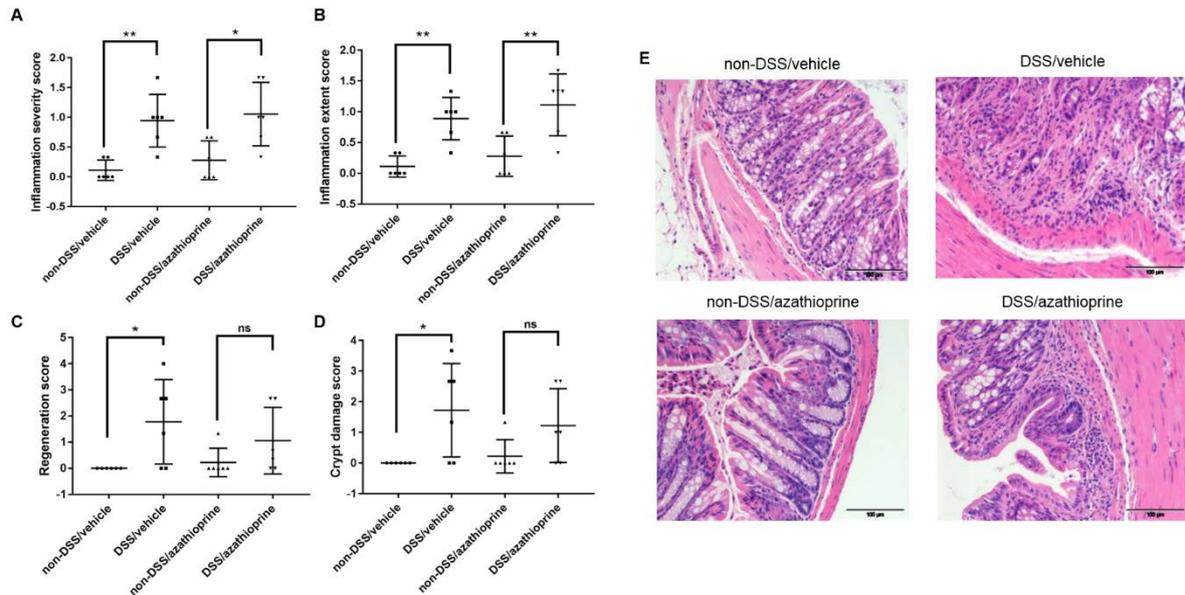
Figure 2. Colon pathology of azathioprine and vehicle treated mice treated with 3% DSS. Histological scoring of colons (**A)** Inflammation severity score (**B**) Inflammation extent score (**C**) Regeneration score (**D**) Crypt damage score (**E**) Representative H&E-stained sections of colon. Data are presented as mean ± S.D. (n=6/group). P<0.05*, P<0.01**. Scale bar = 100μm.

