Abstract

Aims/ Objectives

To evaluate current literature via systematic review to ascertain whether amino acids/ vitamins provide any influence on musculotendinous healing, and by which physiological mechanisms.

Methods

EBSCO, PUBMED, Science Direct, Embase Classic/ Embase, and MEDLINE were searched using terms including “vitamins”, “amino acids”, “healing”, “muscle” and “tendon”. The primary search had 479 citations, 466 of which were excluded predominantly due to non-randomised design. Randomised human and animal studies investigating all supplement types/ forms of administration were included. Critical appraisal of internal validity was assessed using the Cochrane risk of bias tool or the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) risk of bias tool for human and animal studies, respectively. 2 reviewers performed duel data extraction.

Results

Twelve studies met criteria for inclusion: 8 examined tendon healing, 4 examined muscle healing. All studies used animal models, except 2 human trials using a combined integrator.

Narrative synthesis was performed via content analysis of demonstrated statistically significant effects, and thematic analysis of proposed physiological mechanisms of intervention. Vitamin C/ taurine demonstrated indirect effects on tendon healing through anti-oxidant activity. Vitamin A/ glycine showed direct effects on extra-cellular matrix tissue synthesis. Vitamin E shows an anti-proliferative influence on collagen deposition. Leucine directly influences signalling pathways to promote muscle protein synthesis.

Discussion

Preliminary evidence exists demonstrating vitamins and amino acids may facilitate multi-level changes in musculotendinous healing; however recommendations on clinical utility should be made with caution. All animal studies and one human study show high risk of bias with moderate inter-observer agreement (k=0.46).

Currently, there is limited evidence to support the use of vitamins and amino acids for musculotendinous injury. Both high quality animal experimentation of the proposed mechanisms confirming the physiological influence of supplementation; and human studies evaluating effects on tissue morphology and biochemistry are required before practical application.

[299 words]

Introduction

Dietary supplements are defined as any concentrated source of a nutritional compound demonstrating a physiological effect (Department of Health, 2011). A meta-analysis (n=159) shows greater usage of supplements in athletes compared to non-athletes with increasing frequency in elite performers (Knapik et al., 2016). Despite heterogeneity between studies, a pooled prevalence estimate of 60% indicates high usage in the athletic population. Furthermore, supplement use guidelines are inadequate; athletes may misunderstand supplement effects; and display insufficient knowledge to independently plan their diet (Molinero & Márquez, 2009; Petróczi et al., 2007; Torres-McGehee et al., 2012). This may lead to disordered eating behaviours and in extreme cases may leave them at heightened risk of musculoskeletal injury such as fracture (Bonci et al., 2008). Dietary management strategy can also be misinterpreted by coaches who discourage supplement use due to the potential risks of contamination and inadvertent doping (Judkins & Prock, 2012), but who have inadequate knowledge in nutritional practices to plan effectively during injury recovery (Tipton, 2011). Thus, leaving athletes reticent to supplement their diet during rehabilitation (Tack, 2016). This is despite evidence from randomised controlled trials that supplements can provide therapeutic effects on muscle morphometry and strength following injury (Hespel et al., 2001; Holm et al., 2006); and muscular metabolic efficiency following immobility (Eijnde et al., 2001). A survey of 145 athletes demonstrated only 34% considered supplementation to improve musculoskeletal tissue repair (e.g. chondroitin, glucosamine, methyl-sulfonyl-methane and omega-3 fatty acids) (Malinauskas et al., 2007). In similar surveys, maintaining strength/endurance and avoiding sickness were more commonly cited reasons for supplementation (Petróczi et al., 2007a/b; Petróczi et al., 2008).

Animal experiments investigating skin wound healing indicate supplementation can elicit positive effects on collagen synthesis (Ejaz et al., 2009; Uzgare et al., 2009) and tensile breaking-strength (Shukla et al., 1999). More specifically, vitamins and vitamin-related-compounds can increase growth factor release (retinoids) (Wicke et al., 2000); as well as enhancing tensile breaking-strength (vitamin E-like antioxidant, Raxofelast) (Galeano et al., 2001). Other supplements, such as olive oil, can reduce oxidative damage during healing (Rosa et al., 2014); whilst the amino acid arginine can improve wound angiogenesis (Raynaud-Simon et al., 2012). The choice of supplement is therefore critical. Additionally, there is evidence of negative effects through excessive facilitation of pro-inflammatory pathways by omega-3 fatty acids (McDaniel et al., 2008); and in the case linseed and fish oils, of reduced tissue angiogenesis (Otranto et al., 2010). Human trials with arginine demonstrate improvements in blastogenic response to injury (Barbul et al., 1990; Sax, 1994); and improved collagen deposition seen following supplementation with amino acid mixture containing arginine, beta-hydroxy beta-methylbutyrate and glutamine (Williams et al., 2002). Additionally, time to wound healing is reduced following supplementation of a mixture containing protein, zinc, iron and vitamin C (Collins et al., 2005).

