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1 **Original Article**

2 **The impact of complexation or complex coacervation of lactoferrin and**
3 **osteopontin on simulated infant gastrointestinal digestion, intestinal**
4 **inflammation, and in vivo bone development**

5

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Running head:

LF-OPN complex digestion and bioactive potential

Highlights:

- Complexation with OPN delayed gastric proteolysis of LF at low pH
- LF-OPN complexation sustained LF bioactivity after gastrointestinal digestion
- Early life bone strength and structure was improved by LF-OPN complexes
- Complexation may benefit supplementation of LF and OPN in early life

Key words: lactoferrin, osteopontin, complex coacervate, digestion, bone

Abstract

Lactoferrin (LF) and osteopontin (OPN) are bioactive milk proteins which can form heteroprotein complexes and complex coacervates. This research studied the effect of LF-OPN complexation and complex coacervation on the simulated infant gastrointestinal digestion of LF with subsequent examination of gut and bone health bioactivities in preclinical models. In an infant digestion model, the proteolytic profile of LF was unaltered by the pre-association of LF and OPN. Gastric proteolysis of LF

was increased when the model gastric pH was reduced from 5.3 to 4.0, but less so when complexed with OPN. In a model of intestinal inflammation, undigested (79% inhibition) and gastric digestates (26% inhibition) of LF, but not gastrointestinal digestates, inhibited lipopolysaccharide (LPS)-induced NF-κB activation in intestinal epithelial cells. LF-OPN complexation sustained the inhibitory effect (21-43% of the undigested effect, depending on the type of complex) of LF after gastrointestinal digestion, suggesting that the peptides produced were different. In a neonatal rodent model used to study bone development, coacervating LF and OPN improved bone structures with a significant increase of trabecular proportion (BV/TV increase by 21.7 %). This resulted in an 11.3 % increase in stiffness of bones. Feeding the LF and OPN proteins in coacervate format also increased the levels of OPN, P1NP and M-CSF in blood, signifying a more pronounced impact on bone development. This research demonstrated that LF-OPN complexation and complex coacervation can delay simulated infant gastrointestinal digestion of LF and protect or improve the bioactivity of the proteins.

1. Introduction

Lactoferrin (LF) and osteopontin (OPN) are bioactive milk proteins that are important for infant nutrition.[1] LF is a ~78 kDa glycoprotein, whose biochemical properties have been well characterised.[2,3,4] LF is found in the milk of most mammalian species and typically occurs in human milk at ~10-fold higher concentrations (5.1 g/L in colostrum or 1.4 g/L beyond 90 d of lactation) than in bovine milk (0.03 – 0.5 g/L).[5,6,3] The biological functions of LF include anti-microbial, bifidogenic, immunomodulatory, anti-inflammatory, anti-oxidation, and anti-infectious activities.[4,6] Moreover, LF has been shown to promote bone growth by potently stimulating the proliferation and differentiation of primary osteoblasts, to act as a survival factor by inhibiting apoptosis induced by serum withdrawal, and to influence osteoclast metabolism by affecting formation and potently inhibiting osteoclastogenesis.[7] Recent clinical data suggests that the manufacture, form, and dose of bovine LF used in infant nutrition requires deeper consideration as studies show inconsistent findings.[8,9,10,11,12] The interactions between LF and high affinity ligands (e.g., OPN) may also impact the clinical functions of LF.

OPN is a minor, acidic, highly phosphorylated glycoprotein, also present at higher concentrations in human milk (varying between 0.1 and 0.3 g/L, corresponding to 1.3-2.7% of total protein) compared with bovine milk (~0.02 g/L).[13,14] Beyond its nutritive amino acid contribution, OPN has been linked with several bioactivities including contributions to biomineralization, immune, brain, and intestinal development.[4,15,16,17] In particular, its role in bone metabolism and homeostasis has become a focus of research, since OPN has been demonstrated to be closely related to the occurrence and development of many bone-related diseases, including osteoporosis.[18]

Fortification of current infant formula with LF and OPN proteins is possible using suitable bovine milk-derived ingredients that have high levels of protein sequence homology with human LF and OPN ((67 % and 61 %, respectively).[11,19,20] The most clinically effective approach to add LF and OPN to infant formula requires consideration to ensure that the proteins remain functional when digested in the infant gastrointestinal tract.

Heteroprotein complexes of LF and OPN are known to naturally occur in milk and the chemistry of LF-OPN protein-protein interactions has been intensively studied.[21,22,23] Recently, LF and OPN have been shown to undergo heteroprotein complex coacervation (associative liquid-liquid phase separation) under specific combinations of pH, ionic strength, stoichiometry, and total protein concentration, co-localising in a highly ordered, structural network of protein (~30%, w/w) and water (~70%, w/w), which occurs spontaneously within LF:OPN ratios naturally found in human milk.[24]

Some *in vitro* biochemical analyses on LF-OPN complexes isolated from human and bovine milk demonstrated an apparent protection of the proteins against gastrointestinal proteolysis, leading to more effective binding, uptake, cell proliferation, and bioactivity than LF and OPN alone.[25,26] For these reasons, it can be hypothesized that bioactivities relating to the intactness of the individual proteins could be preserved if presenting the proteins as a complex whereby digestive proteolysis is reduced. Adding a bovine LF-OPN complex to a matrix of whey protein hydrolysate and intact α-lactalbumin sustained selected *in vitro* bioactivities of the individual proteins and partially resisted *in vitro* digestion.

Addition of complexed forms of LF and OPN to infant formula as powders of soluble complexes (SC) or complex coacervates (CC) rather than individual bovine milk-

106 derived proteins may delay their digestion, sustain their bioactivity if structural
107 intactness is necessary for efficacy, and render both proteins more bioavailable,
108 potentially resulting in greater health benefits.[19,24,25,26]

109 This research aimed to determine if complexation or complex coacervation with OPN
110 altered gastrointestinal digestion of LF and its bioactivity in two preclinical models,
111 an *in vitro* model of intestinal inflammation and a neonatal rodent model to assess
112 bone development. It was hypothesized that the OPN-LF heteroprotein interactions
113 would lead to altered digestive proteolysis which would impact intestinal epithelial
114 cell responses and subsequently bone development. These new results will help to
115 guide research on how to supplement LF and OPN ingredients in the most biologically
116 effective form.

2. Materials and Methods

2.1 Materials

The LF, OPN, LF-OPN soluble complex (SC), and LF-OPN complex coacervate (CC) powders used in this research were as described by Goulding *et al*.[24] Briefly, the LF and OPN powders (purity of 94.6 and 99.4 % w/w of total protein, respectively) were mixed in the preparation of the SC and CC powders in a LF:OPN ratio of 4:1 (mass protein basis), at a total protein concentration of 5% (w/v), prior to pH adjustment (to pH 6.5 and 4.0 for SC and CC, respectively), phase separation (in case of CC), and freeze-drying. The protein content of the LF, OPN, SC, and CC powders was 98.7 (N x 6.25), 89.7 (N x 7.17), 97.6 (N x 6.38) and 95.7 (N x 6.38) % (w/w), respectively;[27] explanations for nitrogen determination methodology and conversion factors used have been provided in Goulding *et al*.[24] The LF powder had an iron saturation of 9-10 % and the OPN powder had a calcium content of 2.3 % (w/w).

