Characterising the responsiveness of mesenchymal stem cells to arginine, leucine and IGF-1 via the mTORC1 signalling pathway.

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Abstract

As our population ages, diseases of low bone mass, including osteopenia and osteoporosis, are increasing in prevalence. In the elderly, low bone mass is a significant risk for fragility fractures, associated with increased morbidity and mortality. Age-related bone loss is thought to occur due to an imbalance between bone-forming osteoblasts and bone-resorbing osteoclasts as a result of decreased osteoblast differentiation from mesenchymal stem cells (MSCs). Current treatments for low bone mass are limited, where most interventions slow the rate of bone loss rather than rebuilding bone mass. Increasing amino acid (AA) intake through a high protein diet is associated with increased bone formation and decreased fracture risk, thought to occur through increased IGF-1, a potent bone anabolic factor. The mTORC1 complex is a major anabolic signalling pathway, responsive to arginine, leucine and IGF-1. Previous studies have established that mTORC1 dysfunction in osteoblasts causes low bone mass and skeletal fragility in genetic mouse models. The aim of this study was to investigate if AAs (arginine and leucine) and IGF-1 promote bone mass by stimulating osteoblast differentiation in human MSCs via the mTORC1 pathway. AAs and IGF-1 act synergistically to stimulate mTORC1 function in human MSCs. Moreover, under optimised conditions, high levels of these AAs stimulate mTORC1-dependent expression of osteogenic gene markers, *ALPL* (High AA vs. High AA + Rapamycin p=.0048) and *BGLAP*. Characterising the mTORC1-dependent effects of AAs and IGF-1 on osteogenesis, and therefore bone formation, could direct dietary guidelines and identify novel drug targets for treatment of low bone mass.

Word count: 250

Introduction

Diseases characterised by low bone mass, such as osteopenia and osteoporosis, are increasing in prevalence globally, consistent with a growing aged population and a shift towards a more sedentary lifestyle.[1] Low bone mass is associated with significantly higher risk of fractures[2] and considerable loss of quality of life due to disability and loss of independence[3], as well as mortality when compared to age-matched populations.[4] Osteoporosis, osteopenia and associated fractures also impact the Australian economy where an approximately threefold increase in spending was observed between 2007 and 2017, which is only expected to increase in future years.[5]

Age-related bone loss occurs due to an imbalance between the activity of bone-resorbing osteoclasts and bone-forming osteoblasts.[6] Osteoblasts are derived from multipotent mesenchymal stem cells (MSCs), a population that also give rise to several other cell types including adipocytes. The differentiation toward osteoblasts and adipocytes is a mutually exclusive process, spatiotemporally controlled by lineage-specific transcription factors.[7] Adipocyte generation is primarily regulated by transcription factors proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding proteins (C/EBPs) while osteoblast differentiation is controlled through Runt-related transcription factor 2 (RUNX2) and OSTERIX (SP7) expression.[8] With age, there is a shift in MSC differentiation favouring adipocyte formation resulting in a corresponding decrease in osteoblast differentiation and osteoblast numbers[8], thought to be a significant driver of age-related bone loss.[9] Stimulating an increase in osteoblast populations and/or function could mitigate age-related bone loss.

Treatments for bone loss are currently limited, with supplementation of calcium and vitamin D commonly recommended as they reduce bone loss and incidences of non-vertebral fractures in older individuals.[10] Drugs used for the treatment of osteoporosis are also limited with bisphosphonates being the primary therapy. Both interventions act to slow the rate of bone loss, however, patients will continue to deteriorate under these regimes.[11] Anabolic therapies associated with these conditions have suffered drawbacks, particularly due to the sensitivity of osteoblasts to oxidative stress.[8]

Parathyroid hormone and related protein analogues are currently the only anabolic osteoporosis therapy, however, is recommended only to high-risk patients due to the possible risk of osteosarcoma, observed in some animal models.[12] The lack of safe and effective anabolic therapies for long-term treatment represents a clinical need for therapies targeted to increasing bone mass to lessen the incidence and impacts of low bone mass-associated skeletal fragility fractures.[11]

High dietary protein intake has been shown to promote bone anabolism and decrease the risk of fracture.[13] Moreover, protein supplementation has been found to be protective against age-related bone loss while protein under-nutrition is associated with decreased bone mass.[14] A longitudinal, population-based study in aged individuals has also found that lower protein intake is significantly correlated with bone loss.[15] Moreover, individuals with higher dietary protein intake, over the course of the study, maintained their bone mineral density, even after controlling for major confounding factors such as weight, smoking status and alcohol intake.[15] In hip fracture patients, increased protein intake resulted in a reduction in post-surgical bone loss and time spent in hospital[16] as well as promoting better overall recovery.[14] Mechanistically, a positive correlation has been established between dietary protein intake and insulin-like growth factor 1 (IGF-1) levels, indicating that the relationship between protein intake and bone mass may be controlled through IGF-1.[17]

IGF-1 is a potent bone anabolic growth factor that directs MSCs towards an osteogenic cell fate.[18] IGF-1 levels positively correlate with bone mass and bone strength[14] therefore, circulating IGF-1 levels can be used as an informative clinical biomarker, particularly in the monitoring of osteoporotic patients. IGF-1 is synthesised and secreted primarily by the liver[19] but the skeleton[20] has also been identified as a significant source. Matrix-associated IGF-1 has been shown to stimulate osteoblast differentiation from MSCs as knockout of the IGF-1 receptor in pre-osteoblasts resulted in lower bone mass compared to wild-type mice.[20] Hepatic IGF-1 production has been shown to be stimulated in response to amino acids (AAs).[19] Moreover, AAs have been shown to improve bone mineral density[21], with arginine in particular shown to increase IGF-1 production by osteoblast-like cells[22]

and cultured human osteoblasts from healthy[23] and osteopenic[24] populations, inferring that increases in bone mass, due to amino acids, may occur through increased IGF-1 production.

The bone anabolic activity of IGF-1 is mediated by the mechanistic target of rapamycin complex 1 (mTORC1). mTORC1 is the primary nutrient sensor that also responds to other signals to control cell growth and proliferation.[25] IGF-1 activates mTORC1 via the tuberous sclerosis complex (TSC) (Fig. 1). Recently, a second arm of the pathway has been found to respond to AA stimuli. In particular, mTORC1 is activated by arginine and leucine[26, 27], regulated by the CASTOR and Sestrin proteins respectively[28] (Fig. 1).