This review elaborates on a multi-topic feasibility search which incorporated all supplement types and the influence on musculotendinous tissue healing. Subsequently a PICO (population, intervention, comparator, outcome) question was devised: “what is the effect of dietary supplements on musculoskeletal tissue (e.g. cartilage, tendon, muscle, ligament) healing compared to placebo or other control?”, and was used to formulate a search of Google Scholar and PUBMED to evaluate the quality/ volume of existing literature. This search produced 95 papers which when assessed for eligibility was reduced to 24 studies.

Amino acids and vitamins demonstrated sufficient literature to perform a more specific systematic review and consequently these classifications of supplements encapsulated all literature found to evaluate the effect of supplements in muscle and tendon.

The specific research question “what is the effect of vitamins and/ or amino acids on musculoskeletal tissue (e.g. cartilage, tendon, muscle, ligament) healing parameters compared to placebo or other control?” was then devised. This methodology (systematic review via content and thematic analysis) was chosen to synthesise findings of animal and human model experiments, previously undertaken in other areas of medicine (Sallis, 2000; Virmani et al., 2003), and in response to the call for methodologically sound reviews to evolve the research base (Reagan-Shaw et al., 2008).

As such this review aims to identify whether these selected dietary supplements (amino acids and vitamins) provide any influence on muscle and tendon tissue healing in animal and human models of injury (traumatic, degenerative or exercise induced); and if so, what mechanisms underpin this influence.

Methods

This review followed specific methodology guidelines (Centre for Reviews and Dissemination, 2009), and reported in accordance with the Preferred reporting items for systematic review and meta-analysis (PRISMA) statement for reporting systematic reviews (Moher et al. 2015). The review was based upon an *a priori* protocol which described essential procedures to be followed (e.g. the PICO question of issue, comprehensive search strategy and a piloted data extraction pro forma).

Eligibility Criteria

This review followed the following inclusion criteria:

- Randomised controlled trials of human and animal models of tendon and muscle injury. Although it has been reported that randomisation and blinding in animal studies is often not stringently adhered to (Hess, 2011); the choice was made to include these trials but be explicit in critique of these processes. Despite continuing debate around the poor predictive value of animal experiments for humans (McGonigle & Ruggeri, 2014); this review includes animal models to support findings from human trials. Methodological flaws (e.g. insufficient, allocation concealment/ blinding) (Kilkenny et al., 2009) can limit prediction from animals to humans (Hackam

& Redelmeier, 2006; Henderson et al., 2013); leading to problems such as outcome reporting bias (Tsilidis et al., 2013). To attenuate these risks (Sena et al., 2010) explicit application of randomisation and control group use is specified for inclusion.

- Trials examining the influence of vitamins and/ or amino acid compounds applied orally, by injection or topically (either alone or in combination).
- The studies must use outcomes indicative of physiological changes in healing (e.g. ultimate tensile strain, cellular tissue proliferation, and vascularisation).

Non-randomised trials were excluded to reduce selection bias (Hahn et al. 2005). Only English language papers were included due to a lack of translation services. Papers were initially screened by title/ abstract. Potentially relevant abstracts were sourced in full text and assessed independently against distinct inclusion criteria by two reviewers (CT/ FS) for eligibility. A third reviewer was available for consultation and consensus (LK).

Non-randomised experimental or observational studies (including *in vitro* studies), and those investigating pharmaceutical drugs were excluded from the review.

Search

EBSCO, PUBMED, Science Direct and- via the OVID platform- Embase Classic/ Embase, MEDLINE and Global Health databases were searched from inception (1947) to 11th June 2017.