The human milk serum (HMS) used in cell experiments was prepared as previously described in Goulding *et al.* [28]; the concentration of sCD14 in the HMS was 48 μg/mL. Prior informed consent for the use of the donor human milk samples for research purposes was obtained; ethical approval was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland. The lipopolysaccharide (LPS) used in the cell experiments was of the gram-negative *Escherichia coli* O111:B4 serotype. All chemicals used were of analytical grade and provided by Sigma Aldrich (Arklow, Co. Wicklow, Ireland) unless stated otherwise.

139 **2.2. Dry mixing and hydration of meals**

140 Meals undergoing simulated infant gastrointestinal digestion were prepared by
141 hydrating the relevant powders (LF, OPN, SC, and CC) in ultrapure water
142 (18.2 MΩ·cm). All meals had the same total protein concentration of 1.34 % (w/v) to
143 ensure the enzyme:substrate ratio was consistent between samples for simulated
144 digestions.

145 Meals for use in the rodent preclinical study were prepared every two days as fresh
146 solutions at 20 % w/v in distilled water of (1) LF-OPN SC at 1250 mg/kg/day (2) LF-
147 OPN CC at 1250 mg/kg/day and (3) a mixed blend of LF at 1000 mg/kg/day plus OPN
148 at 250 mg/kg/day powder. The respective powders were gradually added to the water
149 in a glass beaker on a magnetic stir plate. The mixture was then stirred until the
150 solution became clear.

151

152 **2.3. Simulated infant gastrointestinal digestion assays**

153 Simulated infant gastrointestinal digestions were performed using a previously
154 described static *in vitro* method (INFOGEST) based on physiological findings in
155 infants born at term (aged 28 days).[29] Briefly, digestions were performed in 40 mL
156 conical, screw capped, amber glass vials which were soaked in 6 % (v/v) nitric acid
157 before each digestion to remove any residues. The vials were placed in a water bath at
158 37°C and agitated at 50 rpm for the duration of the digestion process (1 hour gastric
159 and 1 hour intestinal). Three independent trials with three replicates per trial were
160 performed for gastric (pH 5.3) and gastrointestinal (intestinal stage pH 6.6) digestions
161 of each sample. The gastric lipase (19 U/mL) and pepsin activities (268 U/mL) were
162 achieved using rabbit gastric extract (RGE70, Lipolytech, France) and porcine pepsin.

Continuous timepoint samples were collected which were instantaneously inactivated by different techniques depending on the endpoint analysis.

2.3.1 Apparent protein molecular weight profiling

The apparent protein molecular weight profile of undigested and digested samples was determined by reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using a method previously described.[30] Gastric digestate samples collected for this analysis were preserved by raising the pH to 7.5 using 1 M NaOH (to minimize pepsin activity), while gastrointestinal digestates were preserved by addition of 50 µL of 0.1 M Pefabloc® stock per ml of digestate (to minimize trypsin activity) prior to freezing.[28] This concentration of Pefabloc® was used as the trypsin activity of the static infant digestion model used is 6.25 x lower than the static adult digestion model described by Brodkorb *et al.*, which recommends the addition of Pefabloc® at 5 mM.[31] A non-digested (ND) sample was used as a negative control with each independent gastric and gastrointestinal digestion trial. NDs were prepared by maintaining the pH at 7.5 to ensure that pepsin activity was minimized during the gastric phase, while for gastrointestinal digestion trials, NDs were prepared by pre-mixing porcine pancreatin with Pefabloc® before addition to the reaction vial to minimise trypsin activity.[28] Loading quantity for SDS-PAGE analysis was 6 µg protein/lane. Stained SDS-PAGE gels were scanned using a desktop flatbed scanner (HP Scanjet G4010, HP, Leixlip, Ireland), and densitometric analysis of the bands was carried out using Total-Lab Quant 13.1v software (TotalLab, Newcastle, UK).

Proteins were in-gel digested with trypsin,[32] and resulting peptides were analyzed with liquid chromatography tandem mass spectrometry as previously described[33] using

187 data-dependent acquisition over a 65-minute analysis time. Identification of proteins
188 was performed with Mascot (Matrix Sciences, London, UK) against the SwissProt
189 Bovine database (03/2021 release).

190

191 ***2.3.2 Degree of proteolysis***

192 The O-phthaldialdehyde (OPA) assay was used to determine the degree of proteolysis
193 at each collection timepoint following the modified method of Corrigan and
194 Brodkorb.[34] In brief, gastric and gastrointestinal digestates were inactivated by
195 addition of 24 % (w/v) trichloroacetic acid (TCA) at a volume ratio of 1:1 prior to
196 freezing. After defrosting, samples were centrifuged (4000 x g for 10 min) and 10 µL
197 supernatant (TCA-soluble fraction) or standard (L-leucine) were mixed with 200 µL
198 of OPA reagent. Photometric measurement at 340 nm was performed after a 15 min
199 incubation at 25°C using a Varioskan Flash microplate reader (Thermo Scientific,
200 MA, USA). The concentration of free amine groups was reported as L-leucine (mM)
201 equivalents.

202

203 **2.4. Cell culture assays to model intestinal cell inflammation**

204 HT-29 clone 34 cells (Nestlé Research, Lausanne, Switzerland) were used to assay for
205 activation of the NF-κB signalling pathway, as previously described.[28] HT-29 clone
206 34 cells were selected as a human intestinal epithelial cell model allowing to study the
207 effect of milk bioactive proteins (LF and OPN) on intestinal inflammation induced in
208 response to exposure to lipopolysaccharide (LPS). HT-29 clone 34 are derived from
209 the well-characterized HT-29 cells. HT-29 clone 34 cells were specifically designed
210 for monitoring the NF-kB signaling transduction pathway in a physiologically relevant

cell line. HT-29 clone 34 derived from the HT-29 cell line by stable integration of an NF-kB inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct. As a result, HT-29 clone 34 cells allow the monitoring of NF-kB activation by assessing the activity of SEAP. After cultivation at physiological conditions, cells were seeded at 2 x 10^5 cells/mL in 24-well plates (Greiner Bio-One, Austria) and cultured for 3 days prior to treatment to ensure monolayer confluence. After removal of culture medium, undigested or digested meals in fresh media were added to a final well volume of 1 mL at a concentration of 0.35 µg protein equivalent/mL, which accounted for the dilution factors applied during digestion or sample preservation. Samples for all treatments were filtered using 0.45 µm sterile filters prior to cell incubation. HMS was added at a concentration of 5 % (v/v) of the well volume to provide a source of effector molecules, including soluble CD14 (sCD14). LPS was added to all wells except negative controls, at a concentration of 20 ng/mL. Upon addition of treatments, cells were incubated for 16 h at 37°C and 5 % CO_2. As a measure of NF-κB activation, SEAP activity was quantified in cell culture supernatants by bioluminescence using Phosphalight kits, as per the supplier protocol (Applied Biosystems, MA, USA). Bioluminescence was quantified (according to manufacturer's instructions) as relative luminescence units (RLU) using a Varioskan Flash multiwell plate reader (Thermo Scientific, MA, USA) at an integration time of 1000 ms. For graphical representation of data, the LPS treatment (positive control) was set as 100 relative luminescence units, and all other data were normalized relative to this. A standardized neutral-red (40 µg/mL) uptake assay was used to rule out cytotoxicity.[28]

2.5. Mouse model for preclinical assessment of bone development

Animal studies used the descendants of wild-type B6.129 mouse breeding pairs (Charles River Laboratories Italia, Sant'Angelo Lodigiano, Italy). All experimental procedures were performed accordingly to NIH Guide for the Care and Use of Laboratory Animals, the internal Nestlé Animal Study Committee, the Directive 2010/63/ EU on the protection of animals used for scientific purposes and Italian law (Legislative Decree 26/2014) and were approved by Institutional Animal Survey Board on behalf of the Italian Ministry of Health (licence n. 548/2021-PR to SM).