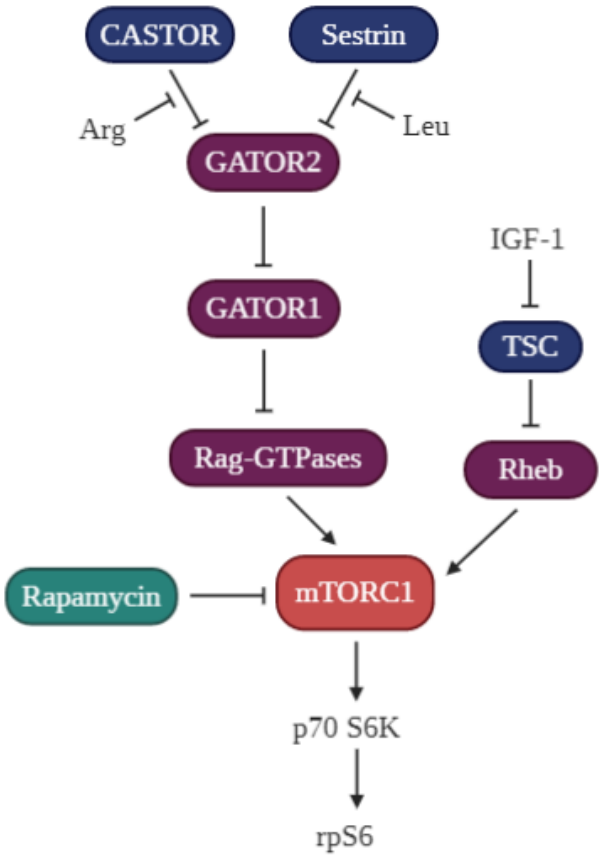

Figure 1: Schematic of IGF-1 and AA-dependent facets of the mTORC1 signalling pathway. mTORC1 is activated in response to arginine and leucine through the Rag-GTPases and IGF-1 through Rheb, and inhibited by rapamycin. Activated mTORC1 drives a signal transduction cascade (via p70 S6K) leading to activation of ribosomal protein S6 (rpS6) to control ribosome biogenesis and translation.[25]

There is a growing body of evidence to suggest that mTORC1 signalling plays a significant role in bone formation. Mice with conditional deletion of either *mTOR* or *Rptor*, components of the mTORC1 complex, in pre-osteoblasts results in impaired osteogenesis, lower bone mass and skeletal fragility compared to controls.[29, 30] Mechanistically, loss of mTORC1 function in osteoblasts leads to a reduction in protein synthesis[29] and downregulation of *Runx2* through phosphorylation of Estrogen Receptor α.[30] Taken together, these results suggest that IGF-1 and AA dependent activation of the mTORC1 pathway may play a critical role in the bone anabolic action of dietary proteins.

A high protein diet has been shown to increase bone mass however, the mechanism by which this occurs is currently not fully known.[1] Different branches of the mTORC1 signalling pathway respond to IGF-1 and the AAs arginine and leucine[31], and inhibition of the mTORC1 complex inhibits IGF-1-mediated osteoblast differentiation.[29] Together, these findings suggest that the mTORC1 complex in MSCs plays a role in directing osteoblast differentiation in response to IGF-1, arginine and leucine. Understanding the mechanism by which this occurs will contribute to creating a stable knowledge foundation to facilitate the development of safe and effective bone anabolic therapies.

This has led to the following hypothesis: An increase in the dietary AAs arginine and leucine stimulates bone mass by directing MSC osteogenic differentiation via IGF-1 and the mTORC1 pathway. This question will be investigated in the following aims:

Aim 1: To demonstrate that AAs, arginine and leucine, and IGF-1 stimulate mTORC1 activity in human MSCs.

Aim 2: To determine if arginine and leucine work synergistically with IGF-1 to promote osteogenesis via an mTORC1-dependent pathway.

Materials and Methods

Reagents

All reagents were purchased from Sigma Aldrich unless otherwise specified.

Subjects and cell culture

Human MSC cultures, previously isolated from bone chips recovered from the posterior iliac crest of healthy adult volunteers, were cultured to passage 3 in α-modified MEM, supplemented with 10% foetal calf serum (FCS), 0.1 mM Ascorbate-2-Phosphate and additives (2 mM L-glutamine, 100 μg/mL Streptomycin, 100 U/mL Penicillin, 1 mM Sodium pyruvate, 15 mM HEPES), subsequently referred to as growth medium.

UE7T-13 cells, an immortalised human mesenchymal stem cell line over-expressing human telomerase reverse transcriptase (hTERT) and Human papillomavirus transforming protein E7, were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan) and cultured in growth medium.

All cells were cultured in a humidified incubator with 5% CO_2 at 37ºC and expanded (1:3) every 3 days.

Cell treatments

Cells were treated with insulin, IGF-1, arginine, leucine or a combination thereof. Physiological reference values for the concentration of insulin was selected based on Li *et. al.* 2019[32]; IGF-1 on Zhu *et. al.* 2017[33] and arginine and leucine on Trabado *et. al.* 2017.[34]

Cell viability

Cell viability was assessed by measuring DAPI (4',6- Diamidino-2-Phenylindole, Dihydrochloride) uptake by flow cytometry. Briefly, treated UE7T-13 cells were trypsinised, pelleted at 400 x *g* and resuspended in flow assay buffer (phosphate buffered saline, 1% FCS, 20mM HEPES and 5mM

EDTA, pH 7.2). DAPI was added to a final concentration of 0.2 μM 10 minutes prior to analysis by flow cytometry (BD FACSCanto™ II Cell Analyzer, BD Biosciences, USA).

Western blot analysis

UE7T-13 cells were seeded onto 60 mm dishes at $3x10^5$ cells in 3 mL of growth medium overnight to allow for adhesion. Cells were then washed with 2 mL of DMEM Ham's F-12 without Arginine, Leucine and Lysine with 0.1 mM Ascorbate-2-Phosphate and additives (referred to as nutrient deplete medium) and starved in 3 mL of nutrient deplete medium for between 24 and 72 hours. Cells were pulsed with indicated factor(s) prior to lysis in 100 μl of modified radioimmunoprecipitation assay (RIPA) buffer as previously described.[29] Total protein (25 μg) from each sample was resolved by 11% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred at 100V for 1 hour onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% (w/v) bovine serum albumin (BSA) in 1X Tris-buffered saline containing 0.1% Tween-20 and 0.05% sodium azide (TBS-T) for >1 hour, immunoblotting was performed using the antibodies described (Table 1). Membranes were imaged using LI-COR Odyssey CLx imaging system and Image Studio Lite software (version 5.2, LI-COR).

Table 1: Antibodies used for immunoblotting.