The following search field was used: (("muscle"[All Fields] OR "musculoskeletal"[All Fields] OR "tendon"[All Fields] NOT "cartilage"[All Fields] NOT "skin"[All Fields] NOT "cutaneous"[All Fields] NOT "bone"[All Fields]) AND ("Amino acid"[All Fields] OR

"vitamin"[All Fields] OR "arginine"[All Fields] OR "leucine"[All Fields] OR "phenol"[All Fields] OR "beta-Hydroxy beta-methylbutyric acid"[All Fields] OR "retinol"[All Fields] OR "ascorbic"[All Fields] OR "taurine"[All Fields])) AND ("healing"[All Fields] OR "repair"[All Fields] OR "collagen"[All Fields]) AND dietsuppl[sb].

Search terms were informed by a feasibility search, and in conjunction with a health information librarian. Citations of key articles found in the feasibility search were evaluated as a grey literature search.

Data Extraction

Data extraction was undertaken by two reviewers using a piloted form. Reasons for exclusion were noted. Data extraction included: date of study, animal or human model, type of tissue, subject details (animal type, age, sex), dosage and type of supplement (compound, administration or consumption), dosage and type of control or comparator, duration of supplementation, follow up times and losses to follow up, outcomes.

Primary outcomes were pre-determined to be:

- Histological changes to tissue collagen content or rate of synthesis
- Biochemical changes in serum composition
- Biomechanical alterations in force tolerance of healing tissue (e.g. ultimate tensile strength)

Secondary outcomes were examined for clinical relevance and collected as appropriate (e.g. radiographic evidence of morphological change).

Data analysis

Although the optimal synthesis method to strengthen findings of treatment effect from randomised trials is considered to be meta-analysis (DerSimonian & Laird, 1986) in this case it was deemed inappropriate as there was insufficient homogeneity of data across tissue types, interventions and outcomes to conform to data pooling. Instead a narrative synthesis was undertaken as per the method described by Popay et al. (2006) combining content analysis of the frequency of outcomes demonstrating statistically significant treatment effects, and thematic analysis of the mechanisms of identified effects. This method provides a framework appropriate for the analysis of intervention effectiveness when statistical means are not applicable (Rodgers et al., 2009); providing flexibility in structure to develop a theory of intervention effect by exploring relationships in the evidence base. This method has been applied previously with success (Arai et al., 2007) with conclusions similar to meta-analysis on the same group of randomised trials (DiGuiseppi & Higgins, 2001). The comparison of narrative synthesis to meta-analysis on the same topic demonstrates the importance of using structure to maintain transparency of the process and establishes trustworthiness of the synthesis product by reducing selection bias (Rodgers et al., 2009).

Critical Appraisal

Evaluation of risk of bias was undertaken using the Cochrane risk of bias tool (Higgins et al., 2011) for human studies or the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) risk of bias tool for animal studies (Hooijmans et al., 2014). Two reviewers examined the studies for risk of bias and internal validity. Specifically the

appraisers evaluated the studies relative to sources of bias (e.g. allocation concealment, blinding, selective outcome reporting) and classified to low, high or unclear risk of bias. In order to assess inter-observer agreement of the risk of bias, the Kappa Statistic was used (Cohen, 1960).

Results

Search

Figure 1 presents the study selection process. Primary search results found 479 papers. 416 articles were excluded after comparison against the research question, on the basis of title and abstract, with 63 remaining for evaluation of full text. 44 of these were excluded due to methodology inclusion criteria (non-randomised studies, narrative and systematic reviews); or due to examining incorrect tissue type (bone, cartilage, ligament, skin wounds). 7 were excluded on the basis of examining a supplement type different to amino acids/ vitamins (e.g. fatty acids), however these studies additionally had non-randomised designs. 12 articles fulfilled the inclusion criteria.

ADD FIGURE 1: PRISMA Flow diagram with the following:

Figure 1. PRISMA flow diagram showing study selection process (Moher et al. 2009)

Data Extraction

Tables 1 and 2 provide a summary of the characteristics and findings of included studies. 8 studies examined tendon healing (Table 1): 1 assessing vitamin A and E (Greenwald et al., 1990); 2 assessing vitamin C (Hung et al., 2013; Ömeroğlu et al., 2009); 1 assessing taurine

(Akdemir et al., 2015); 2 assessing glycine (Vieira, Guerra et al., 2015; Vieira, De Oliveira et al., 2015), and 2 assessing nutrient complexes which included various supplements, (e.g. arginine, methylsulfonylmethane and vitamin C) (Gumina et al., 2012; Notarnicola et al., 2012). Only the integrator articles used human models. The animal experiments examined models of various injury types (e.g. incision/ repair, collagenase-induced, partial tear). Human studies examined surgical repair and tendinopathy models.