2.5.1 Supplementation procedure

Mice (N=12 per treatment) were supplemented orally in two daily sessions from postnatal day (PND) 1 to PND 16. The meals were prepared as described in Section 2.2. In short, LF-OPN SC treatment was at 1250 mg/kg/day, LF-OPN CC treatment was at 1250 mg/kg/day and the mixed blend was treated as LF at 1000 mg/kg/day plus OPN at 250 mg/kg/day powder. The dam was separated from the pups for the duration of the session. Each pup received the respective meal individually by suckling the solutions from a mechanical pipette (Gilson Inc., Middleton, USA) that was gently placed on their lips. After the supplementation period, the experimental subjects were left undisturbed until weaning (PND 28). From PND 28 to 170 all mice received the same amount of a standard diet (*ad libitum* water and food pellets (Mucedola, Settimo Milanese, Italy)). At the end of the study, mice were rapidly decapitated, and femurs were collected as previously described[35] and stored at −80 °C until evaluation of bone health parameters.

2.5.2 Assessment of bone health parameters

Micro-computed tomography (μCT UCT35, Scanco Medical AG, Basserdorf, Switzerland) was used to assess trabecular and cortical microstructure, respectively, at distal metaphysis and midshaft diaphysis femur as previously described.[36] Briefly, trabecular and cortical bone regions were evaluated using isotropic 6 μm voxels. For the femur trabecular region, to eliminate the primary spongiosa, 30 slices of bone under the distal growth plate were not considered. The 80 slices of secondary spongiosa directly below were analysed. Femur cortical structure was assessed using 60 continuous CT slides located at the femur midshaft. Morphometric variables were computed from binarized images using direct, three-dimensional techniques that do not rely on prior assumptions about the underlying structure.[37]

For the trabecular bone regions, bone volume (BV, mm^3), tissue volume (TV, mm^3), and BV/TV fraction (%) and trabecular bone mineral density (Tb.BMD, mg HA/ccm) were assessed. For cortical bone at the femoral midshaft, the cortical BV and TV, as well as antero-posterior (AP) and medio-lateral (ML) diameters (mm) (**Figure 4**) were reported. A three-point bending test was performed to test the biomechanical properties of the femur as previously described.[38] The load was applied in a compression mode at a nominal deformation rate of 0.08 mm/s until fracture. Load-displacement curves were recorded to determine force yield (N) and stiffness (N/mm).

Blood sampling was performed on day 170 and allowed for analyses of serum OPN content as a measure of biodisponibility. Macrophage colony stimulating factor (M-CSF) and Pro-collagen type 1 N terminal (P1NP) were determined in plasma as a measure of bone resorption and bone formation, respectively. M-CSF and OPN were measured by the Luminex 200 assay (Bio-Techne AG, Zug, Switzerland) as per

supplier instructions. Pro-collagen type 1 N terminal (P1NP) was measured using the Mouse Procollagen Type 1 N-Terminal Propeptide ELISA Kit (Bio-Techne AG, Zug, Switzerland) as per supplier instructions.

2.6 Statistical analysis

Data are reported as mean ± standard error. To compare cell culture data to the LPS positive control, a one-way analysis of variance (ANOVA) was performed with a Dunnett's post hoc test using GraphPad Prism software. An unpaired t-test was used to compare the cell culture data of the LF treatment to the SC and CC treatments. For preclinical data, Mann-Whitney test was performed to compare treatment effects. Graph Pad Prism software was used to plot bone health results. Values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Simulated infant gastrointestinal digestion of LF, OPN, and LF-OPN soluble complexes or complex coacervates

LF was resistant to simulated infant gastric digestion (pH 5.3), whether presented alone or as SC or CC, and survived the gastric phase largely intact **(Figure 1)**. LF was subsequently hydrolysed in the intestinal phase of the model, regardless of the presence of OPN or the form of LF-OPN complex (i.e., SC or CC). Whether pre-complexed or not, the intact LF band (~75 kDa) in SDS-PAGE gels was hardly visible after 3 min of intestinal digestion with pancreatin and enzyme inhibition with Pefabloc®. LF was broken down to low molecular weight material (<10 kDa) and an intense ~41 kDa band, which was positively identified (by shotgun mass spectrometry) as a large LF proteolysis product. The OPN (~60 kDa) and OPN N-fragment (~40 kDa) bands were also resistant to gastric, but not intestinal digestion (**Supplementary Figure 1**). Gastric resistance of the proteins was also evident in measurements of free amine group concentration (**Figure 1**). The release of free amine groups in the gastric phase was negligible, whether LF was pre-complexed with OPN or not, while it correlated for all samples with the considerable intestinal proteolysis seen on SDS-PAGE gels. SC and CC digestates led to free amine group concentrations intermediate between LF and OPN digestates. By changing the pH of the gastric phase from 5.3 to 4.0, LF was more sensitive to pepsin proteolysis and was poorly visible in SDS-PAGE gels at the end of gastric digestion (**Figure 1**). Densitometric analysis of the gels revealed that LF proteolysis occurred faster with LF alone than pre-complexed (SC) or pre-coacervated (CC) with OPN (data not shown).

318 The experiments using the model of Ménard *et al.*[29], convey the mild conditions of the
319 infant stomach and the resistance of LF and OPN to gastric proteolysis (pH 5.3).
320 Previous studies on LF and OPN infant digestion have also described their resistance
321 to gastric, but not intestinal, digestion.[11,19,25,26,28,39,40,41,42] Although in the current
322 research, LF was sensitive to 1 h of infant gastric proteolysis at pH 4.0, the digestate
323 gels at 15 and 30 min concur with previous findings that reported a partial resistance
324 to gastric proteolysis at those early timepoints.[11,19,25,26] The presence of a ~48 kDa
325 pepsin-induced LF proteolysis product at pH 4.0 is also consistent with earlier work.[11]