Type	**Manufacturer**	**Catalogue Number**	**Name**	**Dilution**
Primary	Cell Signaling Technology	#2215	Rabbit anti-phospho-ribosomal protein S6 at serine 240/244 (pS6 (S240/244),	1:1000
Secondary	Thermo Fisher	#SA5-35571	Goat anti-rabbit IgG (H+L) Secondary Antibody, DyLight 800 4X PEG	1:20000
Primary	Abcam	#ab6160	Rat anti-tubulin YL1/2 (α-tubulin)	1:2500
Secondary	Thermo Fisher	#SA5-10022	Goat anti-rat IgG (H+L) Cross-Adsorbed Secondary Antibody, DyLight 680	1:20000

Differentiation of MSCs

Primary human MSCs (n=3) were seeded into flat-bottomed 96-well plates at $8x10^3$ cells/well in growth media and allowed to adhere overnight. For induction, media was changed to osteogenic media (DMEM low glucose (#D9443) with 5% FCS dialysed as per manufacturer's instructions (Fisher Scientific), 0.4 mM Lysine-HCl, 2.64 mM KH_2PO_4, 0.1 μM dexamethasone sodium phosphate (Mayne Pharma Pty Ltd, South Australia, Australia), 0.1 mM Ascorbate-2-Phosphate and additives supplemented with either: 5 ng/mL IGF-1 (-AA); 10 μM arginine, 10 μM leucine and 5 ng/mL IGF-1 (Low AA); 150 μM arginine, 200 μM leucine and 5 ng/mL IGF-1 (High AA) or 150 μM arginine, 200 μM leucine, 10 nM rapamycin and 5 ng/mL IGF-1 (High AA + Rapamycin). Media was changed twice weekly for up to 4 weeks.

Mineral Quantitation

Mineral quantitation was performed using Calcium Arsenazo III, after solubilising mineral in 0.6 M HCl overnight at 4°C and normalised to total DNA content using fluorometric DNA quantitation (Invitrogen Quanti-iT Pico Green dsDNA Assay Kit).

RNA Extraction and Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

MSCs were seeded into 12 well plates at $6x10^5$ cells/plate and cultured under conditions specified in Differentiation of MSCs. RNA was extracted with 1mL/well TRIzol (Invitrogen) as per manufacturer's instructions (Thermo Fisher). Isolated RNA was quantitated by Nanodrop 8000 spectrophotometer (Thermo Fisher). 1 μg of total RNA was reverse transcribed into complementary DNA (cDNA) using random hexamers and SuperScript IV Reverse Transcriptase (Invitrogen) as per manufacturer's instructions (Thermo Fisher). PCR reactions were performed with gene specific primers, outlined in Table 2, and SYBR® Green using BioRad CFX 9000qPCR machine (BioRad, Hercules, CA, USA). Gene expression, relative to the housekeeping gene β-actin, was determined by the $2^{-\Delta Ct}$ method.[35]

Table 2: Gene-specific primer pairs for qRT-PCR.			
Gene	**Accession number**	**Forward (5' – 3')**	**Reverse (5' – 3')**
ALPL	NM_000478	GTTCCCGGTGCAACACCAC	CTCGTTGTCTGAGTACCAGTCCC
BGLAP	NM_199173	ATGAGAGCCCTCACACTCCTCG	GTCAGCCAACTCGTCACAGTCC
ACTB	NM_001101	GATCATTGCTCCTCCTGAGC	GTCATAGTCCGCCTAGAAGCAT

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0. Values reported as mean ± standard deviation (SD) or standard error of the mean (SEM). Data were analysed using One-way ANOVA with Tukey's post-hoc test. P-values less than 0.05 were considered statistically significant.

Ethical Conduct of Research

In vitro experiments were conducted in accordance with SAHMRI ethics SAM282 and primary human MSCs were obtained from healthy adults following informed consent in accordance with procedures approved by the Royal Adelaide Hospital Ethics Committee.

Results

Activation of the mTORC1 complex in response to arginine, leucine and IGF-1 in MSCs is poorly described. To investigate this, an immortalised human MSC cell line, UE7T-13, was used as a model system. These cells express all the cell surface markers indicative of an immature MSC including STRO-1, STRO-3, STRO-4, CD73, CD90, CD105, CD106 and CD166.[36] To measure mTORC1 activation, phosphorylation of rpS6, a downstream effector of mTORC1, was used.

UE7T-13 cells remain viable after starvation of arginine, leucine, lysine and FCS for 72 hours.

To observe differences between basal and stimulated rpS6 phosphorylation, it was important to reduce basal levels by starving the cells. To optimise the starve duration of UE7T-13 cells, cultures were starved of arginine, leucine, lysine and FCS for 24, 48 or 72 hours. A starve duration of 72 hours showed the greatest reduction in basal rpS6 phosphorylation (Fig. 2A-C), without affecting cell survival (Fig. 2D). Therefore, all subsequent pulse experiments were conducted following a 72-hour starvation.