4 studies examined the effect of leucine supplementation of muscle healing in animal models (Table 2). 2 studies examined healing following cryolesion, with the other studies examining exercise-induced muscle exhaustion and electrically-stimulated contraction-induced injury.

Tissue healing outcomes included biomechanical measures of tissue integrity; histopathological assessment of tissue via various procedures (e.g. immunostaining, biochemical assay, birefringence analysis); blood serum testing; and morphometric analysis (e.g. gross analysis of limb, microscopic examination of tissue).

Quality Assessment

All animal model studies demonstrated a high risk of bias. This was due to a number of common deficiencies in the methodological quality of these studies. For example, allocation concealment was not described in any of the papers, and as per the review decision rules lack of reporting was assumed to demonstrate failure to complete. Blinding was only undertaken in 1 paper (Kato et al., 2016), however this study did not randomise at the point of allocation and only prior to histochemical analysis. 2 studies (Vieira, De Oliveira et al., 2015; Vieira, Guerra et al., 2015) randomised only prior to light microscopy analysis.

Of the two human studies, one was measured as a high risk of bias (Gumina et al., 2012) due to failure to blind assessors and complete intention-to-treat analysis. However, this study did demonstrate excellent randomisation and allocation concealment and undertook a power calculation to reduce likelihood of type II error. The other human study (Notarnicola et al., 2012) showed low risk of bias, due to computer-assisted randomisation/ allocation concealment, double-blinding, group matching of homogeneity and intention-to-treat analysis.

Inter-observer agreement regarding the risk of bias was considered 'moderate' (k=0.46) according to the Viera and Garrett (2005) Kappa interpretation model. Moderate interrater reliability may suggest an incorrect representation of the studies' risk of bias (McHugh, 2012). However, with most studies demonstrating high risk of bias, caution is already suggested when generalising the results.

		Outcomes				Summary	
		Biomechanical	**Histopathological**	**Biochemical**	**Morphological**	**FINDINGS**	**RISK OF BIAS**
ANIMAL STUDIES							
Akdemir et al., (2015) Model: Rodent [Achilles tendon incision/repair] N= 16 Supplement: Taurine	Dosage: 200mg Taurine Route: Injection (x 1 post repair) Control: Saline Follow up: 42 days	↑Mean max load (p=0.025) ↑ Mean max stress (p=0.025) ↑ Mean energy uptake (p <0.05)	(Verhodstd- modified score) ↓ mean fibrosis, fibroblast proliferation (p <0.05), oedema. ↑neutrophil infiltration (p <0.05)			Efficiency of tendon gliding improved over 6 weeks with a reduction in tissue adhesion due to fibrosis, and increased biomechanical integrity.	R1- High R2- High
Greenwald et al., (1990) Model: Chicken [flexor profundus tendon incision/repair] N= 96 Supplement: Vitamin A, E and beta-carotene	Dosage: Vitamin A (150,000 IU/kg) (once daily for 45 days) Route: Oral Control: Standard chow (vit. A = 22,000 IU/kg; vit. E = 35 IU/kg; betacarotene = 1 mg/kg) Follow up: 7/ 45 days	↑ UTS at 7 days (p=0.007), 30 days (p=0.06), 45 days (p<0.001)				Vitamin A elevates UTS during early healing, but perhaps at the risk of preventing true internal tendon strength. Conversely, vitamin E reduces UTS perhaps due to the reduction in peri-tendon adhesions by anti-oxidant activity. Beta-carotene shows margin elevation of UTS.	R1- High R2- High
	Dosage: Vitamin E (1000 IU/kg) [Otherwise as above]	↓ UTS at 7 days (p=0.004), 45 days (p<0.001)					
	Dosage: Beta-carotene (90mg/kg) [Otherwise as above]	Margin ↑ UTS (p>0.05)					
Ömeroğlu et al., (2009) Model: Rodent [experimental Achilles rupture] N= 42 Supplement: Vitamin C	Dosage: 150mg vitamin C Route: Injection (post rupture and then every 2 days for 3- 21 days Control: 1.5cc saline Follow up: 3/10/21 days		↑ revascularisation at day 3 (p=0.01) ↑ collagen production at day 10 (p=0.021), day 21 (p=0.103) ↑ collagen diameter at day 3/10/21 (p<0.001) ↑ fibroblast proliferation at day 3 (p=0.042)			Vitamin C facilitates histological changes in increased collagen proliferation and collagen fibre diameter, as well as increased numbers of fibroblasts.	R1- High R2- High
Vieira, De Oliveira et al. (2015) Model: Rodent [collagenase induced Achilles tendon injury]	Dosage: 5% glycine diet, daily for 7-21 days Route: Oral Control: Standard chow and water	↑ UTS and maximum displacement load at 21 days (p<0.05)	↑ birefringence of collagen at 7 days ↑ epitenon thickness at 7 days (p<0.05)	↑ GAGs at 7 (p<0.01) and 21 days (p<0.05) ↑ hydroxyproline at 7 and 21 days (p<0.05)		Glycine enhances tissue levels of GAGs, hydroxyproline and NCPs during early healing, leading to greater collagen	R1- High R2- High