Effect of azathioprine on bone phenotype in DSS treated mice

DSS-treated mice showed worsened trabecular microarchitecture compared to non-DSS treated mice as demonstrated by micro computed-tomography (CT) (Fig. 3A). Specifically, DSS-treated mice exhibited a significant decrease in trabecular thickness (Fig. 3D, P<0.05). Non-significant decreases in BV/TV (Fig. 3B), and trabecular number (Fig. 3C), and increases in trabecular separation (Fig. 3E) and pattern factor (Fig. 3F) were also observed in DSS-treated mice. Treatment with azathioprine alone had a significant detrimental effect on the trabecular bone microarchitecture, independent of DSS treatment. Indeed, significant decreases were observed in BV/TV (Fig. 3B, P<0.01), and trabecular number (Fig. 3C, P<0.01), with a concurrent significant increase in trabecular pattern factor (Fig. 3F, P<0.05) indicative of a more disorganised trabecular structure. No effects of DSS or azathioprine treatment were observed on trabecular BMD (Fig. 3G).
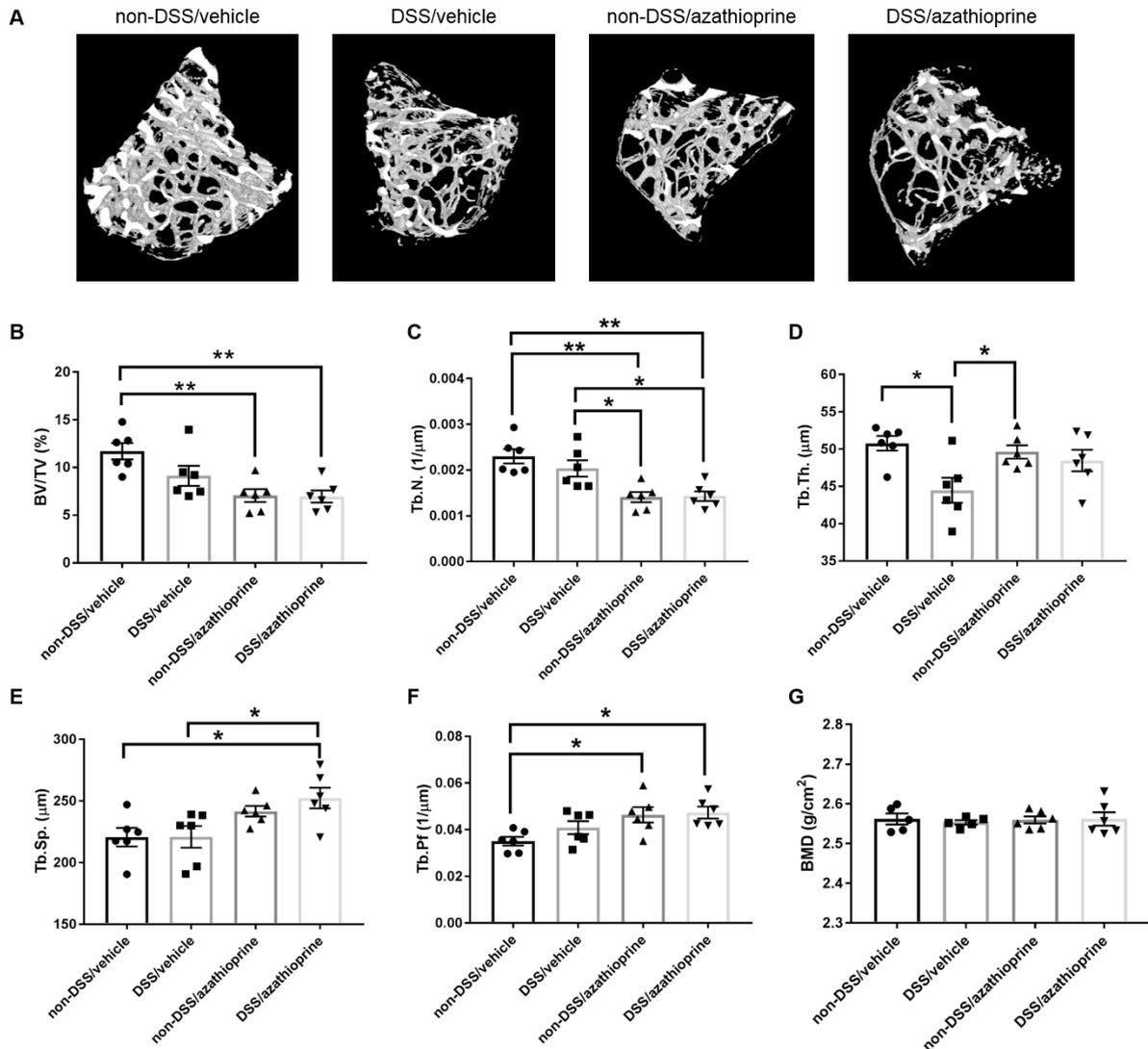
Figure 3. Trabecular bone microarchitecture of azathioprine and vehicle treated mice treated with 3% DSS. (**A**) Representative 3D microCT reconstructions. Trabecular bone parameters between treated and control groups (**B**) bone volume/tissue volume (BV/TV), (**C**) trabecular number (Tb. N.), (**D**) trabecular thickness (Tb. Th.), (**E**) trabecular separation (Tb. Sp.) (**F**) trabecular pattern factor (Tb. Pf.), (**G**) trabecular bone mineral density (BMD). Data are presented as mean ± S.D. (n=6/group). P<0.05*. P<0.01**.