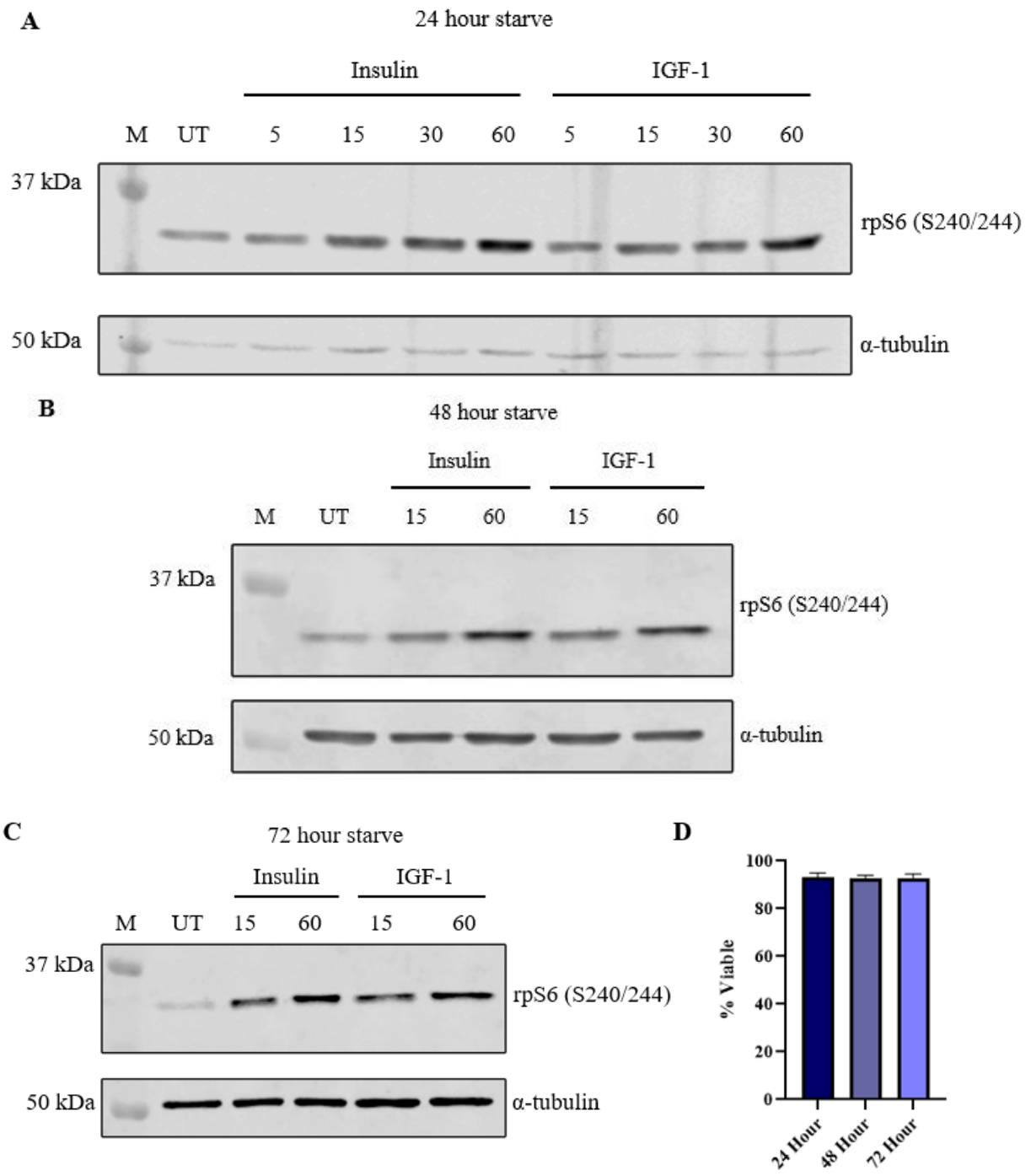

Figure 2: UE7T-13 cells remain viable after starvation of arginine, leucine, lysine and FCS for up to 72 hours. UE7T-13 cells were treated with 100nM insulin or 100 ng/mL IGF-1 for up to 60 minutes after starvation in nutrient deplete medium for (A) 24, (B) 48 and (C) 72 hours. Cell lysates were collected, then analysed by SDS-PAGE and Western blotting using antibodies as indicated. M = protein markers, UT = untreated. (D) Viability of UE7T-13 cells after 24, 48 or 72 hr starvation. Data presented as mean ± SD, One-way ANOVA.

IGF-1, arginine and leucine stimulate rpS6 phosphorylation in human MSCs.

Western blotting was performed to determine whether human MSCs respond to insulin, IGF-1, arginine and leucine. Both insulin and IGF-1 stimulate rpS6 phosphorylation in UE7T-13 cells after 15 or 60 minutes, irrespective of the starve period (Fig 2A-C), with 60-minute stimulation, following 72 hours starvation, showing the greatest differential from basal levels (Fig. 2C).

Physiological levels of arginine and leucine individually do not increase rpS6 phosphorylation compared to the untreated control. However, when administered in combination, they act synergistically to increase rpS6 phosphorylation of human MSCs, suggesting that both arginine and leucine are required to promote mTORC1 activation (Fig. 3).

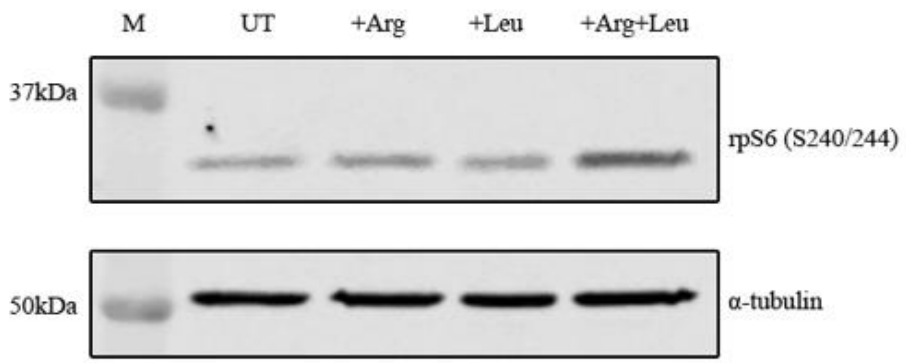

Figure 3: Arginine and leucine stimulate rpS6 phosphorylation in human MSCs. UE7T-13 cells were starved for 72 hours, then treated with 300 μM arginine, 200 μM leucine or both for 60 minutes. Cell lysates were collected, then analysed by SDS-PAGE and Western blotting using antibodies as indicated. M = protein markers, UT = untreated.

Sustained rpS6 phosphorylation of UE7T-13 cells by arginine, leucine and IGF-1 is mTORC1-dependent.

To characterise the response of UE7T-13 cells to arginine, leucine and IGF-1, a time course was performed. A time-dependent increase in rpS6 phosphorylation was observed between 5 and 60 minutes in +Arg+Leu treatment, which decreased by 120 minutes. A similar time-dependent increase was observed in the +Arg+Leu+IGF-1 group, however IGF-1 promoted further rpS6 phosphorylation compared to the same timepoints in the +Arg+Leu group (Fig. 4A). Moreover, rpS6 phosphorylation was sustained at 120 minutes when treated with AAs and IGF-1 together (Fig. 4A), indicating that AAs and IGF-1 work synergistically to promote rpS6 phosphorylation. In UE7T-13 cells pre-treated with rapamycin, rpS6 phosphorylation was reduced to below starvation levels in the presence and absence of stimuli (Fig. 4B), suggesting that rpS6 phosphorylation by AAs and IGF-1 is mTORC-1 dependent.