326 The persistence of the ~41 kDa LF proteolysis product in the intestinal phase of our
327 simulated infant digestion may explain *in vivo* findings whereby small proportions of
328 dietary bovine LF have been detected in infant stool using ELISA techniques.[43,44] It is
329 likely that the ~41 kDa proteolysis product contains the epitope(s) specific for such
330 LF ELISA techniques, as it has been detectable using LF-specific western blot
331 analysis.[45] Dietary LF has also been detected in infant urine and plasma, which may
332 be explained by the proteolytic resistance of LF and the presence of LF-specific
333 receptors on enterocytes which facilitate LF uptake by endocytosis.[44,45] It should be
334 noted that human LF is more resistant to digestion than bovine LF, despite apo-LF
335 being less resistant than holo-LF.[45] This should result in greater persistence of intact
336 LF in breast-fed infants compared with formula-fed infants. The partial resistance of
337 LF to infant digestive conditions (*in vitro* models, preclinical models and clinical
338 findings) differs from adult LF digestion which results in more complete proteolysis
339 due to the more developed, harsher adult gastric conditions (i.e., higher enzymatic
340 activity, longer residence time, lower pH).[3,45] Such conditions have a lesser effect on
341 the digestive resistance of OPN, as it seemed to withstand adult gastric digestion at
342 pH as low as 2.[39,46,47] The resistance of OPN to digestion has been attributed to its

post-translational modifications which protect the disordered structure of OPN from proteolytic cleavage and is likely to be of biological significance as the integrin-binding regions of OPN survive *in vitro* gastric digestion (pH 2.5, 1 h) in an active form.[46] Furthermore, small amounts of dietary OPN have been detected in infant plasma using ELISA, indicating that it, at least partially (depending on epitopes), resisted digestion *in vivo* and was absorbed intestinally.[16]

The gastric resistance of LF and OPN to simulated infant digestion was expected, but it was hypothesized that pre-complexation (as in SC) or pre-coacervation (as in CC) of LF with OPN would enable LF to remain intact for longer during intestinal digestion. This has been suggested as a potential benefit of heteroprotein complex coacervation due to the shielding of enzyme-docking sites at the charged groups of globular proteins.[48] In addition, LF-based complex coacervates have previously been shown to modulate digestion kinetics, digestate peptide profiles, and bioactivity.[49] However, the intestinal phase persistence of SC or CC powders was not increased in the current study as both proteins were sensitive to proteolysis by pancreatic proteases. The extent of proteolysis was intermediate between LF and OPN, thereby not showing any signs of reduced proteolysis compared with un-complexed LF and OPN. The low concentration of free amine groups produced by OPN in the intestinal phase compared with LF, SC, or CC, was somewhat unexpected as all samples had the same undigested total protein concentration. The reason for this is likely two-fold as OPN is partially resistant to proteolysis, but also less efficiently precipitated by trichloroacetic acid (used during end point analysis) as it is an intrinsically disordered protein.[50] The delayed digestion kinetics of SC and CC when gastric pH was reduced to 4.0 warrant further investigation as the infant gastric pH is not constant during digestion but displays wide pH ranges due to differences in buffering between fasted ($pH \leq 3$) and

fed (pH ≥ 5) states.[29] The gastric pH of 5.3 and enzyme activities used in the current study represent averaged digestive parameters to mimic the postprandial state, based on the gastric emptying half-time of term infants.[29] However, continuous enzyme secretions and gastric emptying are not simulated in static digestion models, which are important when studying the relationship between protein structure and digestion kinetics.[31] Accounting for these conditions, and the different digestion fluid species origins could result in different outcomes. Further, the impact of complexation or complex coacervation with OPN on the LF digestate peptidome also remains unknown and merits further research.

3.2. Performance of undigested and digested LF, OPN, and LF-OPN soluble complexes or complex coacervates, in a model of human intestinal epithelial cell inflammation

LF, OPN, and combinations thereof, resulted in a statistically significant inhibition of LPS-induced NF-κB activation, when in an undigested, intact form at 0.35 mg protein/mL (**Figure 2**). The bioactivity of SC and CC was intermediate between individual LF and OPN treatments and both had a significantly different bioactivities compared with LF alone. The gastric digestates of LF, SC, and CC also caused a statistically significant inhibition of LPS-induced NF-κB activation (**Figure 2**), although with reduced bioactivity compared with their undigested equivalents. SC and CC gastric digestates also led to intermediate bioactivity between LF and OPN gastric digestates, and both were significantly more bioactive compared to LF gastric digestate alone. Gastrointestinal digestates of SC and CC both caused a significant inhibition of LPS-induced NF-κB activation while LF and OPN alone showed no

inhibitory effect (**Figure 2**). The inhibition of LPS-induced NF-κB activation by gastrointestinal digestates of SC and CC were not significantly different from each other but were when compared to the LF gastrointestinal digestate alone.

These results imply that the ability of LF to inhibit LPS-induced NF-κB activation requires that LF is not completely proteolyzed. It also demonstrates that digesting a pre-complexed or pre-coacervated LF-OPN powder resulted in an altered gastrointestinal digestate bioactivity in this model of intestinal epithelial cell inflammation. It should be noted that the inhibition of NF-κB activation measured in these experiments is not due to the impact of treating the cells with the undigested or digested proteins alone, as cell viability was maintained in all cases as demonstrated using a nuclear-red assay (data not shown).

The ability of intact LF to inhibit LPS-induced NF-κB activation in this model of human intestinal epithelial cells has previously been demonstrated, whereby LF was shown to have a dose-dependent impact.[28] The mechanism of action is attributable to the ability of LF to inhibit bacterial sensing by the cells.[28] LF achieves this by acting as a decoy which disrupts LPS binding to the toll-like receptor 4/myeloid differentiation protein 2 (TLR4–MD-2) pathogen-recognition-receptor complex expressed on the cell surface, thereby ceasing the intracellular signalling cascade, causing NF-κB activation.[28,51,52] This decoy effect occurs due to the known electrostatic interaction between LF and the lipid A moiety of LPS, the LPS-binding protein (LBP), CD14, or the LPS/CD14 complex. In this model of human intestinal epithelial cell inflammation, intact OPN was also shown to inhibit LPS-induced NF-κB activation in a dose-dependent manner (data not shown), although the mechanism of action is unknown. This finding supports a recent *in vivo* study showing that milk

OPN reduced inflammation in mouse pups injected with the same *Escherichia coli* LPS serotype O111:B4 as used in the current study.[17]

The bioactivity of gastric digestates of LF, SC, and CC was unsurprising due to the high levels of resistance to gastric digestion displayed by both LF and OPN. The reduced bioactive efficacy in gastric digestates compared with undigested LF, SC, and CC, may be related to the presence of other anionic molecules (e.g., gastric lipase, pepsin) during the gastric phase, which reduces the levels of unbound LF available to interact with, and disrupt, LPS. The lack of inhibition of LPS-induced NF-κB activation observed with gastrointestinal digestates of LF suggests that the ~41 kDa LF intestinal proteolysis product detected in the proteolysis assays was not sufficient to maintain the LPS disrupting activity of intact, undigested LF. In contrast, the significant inhibition of LPS-induced NF-κB activation by the gastrointestinal digestates of both SC and CC suggests that the peptides released during gastrointestinal digestion of SC and CC were different to that of LF and OPN alone.