Induction of autophagy by azathioprine in DSS treated mice

Histological analysis of the tibia sections appeared to confirm the results from the microCT analysis, with an apparent reduction in bone volume (indicated by increased red osteoid staining) in the trabecular bone in both the DSS treated mice (Fig. 4A ii, in comparison to Fig. 4Ai) and those treated with azathioprine (Fig. 4A iii & iv, in comparison to Fig. 4Ai). To examine whether azathioprine affects autophagy activity in the skeleton of mice, immunohistochemistry for the autophagy marker LC3 was conducted. LC3 labelling was observed in the osteoblasts lining the trabecular bone, in the osteocytes and throughout the bone marrow. No differences were observed between non-DSS and DSS treated mice (Fig. 4Ci, in comparison to Fig. 4Cii). However, azathioprine treatment appeared to modestly increase the intensity of LC3 labelling, independent of DSS treatment and in particular within the bone marrow, although no significant differences were observed upon quantification of immunolabelling intensity (Fig. 4C iii & iv, in comparison to Fig. 4C i and ii, Suppl. Fig. 2).
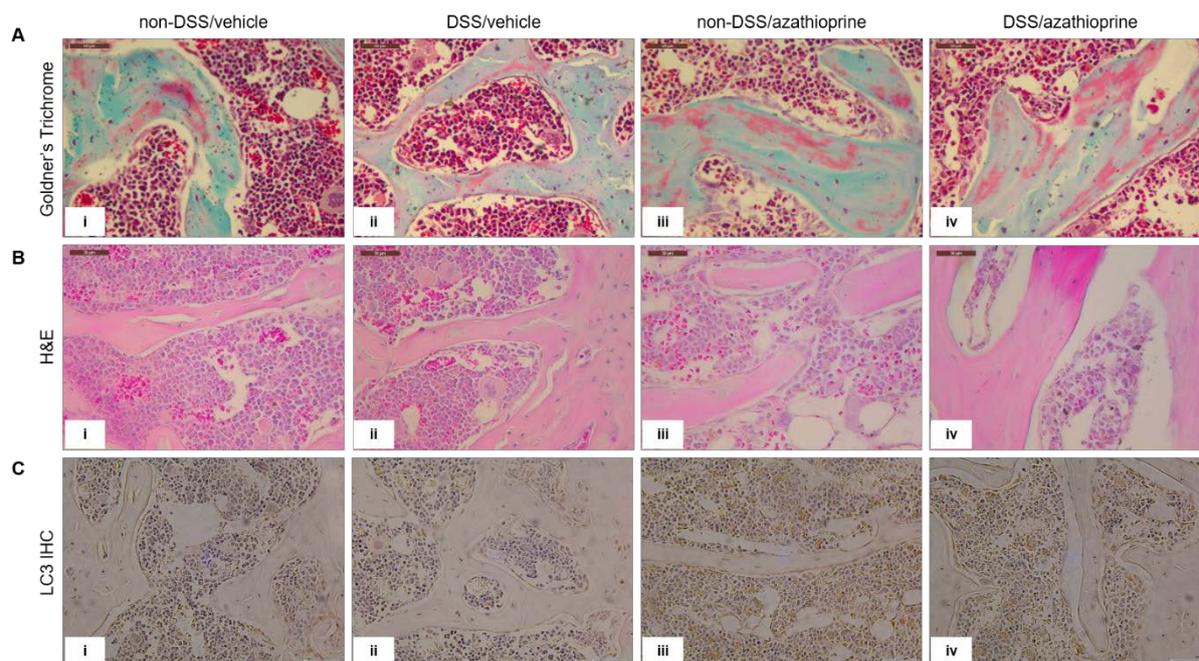
Figure 4. Histological staining and immmunohistochemical labelling of tibia trabecular bone in sections of the tibia. (**A**) Goldner's Trichrome (**B**) H&E (**C**) LC3 immunolabelling. LC3-positive immunolabelling is presented as brown staining. Scale bar = 50μm. Images are representative of 4 different mice/group.

Discussion

Patients with IBD often present poor bone health and are 40% more at risk of bone fracture [3]. Azathioprine is widely used in the treatment of IBD and has been proven to be highly effective, however it has previously been linked to an increase in fracture risk in humans [27]. Here we utilised the DSS model of colitis in mice to delineate the effects of the azathioprine on the skeleton. Histopathological analysis of the colon revealed successful induction of colitis in DSS treated mice, however, it also revealed no differences in the severity or extent of inflammation in azathioprine treated mice compared to vehicle-treated mice. This suggests that any noted effects of azathioprine on the skeleton may be direct and not a consequence of altered nutrition through malabsorption of nutrients.