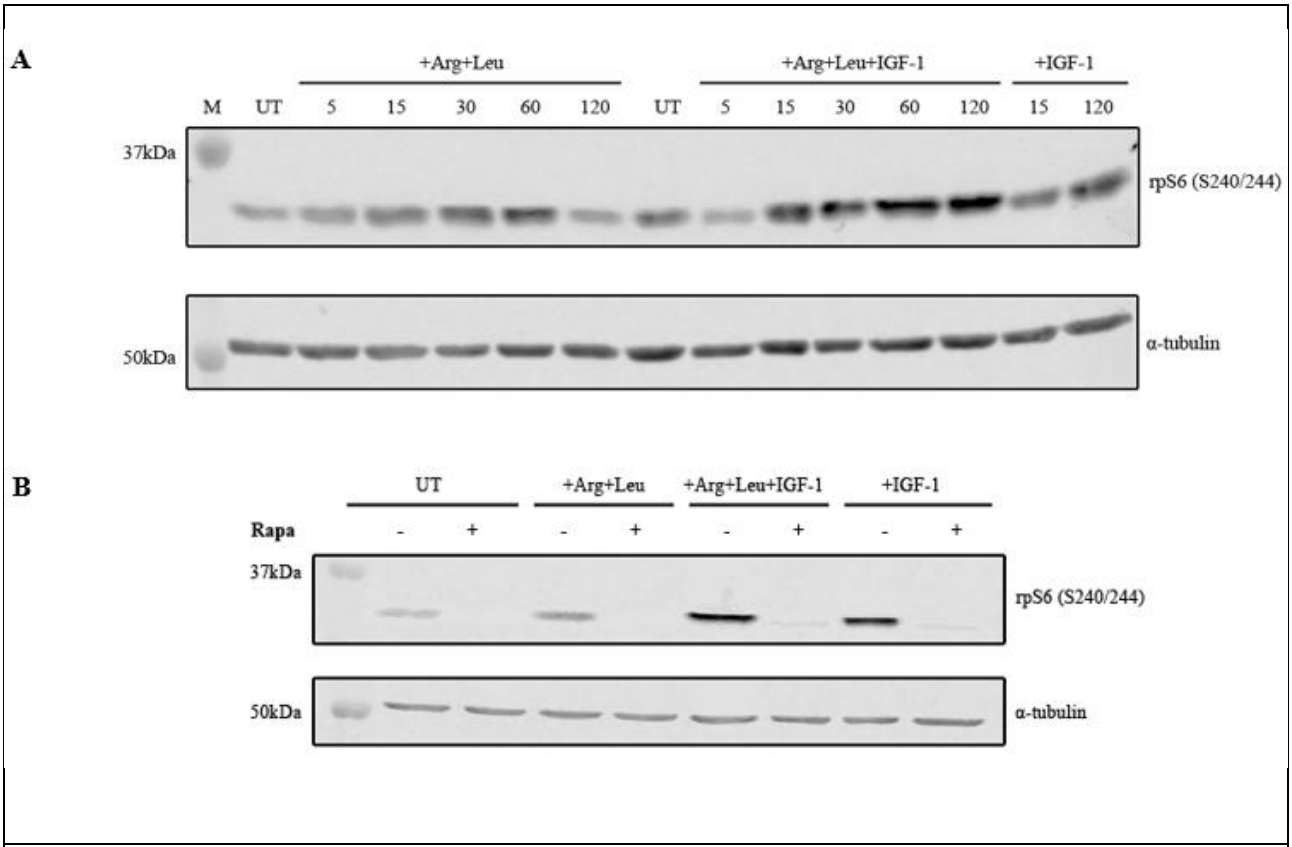

Figure 4: Sustained rpS6 phosphorylation of UE7T-13 cells by arginine, leucine and IGF-1 is mTORC1-dependent. UE7T-13 cells were starved for 72 hours then treated with 300 μM arginine and 200 μM leucine, 100 ng/mL IGF-1 or both treatments together for (A) between 5 and 120 minutes or (B) 60 minutes with or without 60-minute pre-treatment with 10 nM rapamycin. Cell lysates were collected, then analysed by SDS-PAGE and Western blotting using antibodies as indicated. M = protein markers, UT = untreated.

Arginine and leucine exhibit a titratable effect on rpS6 phosphorylation at 5ng/mL IGF-1.

To test the hypothesis that arginine and leucine work synergistically with IGF-1 to stimulate mTORC1-dependent osteogenesis, it was important to establish culture conditions in which the concentrations of arginine and leucine were limiting. Firstly, a titration of IGF-1 without AAs was performed (Fig. 5A). It was determined that between 3.125 ng/mL and 12.5 ng/mL would be ideal as rpS6 phosphorylation appeared to be saturated at 25 ng/mL IGF-1. 10 ng/mL IGF-1 was selected to perform titrations of arginine and leucine (Fig. 5B) however, differences between AA concentrations was difficult to determine. Moreover, in the absence of arginine and leucine, 10 ng/mL IGF-1 alone stimulated robust rpS6 phosphorylation. Consequently, this concentration was halved to 5 ng/mL IGF-1, and a second AA titration was performed. As shown in Fig. 5C, distinct AA concentration-dependent differences in rpS6 phosphorylation were observed enabling the nomination of a 'Low AA' group (10 μM arginine and 10 μM leucine) and a 'High AA' group (150 μM arginine and 200 μM leucine).