Various studies reported that *in vitro* bioactivities of LF are associated with intact, undigested LF.[4,6,28] While important in understanding the mechanisms of LF bioactivity, and how various experimental changes can modulate the bioactivity, the physiological relevance of such research needs to be considered due to the structural transformations which occur to proteins during digestion. Where possible, it should be determined if the bioactivity associated with intact LF is maintained during digestion. During early lactation, human milk LF concentrations are typically highest while infant gastrointestinal tract maturity is lowest, meaning that the intact LF concentration in the upper gut may be highest during this period. Bioactivity of LF *in vivo* might combine LF and LF digestion products, so understanding both would be beneficial.[4,6,53,54] Two well studied peptides of LF, lactoferricin and lactoferrampin,

441 are known to have potent anti-bacterial activity.[51] Other functions of LF peptides
442 reported in the milk-bioactive-peptide-database (MBPD) include ACE-inhibitory,
443 DPP-IV inhibitory, and osteoanabolic activities.[55]

444

445 **3.3. Impact of the LF-OPN complexation on bone development, growth, and**
446 **strength**

447 In the preclinical healthy rodent study, all mice showed similar weight development
448 during the supplementation period and beyond. Overall, mice that were supplemented
449 with the LF-OPN CC showed improved bone development (quality & strength)
450 compared to mice supplemented with other forms of LF-OPN (SC or mixed blend).
451 The LF-OPN CC supplemented mice had higher tissue volume (TV) and bone volume
452 (BV) (**Figure 3AB**), and a significant (21.7 %) increase of trabecular BV/TV
453 compared to mice which were supplemented with LF-OPN SC (**Figure 3C**).
454 Supplementation with a LF-OPN mixed blend also increased BV/TV compared with
455 LF-OPN SC but to a lower extent. Trabecular bone mineral density (**Figure 3D**) was
456 also highest in the LF-OPN CC group. Similar results were obtained in cortical bone
457 in terms of TV and BV (**Figure 4AB**). Antero-posterior diameter and medio-lateral
458 diameter were larger in mice supplemented with LP-OPN complexes compared to
459 control (**Figure 4CD**).

460 LF-OPN CC supplementation of mice resulted in a 3.6 and 11.3 % increase in force
461 yield and stiffness of bones, respectively (**Figure 5AB**). Figures 3E and
462 Supplementary Figure 2 show exemplary trabecular and cortical structures,
463 respectively, after supplementation with the two different LP-OPN complexes vs
464 soluble as obtained by micro-CT.

Blood levels of OPN were highest in mice supplemented with LF-OPN CC. The difference in OPN levels reached statistical significance when compared with the LF-OPN mixed blend supplementation (**Figure 5C**). Similarly, the blood level of both M-CSF and P1NP was significantly higher in LF-OPN CC compared to LF-OPN mixed blend supplemented mice (**Figure 5DE**). These results demonstrated that mice supplemented with the LF-OPN CC had significantly higher microarchitecture trabecular and cortical parameters compared to soluble, suggesting a better bone development and quality. Blood levels of the biomarkers P1NP and M-CSF further indicated that the bone effects of LF-OPN CC are due to an increased bone turnover in favour of bone formation (+466%) versus bone resorption (+125%), respectively.

Both LF and OPN have previously been associated with bone homeostasis and/or bone remodelling in preclinical trials.[56,57.] As aforementioned, the form in which the proteins are consumed is an important consideration prior to translating preclinical findings into physiologically relevant hypotheses but also prior to planning clinical trials. The primary reason for such considerations is due to the impact of gastrointestinal digestion on the proteins and the subsequent absorption of the proteins and/or their breakdown products. In this research, it was hypothesized that pre-complexation or pre-coacervation of LF and OPN could result in more intact proteins persisting during gastrointestinal digestion and increasing the levels of the proteins available to be absorbed in intact form by transcytosis. Although the digestion (reported in Section 3.1) and gut model findings (reported in Section 3.2) were *in vitro*, mechanistically they can help understand why altered bone development was observed. The methods used for evaluation of bone quality were well established and included techniques for characterization of bone mechanical properties and microarchitecture.[58] Normal bone growth and/or strength is typically determined using

individual or combined bone parameters. The resulting preclinical assessment of different parameters relating to bone health of infant mice revealed advantages of pre-coacervation of LF with OPN as opposed to pre-mixed or pre-complexed forms of these proteins.

While the present work cannot clarify whether the beneficial effect on bone quality is due to LF or OPN, serum levels of OPN in the mice were higher after LF-OPN CC supplementation than other forms suggesting good bioavailability of OPN from this type of complex. The *in vitro* digestion and gut model findings can help explain why the fate and function of the proteins differs when presented in complex form. It should also be noted that given that biological sampling was performed in adult mice (170 days old) after the intervention at pre-weaning stage (from day 1 to 16), these results indicate a long-term effect.

4. Conclusions

This research studied the effect of LF-OPN complexation and complex coacervation on the simulated infant gastrointestinal digestion of LF. Their bioactive properties were subsequently examined in two preclinical models, an *in vitro* model of intestinal inflammation and a neonatal rodent model to assess bone development. In the static digestion model used, the proteolytic profile of LF was unaltered by the pre-association of LF and OPN. LF gastric proteolysis was increased when the model gastric pH was reduced from 5.3 to 4.0, but less so when complexed with OPN. In an *in vitro* model of intestinal inflammation, undigested LF-OPN complexes inhibited LPS-induced NF-κB activation in epithelial cells in-between that of LF and OPN alone. Undigested and gastric digestates of LF, but not gastrointestinal digestates, also

514 inhibited LPS-induced NF-κB activation in this model. Interestingly, complexation
515 with OPN sustained the LF inhibitory capacity after gastrointestinal digestion unlike
516 un-complexed LF, suggesting that the peptides produced were different. In a neonatal
517 rodent model, differences were detected between the bioactive impact of LF-OPN
518 complexes on bone development whereby coacervating the proteins demonstrated
519 improved bone trabecular and cortical structure. Feeding the proteins in coacervate
520 format also increased the levels of OPN, P1NP and M-CSF in blood, signifying a more
521 pronounced impact on bone development. The findings from the *in vitro* digestion and
522 intestinal inflammation models help explain why the fate and function of the proteins
523 differs when presented in complex form. **This research demonstrated that LF-OPN**
524 **complexation and complex coacervation can delay simulated infant**
525 **gastrointestinal digestion of LF, and protect or improve the bioactivity of the**
526 **proteins, depending on the target function.** These preclinical insights merit further
527 investigation to assess if presenting bioactive proteins such as LF and OPN in complex
528 coacervate form can be used as a strategy to improve infant health.

529

530 **Conflicts of interest**

531 The authors have no known conflicts of interest to declare.

532

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546 **Figure legends**

547 **Figure 1**. Proteolysis of uncomplexed and complexed lactoferrin (LF) and osteopontin
548 (OPN) during simulated infant gastrointestinal digestion (A) at pH 5.3 (gastric) and
549 pH 6.6 (intestinal) whereby presented is the concentration of free amine groups (as
550 mM L-leucine equivalents) in the supernatants of trichloroacetic acid-precipitated
551 digestates, as measured using the o-phthaldialdehyde (OPA) assay. The legend is
552 annotated on the graph. Panel (B) presents the proteolysis of uncomplexed and
553 complexed LF and OPN during simulated infant gastric digestion at pH 4.0. The
554 apparent molecular weight profile of simulated infant gastric digestates of LF (left),
555 LF-OPN soluble complexes (SC, middle) and LF-OPN complex coacervates (CC,
556 right), as analysed using reducing SDS-PAGE. Loading quantity was 6 μg
557 protein/lane. Each lane contains a digestate collected at a specific timepoint (in
558 minutes) during simulated gastric (G) digestion, which is annotated at the bottom of
559 each lane. ND is the non-digested sample.