There are a number of risk factors associated with IBD-related bone loss including poor nutrient intake/ absorption, chronic inflammation, and use of glucocorticoids [5]. Central to the inflammatory response is the chronic release of the pro-inflammatory cytokines IL-6 and TNF-α. Indeed IL-6 has been identified as the predominant cytokine mediating the bone abnormalities, and genetic variations in IL-6 correlate well with the clinical course of IBD and the extent of bone loss [28, 29]. These pro-inflammatory cytokines are known to promote bone loss directly, but also through altered sensitivity and secretion of growth hormone and insulin-like growth factor in IBD [30–32]. DSS-induced colitis is the result of deterioration of the epithelial barrier, allowing for the influx of antigens and microorganisms, and prompting the increased expression of these pro-inflammatory mediators [23, 24]. Indeed this has previously been shown to have detrimental effects on bone quality [25, 26]. In accordance with these studies, we observed worsened bone trabecular microarchitecture with DSS treatment in our mice. Further, when azathioprine was administered alone it was also found to have a detrimental effect on bone microarchitecture. This is consistent with previous reports of increased overall skeletal fracture risk in individuals prescribed azathioprine [27], although contradicts that in which combination of anti-TNF-α and azathioprine had a positive effect [18]. The anti-TNF-α monoclonal antibody infliximab has on its own been associated with increases in BMD and markers of bone which may provide explanation as to the disparity between these results [15–17]. In addition, it has been suggested that azathioprine can disrupt the bone remodelling process in a rat model by

184 suppressing T lymphocytes causing disturbances in the RANKL system responsible for osteoclast
185 formation and activity [33]. Specifically, the authors found that although the length and diameter of
186 the bones remained unchanged, azathioprine caused an overall reduction in femur and tibia mass,
187 whilst also reducing the calcium content. Further, the thickness of the trabeculae in the femur was
188 found to be reduced in rats when treated with azathioprine in both the distal epiphysis and
189 metaphysis [33]. The bisphosphonate alendronate prevented the development of these skeletal
190 changes when administered in combination with azathioprine [33]. These findings complement our
191 findings here that the administration of azathioprine may contribute to overall bone loss and
192 trabecular bone deterioration. This suggests that azathioprine alone may therefore not be a suitable
193 drug of choice for IBD patients who are more at risk of osteoporotic bone fractures, such as the
194 elderly.

195 We hypothesised that the detrimental effects on bone caused by azathioprine may result in the
196 induction of the autophagy pathway, as azathioprine has already been shown to induce this process
197 in peripheral blood mononuclear cells and colorectal cancer cells [22, 34]. Autophagy is a homeostatic
198 process in which cells degrade protein aggregates and damaged organelles [35]. Upon the induction
199 of autophagy, LC3-I becomes lipidated and becomes LC3-II before inserting into the autophagosome
200 membrane [36]. Because of this, the detection of LC3 within a sample is a recognised marker used to
201 show the presence of autophagy. Here we revealed increased LC3 labelling in our azathioprine
202 treated mice, suggestive of autophagy induction. This suggests that azathioprine is an effective
203 inducer of autophagy activity in the skeleton. The reasons for this are currently speculative, as it is
204 currently not known whether autophagy is indirectly induced as a survival mechanism to cope with
205 the adverse effects caused by azathioprine on bone health. Similarly, the precise effects of
206 azathioprine on osteoblast function are currently unknown. The effects of another autophagy inducer
207 - rapamycin – are however well documented, albeit somewhat controversial in their findings. It has
208 been shown that rapamycin, in the presence of lipopolysaccharides, can promote the differentiation
209 of human embryonic stem cells (hESCs) into mature osteoblasts by modulating mTOR signalling [37,
210 38]. However, it was also found that rapamycin inhibits osteoblast proliferation and differentiation
211 in MC3T3-E1 cells. It was observed that even at low concentrations (0.1-20 nM), rapamycin reduced
212 osteocalcin and osterix mRNA expression in differentiating MC3T3-E1 osteoblasts, as well as
213 reducing their mineralisation capacity [39]. Therefore, this further highlights the need to understand
214 more fully the mechanism of action of azathioprine before a better level of care for patients can be
215 provided.