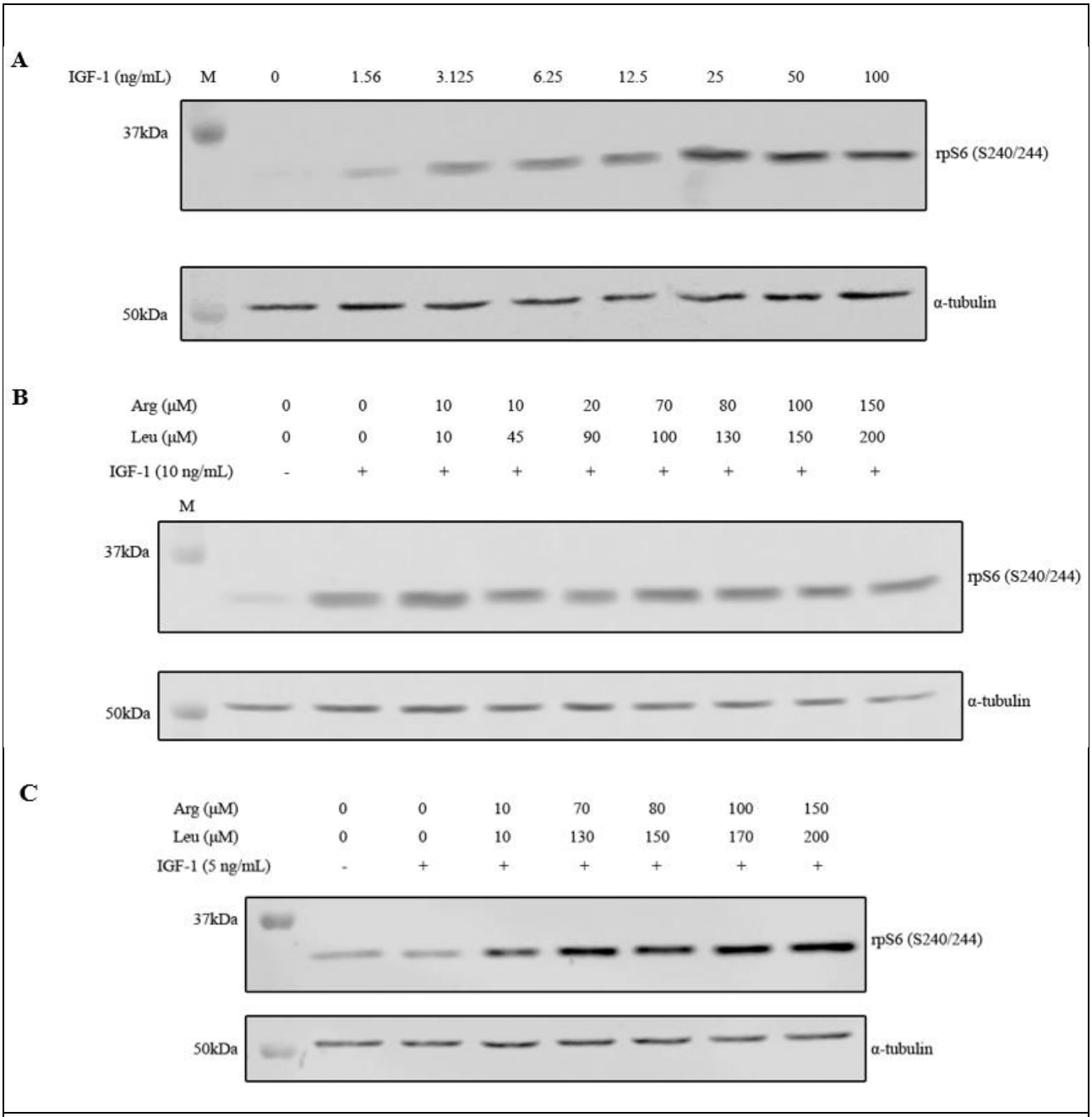

Figure 5: Arginine and leucine exhibit a titratable effect at 5ng/mL IGF-1. (A) UE7T-13 cells were treated with increasing concentrations of IGF-1 for 60 minutes. (B) Increasing AA concentrations were applied for 60 minutes in combination with 10 ng/mL IGF-1 and (C) 5 ng/mL IGF-1. Cell lysates were collected, then analysed by SDS PAGE and Western blotting using antibodies as indicated. M = protein markers.

High amino acid levels stimulate early and late stage markers of osteogenesis.

To determine the effect of arginine and leucine concentrations on osteogenesis, temporal regulation of *ALPL* and *BGLAP* genes, early and late markers of osteogenesis respectively,[37, 38] were measured throughout the 28-day induction period. Consistent with *ALPL* as an early osteoblastic marker, increased levels of expression were observed at Day 7 and Day 14 for all treatment groups when compared to Day 0 (Fig. 6A). Importantly, at Day 14, the High AA group showed a significantly higher fold increase in *ALPL* expression, which was inhibited in the rapamycin treated group, implying an mTORC1-dependent mechanism. Day 7 reflected the same trends without statistical significance (Fig. 6A). At Day 21, the late-stage differentiation marker *BGLAP* was induced at low levels in the -AA and Low AA groups. Moreover, the High AA group showed the greatest expression, which was reduced by administration of rapamycin, suggesting this response is mTORC1-dependent. Despite a lack of statistical significance, this mirrors the trends observed in *ALPL* expression at Days 7 and 14 (Fig 6B). Together, these data suggest that AA availability is a driving factor for osteogenesis, through the mTORC1 pathway.