560

561 **Figure 2.** The impact of **(A)** undigested, **(B)** gastric (digestion performed at pH 5.3)
562 digestates or **(C)** gastrointestinal (intestinal digestion performed at pH 6.6) digestates
563 of lactoferrin (LF), osteopontin (OPN), LF-OPN soluble complexes (SC) or LF-OPN
564 complex coacervates (CC) in an *in vitro* model of human intestinal epithelial cell
565 inflammation. HT-29 clone 34 cells were treated with LPS (20 ng/mL) in the absence
566 or the presence of the experimental samples (0.35 mg protein equivalent/mL) for 16 h
567 at 37°C. NF-kB activation was evaluated by measuring SEAP activity in cell culture
568 supernatants. Data were normalized to give 100 relative luminescence units (RLU) for
569 the LPS treatment. Data are shown as mean values ± standard error of triplicate

570 measurements from three independent experiments. '*' and 'b' represent statistically
571 significant differences ($P < 0.05$) from the corresponding LPS and LF ('a') treatments,
572 respectively.

573

574 **Figure 3.** The impact of lactoferrin-osteopontin soluble complexes (SC), lactoferrin-
575 osteopontin complex coacervates (CC), and a mixed blend of uncomplexed lactoferrin
576 and osteopontin (LF & OPN mix) on trabecular bone structure. (A) trabecular tissue
577 volume (TV, mm^3), (B) trabecular bone volume (BV, mm^3), (C) trabecular BV/TV
578 fraction (%), (D) trabecular bone mineral density (Tb.BMD, mg HA/ccm), (E) micro-
579 CT image of bone trabecular structures following supplementations. Data are shown
580 as mean values ± standard error of triplicate measurements. '*' represents statistically
581 significant differences ($P < 0.05$) between treatments, other p-values compare CC to
582 SC as indicated.

583

584 **Figure 4.** The impact of lactoferrin-osteopontin soluble complexes (SC), lactoferrin-
585 osteopontin complex coacervates (CC), and a mixed blend of uncomplexed lactoferrin
586 and osteopontin (LF & OPN mix) on cortical bone structure. (A) cortical tissue volume
587 (BV, mm^3), (B) cortical bone volume (BV, mm^3), (C) cortical antero-posterior
588 diameter (AP, mm), (D) medio-lateral diameters (ML, mm). Data are shown as mean
589 values ± standard error of triplicate measurements. '*' represents statistically
590 significant differences ($P < 0.05$) between treatments, other p-values compare CC to
591 SC or to LF & OPN mix, as indicated on the figure.

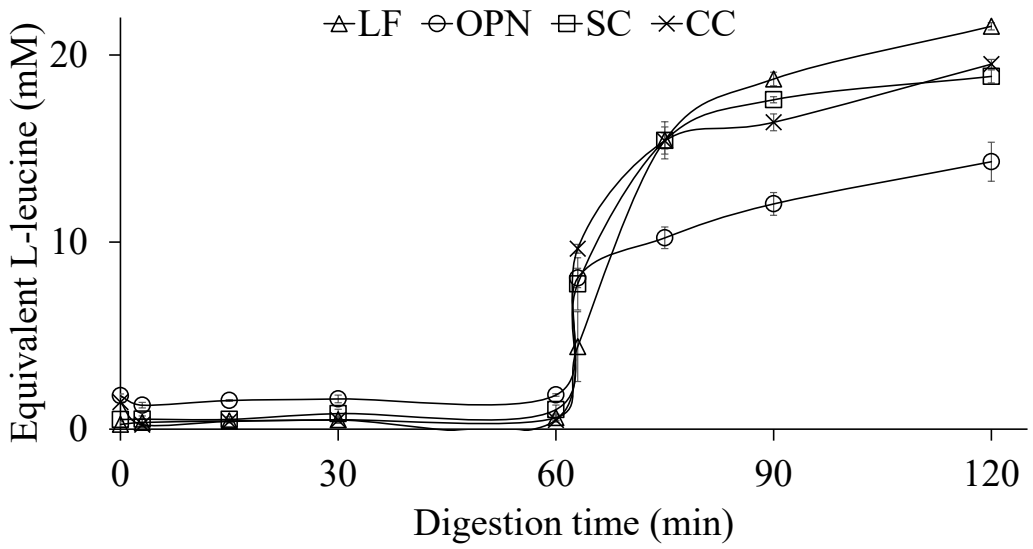
592

Figure 5. The impact of lactoferrin-osteopontin soluble complexes (SC), lactoferrin-osteopontin complex coacervates (CC), and a mixed blend of uncomplexed lactoferrin and osteopontin (LF & OPN mix) on the biochemical properties of bones and blood biomarkers of bioactivity and bone turnover. (A) Bone force yield (N) and (B) bone stiffness (N/mm) from a three-point bending test of the femur; (C) serum OPN levels (pg/ml) obtained by Luminex 200 assay, (D) Macrophage colony stimulating factor (M-CSF) blood levels (pg/ml) measured by Luminex 200 assay (E) Pro-collagen type 1 N terminal (P1NP) blood levels (pg/ml) measured by ELISA assay. Data are shown as mean values ± standard error of triplicate measurements. '*' represents statistically significant differences ($P < 0.05$) between treatments, other p-values compare CC to SC or to LF & OPN mix, as indicated on the figure.