N= 50 Supplement: Glycine	Follow up: 7/21 days			↑ NCPs at 7 days (reduced by 21 days) (p<0.01)		synthesis and enhanced extracellular matrix re-modelling. Thus leading to greater biomechanical strength.	
Vieira, Guerra et al., (2015) Model: Rodent [collagenase induced Achilles tendon injury] N= 35 Supplement: Glycine +/- green tea	Dosage: *Camellia sinensis* (green tea/ GT) [700 mg/kg/day] +/- 5% glycine diet (daily for 7-21 days) Route: Oral, daily for 7-21 days Control: Standard chow and water Follow up: 7/21 days	↑ UTS at 21 days (GT and glycine) (p<0.05)	↑ birefringence of collagen at 7 days (GT and glycine) (p<0.05)	↑ GAGs at 21 days (GT and glycine) (p<0.05) ↑ hydroxyproline at 7 days (GT and glycine) and 21 days (GT only) (p<0.05) ↓ NCP at 7 and 21 days (GT and glycine) (p<0.05)		The combination of glycine and GT shows greater benefits to early collagen organisation at 7 days, leading to enhanced fibre stability and load tolerance.	R1- High R2- High
Hung et al., (2013) Model: Chicken [incisional injury to the flexor digitorum profundus tendon] N= 57 Supplement: Vitamin C	Dosage: Vitamin C (5mg/ml or 50mg/ml) Route: Injection immediately post injury Control: Saline Follow up: 14/ 42 days	↓ tendon gliding resistance at 6 weeks (both dosages) (p=0.010)	↓ swelling at 2 weeks (50ml) ↓ tendon adhesions and fibrosis at 2 weeks (5ml)	↑ glutathione at 2 weeks (p=0.05)	↓ fibrotic size at 6 weeks (5mg) (p=0.013	Vitamin C injection enhances anti-oxidant action, leading to reduced tendon adhesion and restoration of glutathione levels. There is a greater response for gliding resistance and fibrotic size with a lower dose (5mg).	R1- High R2- High
HUMAN STUDIES							
Gumina et al., (2012) Model: Human [Arthroscopic repair of a large postero-superior rotator cuff tear] N= 90 Supplement: Combined integrator (Tenosan: arginine L-alpha-ketoglutarate, methylsulfonylmethane, hydrolyzed type I collagen and bromelain)	Dosage not given. Route: Oral (2 sachets per day for 12 weeks) Control: No supplement Follow up: 84 days				↓ incidence of post repair classification type I-III (Sugaya's classification) (p=0.045) ↓ percentage re-rupture (p=0.111) ↑ percentage of type I-II repair integrity (p<0.05)	There is a higher incidence of type I-II classification of repair intensity and a reduced rate of re-rupture in the intervention group.	R1- High R2- Unclear

Notarnicola et al., (2012) Model: Human [Insertional Achilles tendinopathy] N= 64 Combined integrator (Tenosan: arginine-L-alpha-ketoglutarate (500 mg), MSM (550 mg), hydrolyzed collagen type I (300 mg), Vinitrox (125 mg), bromelain (50 mg), and vitamin C (60 mg).	Dosage: arginine-L-alpha-ketoglutarate (500 mg), MSM (550 mg), hydrolyzed collagen type I (300 mg), Vinitrox (125 mg), bromelain (50 mg), and vitamin C (60 mg [+ extra-corporeal shockwave therapy] Route: Oral (2 sachets per day for 60 days) Control: Placebo [+ extra-corporeal shockwave therapy] Follow up: 6 months	↓ mean oximetry value at 6 months ($p<0.0001$)	Despite small effect sizes, the findings suggest ECSWT and a combined integrator can reduce tissue perfusion at 6 months in Achilles tendinopathy.	R1- Low R2- Unclear