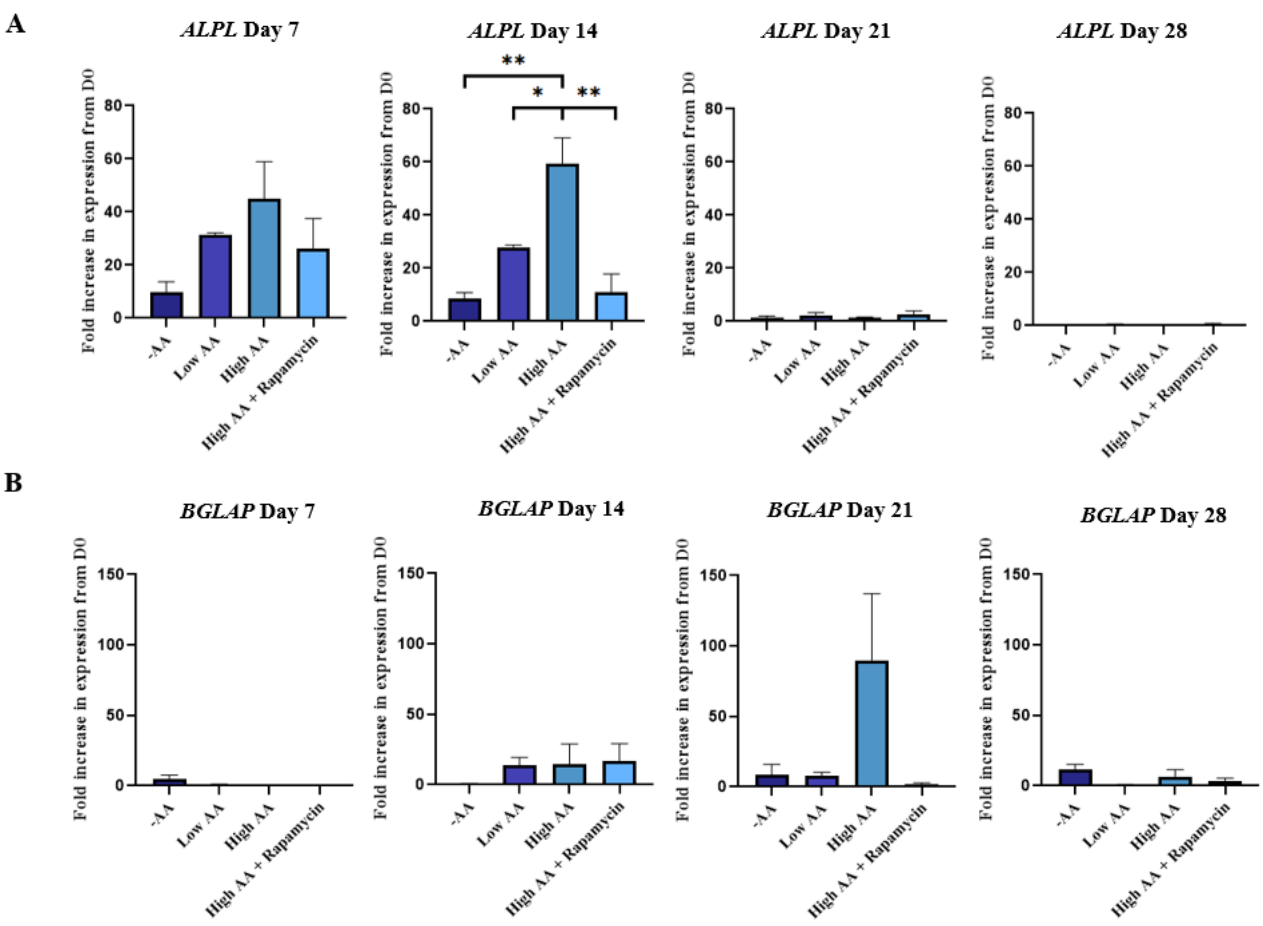

Figure 6: Effect of modulating arginine and leucine on temporal expression of osteo-related genes. Gene expression levels for (A) Alkaline phosphatase (*ALPL*) and (B) Osteocalcin (*BGLAP*) were determined by qRT-PCR. Treatment groups: -AA = 5 ng/mL IGF-1; Low AA = 10 μM arginine, 10 μM leucine and 5 ng/mL IGF-1; High AA = 150 μM arginine, 200 μM leucine and 5 ng/mL IGF-1; High AA + Rapamycin = 150 μM arginine, 200 μM leucine, 10 nM rapamycin and 5 ng/mL IGF-1. Data are presented as mean ± SEM for n=3 donors, performed in triplicate, Statistical significance was determined by One-way ANOVA and Tukey's post-hoc test (*ALPL* Day 14: -AA vs. High AA, p=.0019; Low AA vs. High AA, p=.026 and High AA vs. High AA + Rapamycin: p=.0048).

Arginine and leucine levels do not significantly affect cell mineralisation.

To determine whether calcified mineral deposition reflected the observed trends in osteo-related gene expression, a calcium assay was performed and normalised to DNA content to consider effects on cell proliferation. Modulating the concentration of AAs however, had no significant effect on cell mineralisation at Day 21 or Day 28 post-induction nor does it appear that there were any trends present between treatment groups (Fig. 7).

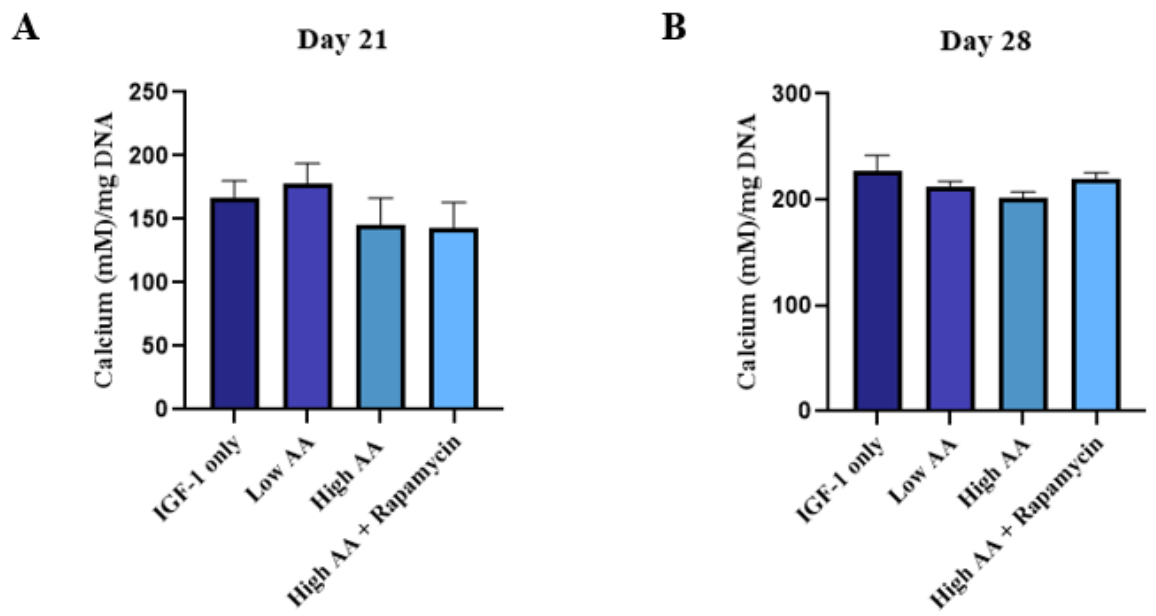

Figure 7: Arginine and leucine levels do not significantly affect cell mineralisation. Calcified mineral normalised to DNA content at (A) Day 21 and (B) Day 28 for n= 3 donors/treatment group. Treatments: IGF-1 only = 5 ng/mL IGF-1; Low AA = 10 μM arginine, 10 μM leucine and 5 ng/mL IGF-1; High AA = 150 μM arginine, 200 μM leucine and 5 ng/mL IGF-1 and High AA + Rapamycin = 150 μM arginine, 200 μM leucine, 10 nM rapamycin and 5 ng/mL IGF-1. No statistical significance observed at Day 21 or Day 28 (One-way ANOVA, mean ± SEM).

Discussion

Diseases of low bone mass are increasing in prevalence globally in keeping with an ageing population. Low bone mass is likely due to an imbalance in MSC differentiation leading to the suppression of osteoblastogenesis in older age.[8] Increasing AA intake, through a high protein diet, has been shown to not only promote increases in bone mass, but also decrease risk of fracture.[13] The mTORC1 signalling pathway has been well-established in anabolic processes, but its role in osteogenic differentiation of MSCs in response to AAs and IGF-1 is unclear. Throughout this study, the mTORC1-dependent effects of AAs and IGF-1 on human MSCs were investigated in an *in vitro* model.

Dietary proteins contain essential and conditionally essential AAs including arginine and leucine, which have been shown to stimulate production of IGF-1 to promote bone anabolism[14, 19]. In this study, we were able to successfully determine that human MSCs are responsive to arginine, leucine and IGF-1 in an mTORC-1 dependent manner, implicating the pathway in the human response to dietary protein. It is interesting to note that the synergistic effect that arginine and leucine had on rpS6 phosphorylation has not been described previously[31], which may indicate that GATOR2 requires inputs in addition to arginine or leucine individually. In keeping with this idea, we also found that, in UE7T-13 cells, rpS6 phosphorylation can be stimulated through the addition of lysine as well as arginine and leucine during pulse experiments (Fig. S1).

Recent studies have found that increased mTORC1 signalling increases osteogenic markers including *Alpl*[39] and *Ocn*[40] *in vitro* as well as increasing bone mass in ovariectomised mice.[39, 40] We were able to show that induction of *ALPL* and *BGLAP*, at early and late stages of osteogenesis respectively, was inhibited through the inclusion of rapamycin, demonstrating that AAs promote osteogenesis via mTORC1. Statistical significance could not be achieved for *ALPL* at Day 7 or *BGLAP* at Day 21 due to high donor variability. Therefore, differentiation assays with additional donors may be required to

produce more conclusive evidence. Together, these data suggest that AAs may influence the differentiation program in human MSCs through mTORC1.