Figures

Figure 1

A



B

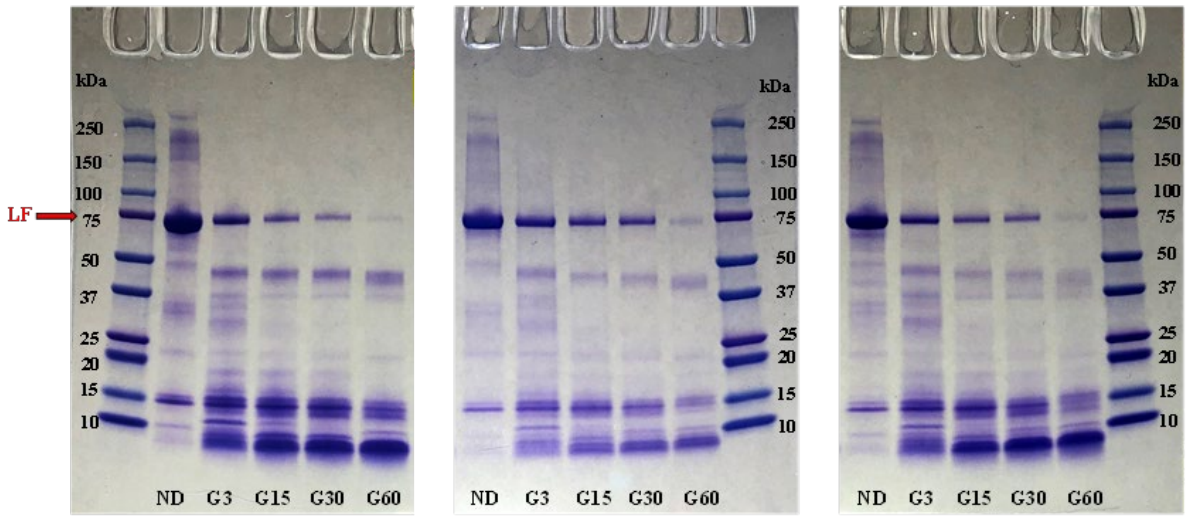


Figure 2

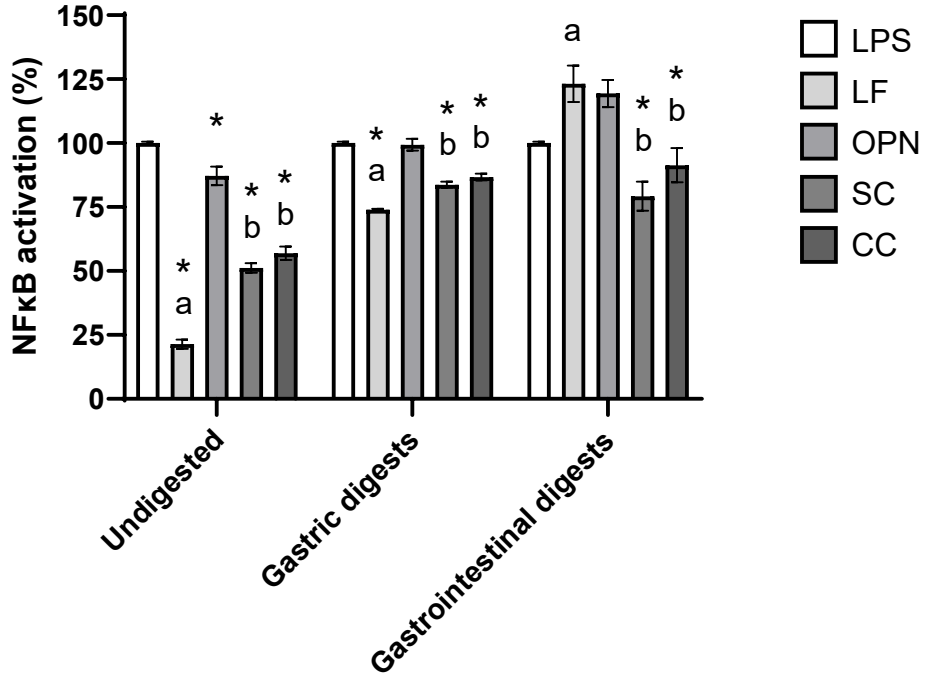

NFκB activation (%)
150
125
100
75
50
25
0
Undigested
Gastric digests
Gastrointestinal digests
LPS
LF
OPN
SC
CC

Figure 3

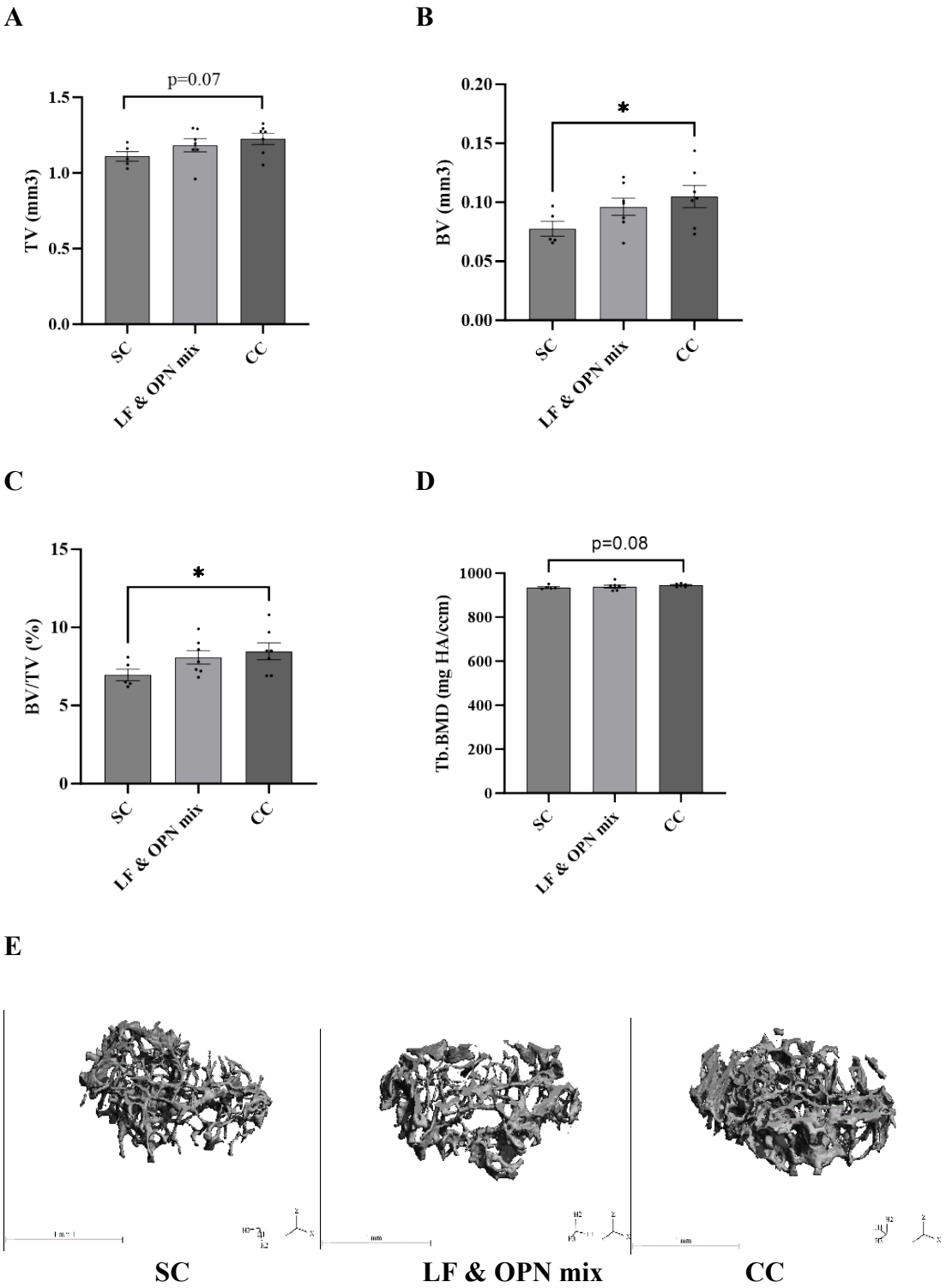

A
p=0.07
TV (mm3)
1.5
1.0
0.5
0.0
SC
LF & OPN mix
CC
B
*
BV (mm3)
0.20
0.15
0.10
0.05
0.00
SC
LF & OPN mix
CC
C
*
BV/TV (%)
15
10
5
0
SC
LF & OPN mix
CC
D
p=0.08
Tb.BMD (mg HA/ccm)
1000
800
600
400
200
0
SC
LF & OPN mix
CC
E
SC
LF & OPN mix
CC