216 In conclusion, the data in this manuscript suggest that azathioprine treatment may have a
217 deleterious effect on bone health in IBD patients who may already be at increased risk of osteoporotic
218 bone fractures and thus will inform on future treatment strategies for patient stratification.

219 **Materials and Methods**

220 *Animals*

221 Male 10-week old C57BL/6J mice (n=6/group) (Charles River, UK) were treated with 3% DSS
222 (molecular mass ~40,000kD; MP Biomedical, UK) in their drinking water *ad libitum* for 4 days,
223 following which they were given normal tap water for a 14-day recovery period. Control (non-DSS
224 treated) male mice received normal tap water for the duration of the study. The dose and duration of
225 the DSS treatment was based on previous studies using the same mouse strain, age and sex [26]. It is
226 important to note that this model is unable to distinguish between ulcerative colitis and Crohn's
227 disease. Mice were treated using an oral gavage daily throughout the experiment with 10mg/kg/day
228 of azathioprine or a vehicle control (n=6/group). The health status of the DSS-treated mice was scored
229 daily, with particular attention paid to their coat condition, mobility, presence of blood in stools and
230 eye clarity. Body weights of all mice were recorded daily. After the 14-day recovery period, the mice
231 were culled and blood, colon and bone samples collected. Mice were kept in polypropylene cages,
232 with light/dark 12-hr cycles, at 21 ± 2°C, and fed ad libitum with maintenance diet (Special Diet
233 Services, Witham, UK). All experimental protocols were approved by Roslin Institute's Animal Users

234 Committee and the animals were maintained in accordance with UK Home Office guidelines for the
235 care and use of laboratory animals.

Colon pathology

237 The colon was dissected from all mice, measured and fixed in 4% paraformaldehyde (PFA) for
238 24 hours. Each colon was divided into 3 transverse segments including proximal, middle and distal
239 portions. Tissue processing, wax embedding, sectioning (5µm thick) and Hematoxylin and eosin
240 (H&E) staining were conducted following routine procedures. Colon pathology was graded blind on
241 sections from all 3 segments of each mouse using an established histological grading scheme (Suppl.
242 Table 2) [40, 41]. Scores from all three segments were averaged to provide an overall pathology score,
243 as well as were analysed in the separate regions of the colon.

Bone histology & immunohistochemistry

245 The tibia was dissected from all mice, fixed in 4% formaldehyde, and decalcified in 10%
246 ethylenediaminetetraacetic acid (EDTA) at 4oC. Tissue processing, wax embedding, and sectioning
247 (5µm thick) were done following routine procedures. Tibia sections were stained for histological
248 analysis using the Goldner's stain, and H&E following standard protocols. For
249 immunohistochemistry, sections were dewaxed in xylene, rehydrated in graded alcohol and
250 incubated for 90 minutes at 70°C in 10 mM citrate buffer for antigen retrieval. Any endogenous
251 peroxidase activity was blocked by using 0.3% H_2O_2 for 30 minutes at room temperature. LC3 (1:500,
252 polyclonal raised in rabbit; MBL) antibodies were used with an appropriate IgG control (Suppl. Fig.
253 2). The Vectastain ABC universal kit (Vector Laboratories, Peterborough, UK) was used according to
254 the manufacturer's instructions. The samples were counterstained with haematoxylin before being
255 dehydrated and mounted using DePex. Quantification of immunolabelling was conducted using
256 reciprocal intensity and the open source software Image J (http://fiji.sc/) [42].

microCT

258 To evaluate trabecular microarchitecture and cortical bone geometry of the tibia from control
259 and DSS-treated mice, we used microCT (Skyscan 1172 X-ray microtomograph, Bruker, Kontich,
260 Belgium) as described previously [43]. In brief, high-resolution scans with an isotropic voxel size of
261 5 µm were acquired (60 kV, 0.5 mm aluminium filter, 0.6° rotation angle). Two images were averaged
262 at each rotation angle. Scan reconstruction was conducted using NRecon software (Bruker) and each
263 bone was analysed using CtAN (Bruker). For the trabecular analysis, the base of the growth plate was
264 used as a standard reference point. A 1.25mm trabecular bone region, located at 5% of the total length
265 beneath this reference point, was analysed. To investigate the changes in the cortical bone geometry,
266 two 0.5mm sections were analysed at 37% and 50% of the total bone length from the reference starting
267 slice (first appearance of the medial tibial condyles). To assess BMD, phantoms were used to calibrate
268 the CTAn software. BMD phantoms of known calcium hydroxyapatite mineral densities of 0.25 and
269 0.75 g/cm^3 were scanned and reconstructed using the same parameters as used for bone samples.