Table 1
Summary of study characteristics and findings of selected supplements on tendon healing

[UTS- Ultimate tensile strength GAGs- Glycosaminoglycans NCP- Non-collagenous proteins MSM- Methylsulfonylmethane R1- Reviewer 1 [CT] R2- Reviewer 2 [FS] ECSWT- extra-corporeal shockwave therapy

		Outcomes				Summary	
		Biomechanical	**Histopathological**	**Biochemical**	**Morphological**	**FINDINGS**	**RISK OF BIAS**
ANIMAL STUDIES							
Anthony et al., (1999) Model: Rodent [exercise induced exhaustion of gastrocnemius/ plantaris] N= 30 Supplement: Leucine	Dosage: a) Leucine + exercise (54g/L), b) Leucine (54g/L) + exercise + carbohydrate (235.5g glucose/ 235.5g sucrose) Route: Oral diet provided immediately post exercise. Control: c) Sedentary control (no supplement), d) Exercise + dietary deprivation, e) exercise + carbohydrates (262.5g glucose/ 262.5g sucrose) Follow up: (outcomes taken 1 hour post eating)		↑ fractional rate of protein synthesis [Protein synthesis elevated to the same as non-exercise controls 1 hour after exercise] (leucine +/- carbohydrates) ($p<0.05$)	↑ recovery of skeletal muscle glycogen 1 hour post exercise (carbohydrate +/- leucine only) ($p<0.05$) ↑ plasma insulin and muscle glycogen (carbohydrate + leucine) vs carbohydrate only ($p<0.05$)		Leucine may stimulate protein synthesis by enhancing muscle sensitivity to available insulin immediately after ingestion. Leucine works independently to stimulate protein synthesis (almost as effectively as leucine combined with carbohydrate) despite not raising insulin levels.	R1- High R2- High
Kato et al., (2016) Model: Rodent [electrically stimulated tibialis anterior contractions] N= 49 Supplement: Leucine	Dosage: Essential amino acids: leucine (1g/kg) Route: Oral (2 days pre-exercise and 1-14 days post) Control: Oral saline gavage Follow up: 1-14 days	↑ speed of return to stronger muscle function (3 days in leucine group) ($p<0.05$) Return of isometric dorsiflexion torque to the level of controls in 14 days (leucine only)	↓ numbers of damaged fibres (leucine) ($p<0.001$)	↓ interleukin-6 expression 1 day post exercise (leucine only) ($p<0.001$)		Provision of leucine enhances muscle healing by suppressing inflammation (indicated by reduced IL-6). Thus leading to attenuation of muscle damage and earlier return of muscle function following exercise.	R1- High R2- High
Pereira, Baptista et al., (2014) Model: Rodent [soleus muscle cryolesion] N=48 Supplement: Leucine	Dosage: Leucine (1.35g/kg) Route: Oral gavage (13 days pre-injury, 10 days post) Control: Oral saline gavage	↓ reduction in strength production between pre and post fatigue tetanic stimulus (compared to the reduction shown in control animals)		↑ myofiber cross sectional area at day 10 ($p<0.05$) ↓ inflammatory cell infiltration at day 10 ($p<0.05$)		Leucine improves recovery of muscle fibre size and function (resistance to fatigue) post injury. The mechanism of these changes involves	R1- High R2- High

	Follow up: 1-10 days			↓ area density of collagen type III at day 10 (p<0.05) ↓ macrophage infiltration on day 3 (p<0.05) ↓ expression of mTOR/ p-mTOR at day 10 (p<0.05) ↓ activation of FOXO3a at day 3 and 10 (p<0.05) Cryolesion-induced increase in ubiquinated protein was attenuated (p0.05)	the protein ubiquination pathway. Changes include a slight reduction in mTOR expression (but no other alteration to the PI3K/Akt/ mTOR pathway); and reduced FOXO3a activation/ ubiquinated protein content. This would indicate reduced post exercise proteolysis.	
Pereira, Silva et al., (2014) Model: Rodent [tibialis anterior cryolesion] N= 22 Supplement: Leucine	Dosage: Leucine (1.35g/kg) Route: Oral gavage (13 days pre-injury, 10 days post) Control: Oral saline gavage Follow up: 10 days	Prevention of reduction in pre-fatigued tetanic strength (however not sustained during fatigue protocol)	↓ expression of phosphorylated TGF-beta receptor type-1 at day 10 (p<0.05) ↓ Smad2/3-positive nuclei at day 10 (p<0.05) ↓ MyHC-n positive regenerating myofibers at day 10 (p<0.05) ↑ MyHC-II at day 10 (p<0.05) ↓ hydroxyproline to levels of control (p<0.05) ↑ fractional rate of protein synthesis (p<0.05)		↓ procollagen/ thin collagen fibers. ↓ inflammatory area (p<0.05)	Leucine supplementation improves muscle contractile performance, without change in myofiber size. There is an accelerated shift from neonatal MyHC to adult MyHC and attenuation of proteins which promote excessive collagen synthesis.	R1- High R2- High