Figure 4

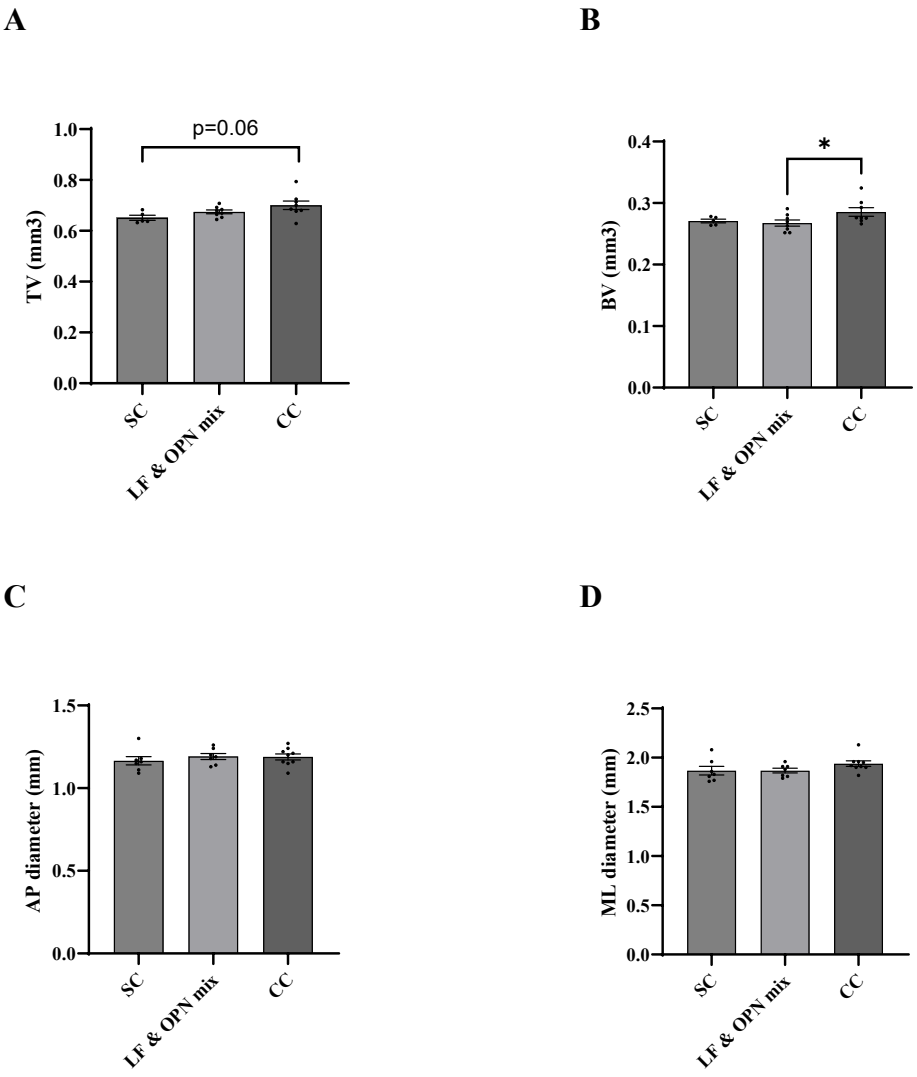
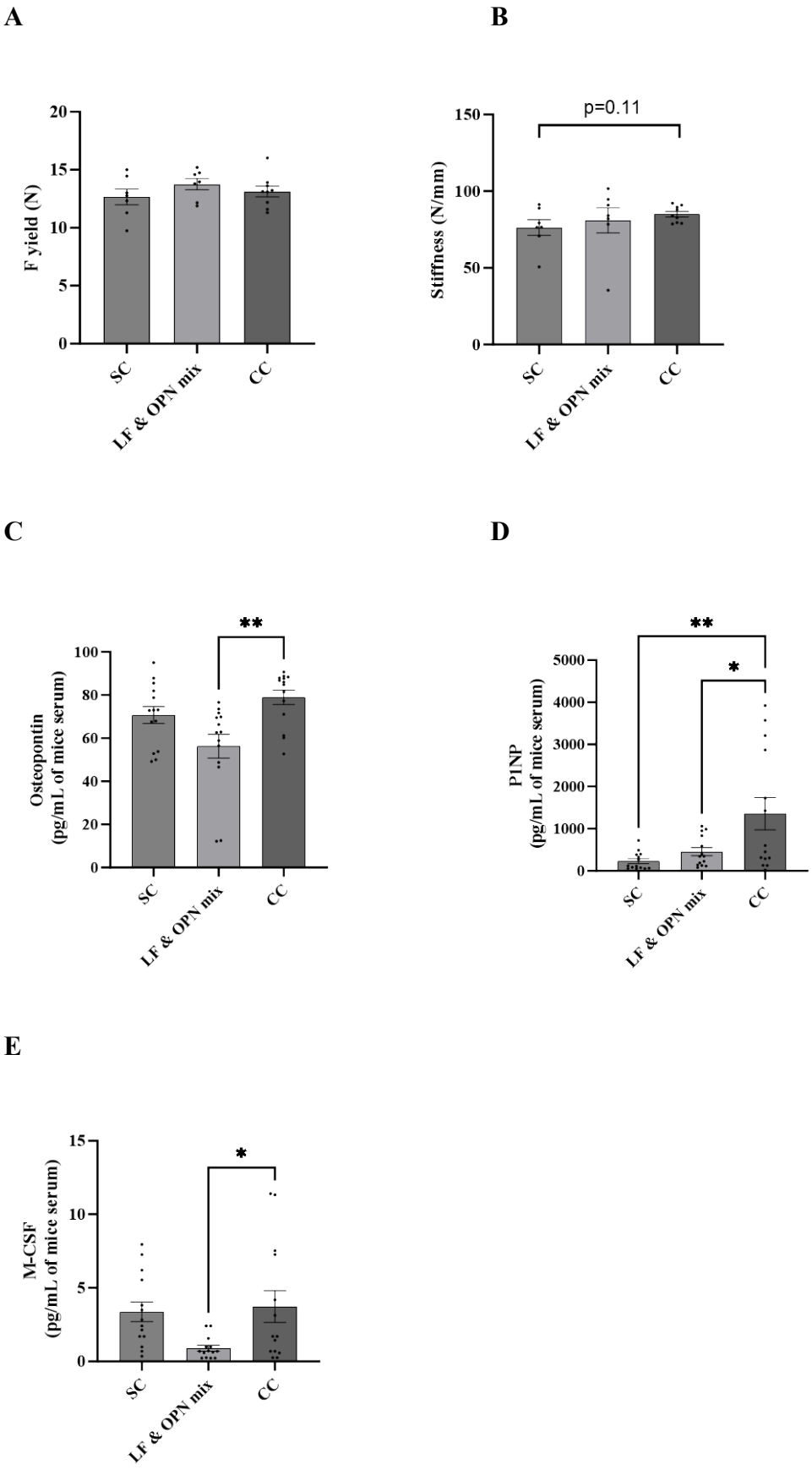

A
B
C
D
p=0.06
*
TV (mm3)
BV (mm3)
AP diameter (mm)
ML diameter (mm)
SC
LF & OPN mix
CC

Figure 5


A
F yield (N)
20
15
10
5
0
SC
LF & OPN mix
CC
B
Stiffness (N/mm)
p=0.11
150
100
50
0
SC
LF & OPN mix
CC
C
Osteopontin (pg/mL of mice serum)
**
100
80
60
40
20
0
SC
LF & OPN mix
CC
D
P1NP (pg/mL of mice serum)
**
*
5000
4000
3000
2000
1000
0
SC
LF & OPN mix
CC
E
M-CSF (pg/mL of mice serum)
*
15
10
5
0
SC
LF & OPN mix
CC

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