Statistical analysis

271 Data are expressed as the mean ± standard deviation (S.D.). A power analysis was conducted on
272 microCT data from a previous study using an identical model and 6 experimental mice per group
273 were required to detect statistically significant differences in the bone trabecular microarchitecture
274 [26]. Analysis was performed by one-way analysis of variance (ANOVA) with appropriate post-hoc
275 tests. $P<0.05$ was considered to be significant and noted as *, with P values of <0.01 and <0.001 were
276 noted as ** and *** respectively.

277 **Supplementary materials:**
278 *Suppl. Table 1: Significance of weight changes between treatment groups of mice in DSS-study taken daily over the 18-day*
279 *treatment period.*

Suppl. Table 2: Scoring criteria of colon pathology.

Suppl. Fig. 1: Colon pathology of azathioprine and vehicle treated mice treated with 3% DSS. Histological scoring of proximal, middle and distal regions of the colon (A) Inflammation severity score (B) Inflammation extent score (C) Regeneration score (D) Crypt damage score. Data are presented as mean ± S.D. (n=6/group). P<0.05*, P<0.01**, P<0.001***.

Suppl. Fig. 2: (A) Appropriate IgG control for LC3 immunolabelling. Scale bar = 50μm. Quantification of immunohistochemistry – reciprocal intensity of (B) osteoblasts (C) bone marrow (D) whole bone slice. Data are presented as mean ± S.D.

Author Contributions: Conceptualization, KS, CS.; Formal Analysis, SM, KM, EM, KS, CS.; Data Curation, SM, KM, EM.; Methodology, KS, CS, CF.; Writing – Original Draft Preparation, SM, KS, CS.; Writing – Review & Editing, SM, KM, EM, CF, CS, KS.; Supervision, KS, CS.; Project Administration, KS.; Funding Acquisition, KS, CS.

Conflicts of Interest: The authors declare no conflicts of interest

References

1. Zhang Y-Z, Li Y-Y. Inflammatory bowel disease: Pathogenesis. World J Gastroenterol. 2014;20:91–9. doi:10.3748/wjg.v20.i1.91.

2. England N. 2013/14 Nhs Standard Contract for Colorectal: Complex Inflammatory Bowel Disease (Adult). NHS Engl. 2014;:1–6.

3. Ali T, Lam D, Bronze MS, Humphrey MB. Osteoporosis in Inflammatory Bowel Disease. American Journal of Medicine. 2009;122:599–604.

4. Bianchi ML. Inflammatory bowel diseases, celiac disease, and bone. Archives of Biochemistry and Biophysics. 2010;503:54–65.

5. Lima CA. Risk factors for osteoporosis in inflammatory bowel disease patients. World J Gastrointest Pathophysiol. 2015;6:210.

6. Blanck S, Aberra F. Vitamin D deficiency is associated with ulcerative colitis disease activity. Dig Dis Sci. 2013;58:1698–702.

7. Jørgensen SP, Hvas CL, Agnholt J, Christensen LA, Heickendorff L, Dahlerup JF. Active Crohn's disease is associated with low vitamin D levels. J Crohn's Colitis. 2013;7.

8. Silvennoinen J, Lamberg-Allardt C, Kärkkäinen M, Niemelä S, Lehtola J. Dietary calcium intake and its relation to bone mineral density in patients with inflammatory bowel disease. J Intern Med. 1996;240:285–92.

9. Moschen AR, Kaser A, Enrich B, Ludwiczek O, Gabriel M, Obrist P, et al. The RANKL/OPG system is activated in inflammatory bowel diseases and relates to the state or bone loss. Gut. 2005;54:479–87.

10. Bernstein CN, Sargent M, Leslie WD. Serum osteoprotegerin is increased in Crohn's disease: a population-based case control study. Inflamm Bowel Dis. 2005;11:325–30. doi:10.1097/01.mib.0000164015.60795.ca.