Table 2
Summary of study characteristics and findings of selected supplements on muscle healing

CSA- cross sectional area FOXO3- Forkhead box O3 GAGs- glycosaminoglycans IL-6- interleukin 6 MDA- malondialdehyde mTOR- Mechanistic target of rapamycin myHC-II- myosin heavy chain II (adult) NCP- non-collagenous proteins TGF-β- transforming growth factor beta]

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Data Analysis

Table 3 illustrates the results of the content analysis showing the common tissue healing outcomes and mechanisms of supplementation effect across studies. Outcomes with statistically significant effect only are displayed (p values <0.05). Whilst there is debate around the appropriate use of p-values as a measure of significance (Wasserstein & Lazar, 2016), these tables aim to represent the key findings of the studies examined, rather than making any judgements as to the degree of intervention effect each supplement provides. This allows presentation of the large variability of heterogenous outcomes upon which the reviewer can base the theoretical examination of underlying mechanisms whilst reducing the probability of chance and providing evidence against the null hypothesis. Interestingly, inclusion of outcomes with no significant effect or negative effect did not influence the final conclusions in this review and were removed to focus the analysis.

Thematic analysis indicates that various characteristics of tissue healing effect are shared across studies. Tables 1 and 2 delineate themes of the treatment effects by the outcomes presented in the studies (e.g. biomechanical, biochemical, etc.). However, as the content analysis displays only the outcomes chosen by the original researchers they may be open to publication bias, and other mechanisms may be possible.

Themes were also extracted relative to when mechanisms were observed within normal healing phases (Watson, 2006) and are displayed in figures 2 and 3. Figure 2 illustrates that the changes in the extra-cellular matrix in response to tendon injury (beyond those seen in controls) occur predominantly throughout the inflammatory and proliferation phases of healing (within the first 21 days), but that changes are sustained into the remodelling phase (beyond 42 days). Morphological benefits remain evident for 6 months. Figure 3

demonstrates that the mechanisms of effect in muscle tissue occur earlier and are commenced almost immediately (1 hour) post injury during the bleeding phase. Modification of muscle protein synthesis balance is then sustained during the inflammatory phases, where inflammation and oxidation is attenuated by supplementation.

Further themes are apparent relative to how healing is influenced by supplementation, and patterns are observed relative to whether there is evidence of direct effects on cellular proliferation, or indirect physiological processes which precede healing. Of course, this observation is dependent upon which outcomes are used, but allows exploration of the effects of supplements on the response to injury. Within tendon tissues, mechanisms include enhanced anti-oxidant action; control of immune response; and modification of elements of the extra-cellular matrix (ECM) content. The former effects (anti-oxidant action and immune response control) could be themed as indirect actions to cellular proliferation as they show changes which facilitate the tissues response to allow improved cellular regeneration, but do not directly cause cell synthesis. The latter mechanism (proliferation of ECM constituent components) includes increases in cellular components of collagen (hydroxyproline); balance of fibroblastic response; increases in glycosaminoglycan levels; reduction in non-collagenous proteins; and increases in the size and amount of collagen produced. These could be categorised as direct changes which facilitate proliferation and remodelling of regenerating tissue. In muscle healing, indirect mechanisms are also apparent (e.g. increased anti-oxidant or inflammatory action). However, rather than alterations to the extra-cellular matrix (as direct actions), biomechanical and muscular efficiency changes occur alongside promotion of plasma insulin sensitivity and a reduction in proteolysis. Following injury this would mean the number of damaged muscle fibres is

reduced, and there is improved functional rate of protein synthesis, leading to improved muscle tissue integrity, function and cross-sectional area.