Observing an increase in expression of *ALPL* at Days 7 and 14 and *BGLAP* at Day 21 in the High AA group compared to other groups led us to hypothesise that cell mineralisation would follow the same trend however, this was not the case. Despite our study finding no significant effect or obvious trend between groups at Days 21 and 28, we acknowledge that dexamethasone, one of the key drivers of osteogenic differentiation of MSCs *in vitro*[41], has been shown to activate REDD1[42] in skeletal muscle, which is an inhibitor of mTORC1 though the TSC.[43] Whether REDD1 inhibits mTORC1 in a similar manner in the bone microenvironment though has not been investigated. As a result, optimisation studies are required to modify osteogenic media to exclude dexamethasone and therefore determine the true effect of modulating AAs on cell mineralisation.

Alkaline phosphatase has been shown to be a critical regulator of bone mineralisation in mice.[44] Throughout the 28-day induction period, neither *ALPL* nor *BGLAP* expression was upregulated in the -AA group suggesting that calcified mineral could not form due to lack of osteoblast differentiation. It is currently unknown whether human MSCs can survive in arginine and leucine free conditions for extended periods, so it is plausible that the observed signal was representative of artefacts from a compensatory mechanism or cell death through apoptotic or necrotic pathways.

A relationship between AAs and IGF-1 production[45] as well as the role of IGF-1 in promoting osteoblastic differentiation of MSCs through activation of mTOR[20] has been established however, whether these events are related has not been described. Data from this study suggest that AAs promote mTORC1 activation inferring that these may be correlated. It is possible that MSCs could be responding to AAs by stimulating mTORC1-dependent IGF-1 synthesis and secretion to act in an autocrine or paracrine manner to induce osteoblast differentiation. Therefore, the effect of AAs on IGF-1 gene expression as well as IGF-1 protein levels warrants enquiry.

Currently, in Australia, National Health and Medical Research Council guidelines recommend higher protein intake for older individuals.[46] A high protein diet has previously been proposed as having detrimental effects on bone health, known as the acid-ash hypothesis.[1] This was speculated due to increased urinary calcium excretion observed in individuals with high dietary protein intake.[47] It was thought that this caused increased renal acid load, subsequently resulting in calcium, as well as other alkaline minerals, to be released from the bone to neutralise it.[48] It has since been shown that an increase in dietary protein causes increased calcium absorption, allowing for acid neutralisation to occur with serum calcium rather than calcium derived from bone.[49] Further elucidation of the mechanism behind how high dietary protein intake influences bone mass could help to inform dietary guidelines, particularly for the elderly and those at risk of diseases characterised by low bone mass. Additionally, it would allow exploration of the possibility of developing anabolic agents for low bone mass treatments.

This is the first study to date to assess the response of MSCs to both the AA sensing and growth factor branches of the mTORC1 pathway in a human context. Other research has primarily investigated each facet of the pathway individually or focussed on substrates that induce anabolic processes through mTORC1. Now that evidence of a mTORC1-dependent relationship between AAs, IGF-1 and human MSCs has been established, further investigation to elucidate the mechanism behind the response of dietary protein intake and MSC differentiation, and therefore bone formation, can be conducted.

In summary, results from this study suggest that arginine, leucine and IGF-1 promote mTORC1 signalling in human MSCs and modulating AA concentration influences gene expression of early and late osteogenic markers, *ALPL* and *BGLAP*. While we could not deduce with certainty whether modulating AA concentration influences MSC mineralisation, optimisation of the protocols used will provide some clarity. Studies in the near future will optimise differentiation protocols, assess IGF-1 production by MSCs and investigate the mTORC1-dependent effects of AAs and IGF-1 on MSC and

osteoblast biology *in vivo,* while longer-term studies will assess the osteoclastic division of bone remodelling.

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Supplementary Material

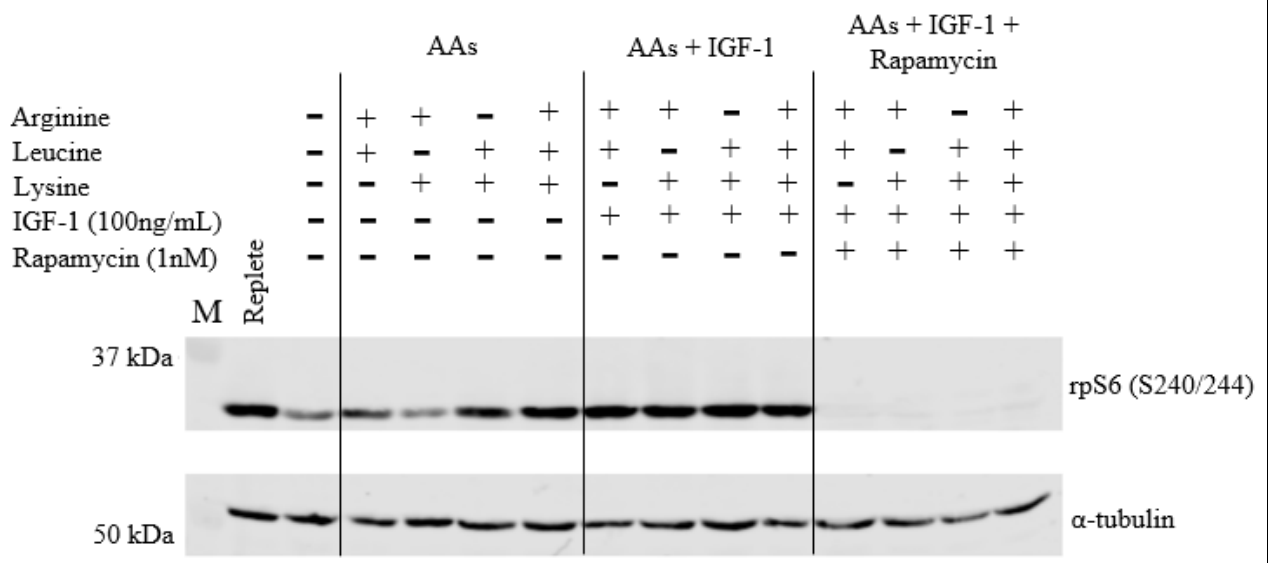

Figure S1. Lysine promotes further synergism of the mTORC1 response to AAs in the absence of IGF-1. UE7T-13 cells were starved for 72 hours, then treated with 300 μM arginine, 200 μM leucine, 300 μM lysine or 100 ng/mL IGF-1 60 minutes, with or without 60-minute pre-treatment with 10 nM rapamycin. Cell lysates were collected, then resolved by 9% SDS-PAGE and Western blotting using antibodies as indicated. M = protein markers, Replete = nutrient deplete media supplemented with 10% FCS, 300 μM arginine, 200 μM leucine, 300 uM lysine and 100 ng/mL IGF-1.