11. Turk N, Cukovic-Cavka S, Korsic M, Turk Z, Vucelic B. Proinflammatory cytokines and receptor activator of nuclear factor κB-ligand/osteoprotegerin associated with bone deterioration in patients with Crohn's disease. Eur J Gastroenterol Hepatol. 2009;21:159–66.

12. Lee N, Radford-Smith GL, Forwood M, Wong J, Taaffe DR. Body composition and muscle strength as predictors of bone mineral density in Crohn's disease. J Bone Miner Metab. 2009;27:456–63.

13. Abraham BP, Prasad P, Malaty HM. Vitamin D deficiency and corticosteroid use are risk factors for low bone mineral density in inflammatory bowel disease patients. Dig Dis Sci. 2014;59:1878–84.

14. Lane NE. Glucocorticoid-Induced Osteoporosis: New Insights into the Pathophysiology and Treatments. Current Osteoporosis Reports. 2019;17.

15. Miheller P, Muzes G, Rácz K, Blázovits A, Lakatos P, Herszényi L, et al. Changes of OPG and RANKL concentrations in Crohn's disease after infliximab therapy. Inflamm Bowel Dis. 2007;13:1379–84.

16. Franchimont N, Putzeys V, Collette J, Vermeire S, Rutgeerts P, De Vos M, et al. Rapid improvement of bone metabolism after infliximab treatment in Crohn's disease. Aliment Pharmacol Ther. 2004;20:607–14.

17. M.T. A, J.L. G, E.A. V, L.Y. K, P. V, L.A. M, et al. Treatment with infliximab is associated with increased markers of bone formation in patients with Crohn's disease. J Clin Gastroenterol. 2006;40:55–63. http://www.ncbi.nlm.nih.gov/pubmed/16340635. Accessed 19 Nov 2019.

18. Krajcovicova A, Hlavaty T, Killinger Z, Miznerova E, Toth J, Letkovsky J, et al. Combination therapy with an immunomodulator and anti-TNFα agent improves bone mineral density in IBD patients. J Crohn's Colitis. 2014;8:1693–701.

19. Denson LA. The role of the innate and adaptive immune system in pediatric inflammatory bowel disease. Inflammatory Bowel Diseases. 2013;19:2011–20.

20. Hooper KM, Barlow PG, Stevens C, Henderson P. Inflammatory bowel disease drugs: A focus on autophagy. Journal of Crohn's and Colitis. 2017;11:118–27.

21. Yu L, Chen Y, Tooze SA. Autophagy pathway: Cellular and molecular mechanisms. Autophagy. 2018;14:207–15. doi:10.1080/15548627.2017.1378838.

22. Hooper KM, Casanova V, Kemp S, Staines KA, Satsangi J, Barlow PG, et al. The Inflammatory Bowel Disease Drug Azathioprine Induces Autophagy via mTORC1 and the Unfolded Protein Response Sensor PERK. Inflamm Bowel Dis. 2019;25:1481–96. doi:10.1093/ibd/izz039.

23. Perše M, Cerar A. Dextran sodium sulphate colitis mouse model: Traps and tricks. Journal of Biomedicine and Biotechnology. 2012;2012.

24. Araki Y, Mukaisyo KI, Sugihara H, Fujiyama Y, Hattori T. Increased apoptosis and decreased proliferation of colonic epithelium in dextran sulfate sodium-induced colitis in mice. Oncol Rep. 2010;24:869–74.

25. Hamdani G, Gabet Y, Rachmilewitz D, Karmeli F, Bab I, Dresner-Pollak R. Dextran sodium sulfate-induced colitis causes rapid bone loss in mice. Bone. 2008;43:945–50.

26. Dobie R, MacRae VE, Pass C, Milne EM, Ahmed SF, Farquharson C. Suppressor of cytokine signaling 2 (Socs2) deletion protects bone health of mice with DSS-induced inflammatory bowel disease. Dis Model Mech. 2018;11. doi:10.1242/dmm.028456.

27. Vestergaard P, Rejnmark L, Mosekilde L. Methotrexate, azathioprine, cyclosporine, and risk of fracture. Calcif Tissue Int. 2006;79:69–75.

28. Schulte CMS, Dignass AU, Goebell H, Roher HD, Schulte KM. Genetic factors determine extent of bone loss in inflammatory bowel disease. Gastroenterology. 2000;119:909–20.

29. Paganelli M, Albanese C, Borrelli O, Civitelli F, Canitano N, Viola F, et al. Inflammation is the main determinant of low bone mineral density in pediatric inflammatory bowel disease. Inflamm Bowel Dis. 2007;13:416–23.

30. Fernandez-Vojvodich P, Zaman F, Sävendahl L. Interleukin-6 acts locally on the growth plate to impair bone growth. Ann Rheum Dis. 2013;72:e24. doi:10.1136/annrheumdis-2013-204112.

365 31. Wong S, Smyth A, McNeill E, Galloway P, Hassan K, McGrogan P, et al. The Growth Hormone -Insulin Like
366 Growth Factor 1 Axis In Children & Adolescents With Inflammatory Bowel Disease & Growth
367 Retardation. Clin Endocrinol (Oxf). 2010. doi:10.1111/j.1365-2265.2010.03799.x.

368 32. Wong SC, Dobie R, Altowati MA, Werther GA, Farquharson C, Ahmed SF. Growth and the growth hormone-
369 insulin like growth factor 1 axis in children with chronic inflammation: Current Evidence, Gaps in Knowledge,
370 and Future Directions. Endocrine Reviews. 2016;37:62–110.

371 33. Cegiela U, Kaczmarczyk-Sedlak I, Pytlik M, Folwarczna J, Nowińska B, Fronczek-Sоќl J. Alendronate
372 prevents development of the skeletal changes induced by azathioprine in rats. Acta Pol Pharm - Drug Res.
373 2013;70:309–15.

374 34. Chaabane W, Appell ML. Interconnections between apoptotic and autophagic pathways during thiopurine-
375 induced toxicity in cancer cells : the role of reactive oxygen species. Oncotarget. 2016;7:75616–34.

376 35. Henderson P, Stevens C. The Role of Autophagy in Crohn's Disease. Cells. 2012;1:492–519.

377 36. Nollet M, Santucci-Darmanin S, Breuil V, Al-Sahlanee R, Cros C, Topi M, et al. Autophagy in osteoblasts is
378 involved in mineralization and bone homeostasis. Autophagy. 2014;10:1965–77.

379 37. Lee K-W, Yook J-Y, Son M-Y, Kim M-J, Koo D-B, Han Y-M, et al. Rapamycin promotes the osteoblastic
380 differentiation of human embryonic stem cells by blocking the mTOR pathway and stimulating the BMP/Smad
381 pathway. Stem Cells Dev. 2010;19:557–68. doi:10.1089/scd.2009.0147.

382 38. Li X, Chang B, Wang B, Bu W, Zhao L, Liu J, et al. Rapamycin promotes osteogenesis under inflammatory
383 conditions. Mol Med Rep. 2017;16:8923–9. doi:10.3892/mmr.2017.7693.

384 39. Singha UK, Jiang Y, Yu S, Luo M, Lu Y, Zhang J, et al. Rapamycin inhibits osteoblast proliferation and
385 differentiation in MC3T3-E1 cells and primary mouse bone marrow stromal cells. J Cell Biochem. 2008;103:434–
386 46. doi:10.1002/jcb.21411.

387 40. Williams KL, Fuller CR, Dieleman LA, DaCosta CM, Haldeman KM, Sartor RB, et al. Enhanced survival and
388 mucosal repair after dextran sodium sulfate-induced colitis in transgenic mice that overexpress growth
389 hormone. Gastroenterology. 2001;120:925–37. doi:10.1053/gast.2001.22470.

390 41. Dieleman LA, Palmen MJHJ, Akol H, Bloemena E, Peña AS, Meuwissen SGM, et al. Chronic experimental
391 colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. Clin Exp Immunol.
392 1998;114:385–91.

393 42. Nguyen DH, Zhou T, Shu J, Mao J-H. Quantifying chromogen intensity in immunohistochemistry via
394 reciprocal intensity. www.CancerInCytes.org. Accessed 19 Nov 2019.

395 43. Staines KA, Javaheri B, Hohenstein P, Fleming R, Ikpegbu E, Unger E, et al. Hypomorphic conditional
396 deletion of E11/Podoplanin reveals a role in osteocyte dendrite elongation. J Cell Physiol. 2017.
397 doi:10.1002/jcp.25999.

398