A final theme is apparent relative to the physiological level at which supplements act. The variety of outcomes show effects on tissue at a molecular biological level (e.g. biochemical or genetic change); a cellular biological level (e.g. protein signalling pathways); a histopathological level (e.g. tissue biopsy and microscopic evaluation); and a morphological level (e.g. radiological or clinical examination). The themes show the supplements contribute to healing at various cellular levels, at various times alongside the healing phases, with both indirect and direct effects on the proliferation of tissue. As such there are a variety of outcomes which can be used to demonstrate their effect on healing and the structural integrity of the healing tissue.

TENDON																
		↓ NCP	↑ hydroxyproline concentration	↑ GAGs	↑ anti-oxidant action (reduced MDA/ elevated glutathione)	↑ fibroblast differentiation	↓ immune cell response (macrophages, lymphocytes)	↓ fibroblast proliferation	↑ collagen diameter	↑ collagen proliferation	↓ swelling	↑ tissue perfusion/ oximetry	↓ UTS	↑ UTS or mean maximum stress	Morphologiical evidence of enhanced healing
							ANIMAL STUDIES									
Akdemir et al. (2015)	Taurine					✓	✓	✓			✓			✓		
Vieira et al (2015a)	Glycine		✓	✓						✓				✓	✓	
Vieira et al. (2015b)	Glycine, green tea	✓	✓	✓						✓				✓		
Greenwald et al. (1990)	Vitamin A													✓		
Greenwald et al. (1990)	Vitamin E												✓			
Ömeroğlu et al. (2009)	Vitamin C								✓	✓		✓				
Hung et al. (2013)	Vitamin C				✓			✓			✓					
							HUMAN STUDIES									
Gumina et al. (2012)	Combined integrator 1														✓	
Notarnicola et al. (2012)	Combined integrator 2											✓				

Table 3

Effects of supplementation on physiological parameters of healing in tendon and muscle tissue (statistically significant changes in response to intervention over control presented)

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MUSCLE

		↑ plasma changes to insulin/ glycogen	↓ interleukin 6	↑ MyHC-II gene expression	↓ transcription factor protein encoding relative to cell proliferation (mTOR, FOXO3)	↓ Smad2/3 + nucleus	↓ expression of phosphorylated TGF-β	↓ hydroxyproline	↓ ubiquinated protein	↓ number of damaged fibres	↑muscle strength or function	↑ functional rate of protein synthesis	↑ muscle CSA
ANIMAL STUDIES													
Anthony et al. (1999)	Leucine	✓										✓	
Kato et al. (2016)	Leucine		✓							✓	✓		
Pereira, Baptista et al. (2014)	Leucine		✓		✓				✓				✓
Pereira, Silva et al. (2014)	Leucine		✓	✓		✓	✓	✓				✓	

Table 3 continued

Effects of supplementation on physiological parameters of healing in tendon and muscle tissue (statistically significant changes in response to intervention over control presented)

[Combined integrator 1- Arginine L-α- ketoglutarate, MSM, hydrolysed collagen I, bromelain Combined integrator 2- Arginine L-α- ketoglutarate, MSM, hydrolysed collagen I, bromelain, Vinitrox, vitamin C

CSA- cross sectional area FOXO3- Forkhead box O3 GAGs- glycosaminoglycans IL-6- interleukin 6 MDA- malondialdehyde mTOR- Mechanistic target of rapamycin myHC-II- myosin heavy chain II (adult) NCP- non-collagenous proteins TGF-β- transforming growth factor beta]

ADD FIGURE 2: Mechanisms of tendon issue change with the following:

Figure 2. Graphical representation of mechanisms of supplement effect on tendon tissue relative to musculoskeletal healing phases

(modified as described by Watson, 2006)

ADD FIGURE 3: Mechanisms of muscle tissue change with the following:

Figure 3. Graphical representation of mechanisms of leucine effect on muscle tissue relative to musculoskeletal healing phases (modified as described by Watson, 2006)

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Discussion

This review shows that specific amino acids (leucine, glycine, taurine) and vitamins (A, E and C) provide particular effects on the physiology of musculoskeletal tissue healing. These mechanisms occur at multiple levels (from molecular physiology to morphological tissue structure) and influence cell regeneration directly (cell proliferation) and indirectly (cellular environment enhancement). These changes occur alongside improvements in biomechanical integrity. The findings are predominantly guided by animal experiments, due to the ethical difficulties of performing histological examination of human tissue. The mechanisms described are indicative of the outcomes which demonstrated a degree of statistical significance, and perhaps do not outline all potential mechanisms. Similar effects in skin and experimental models are provided as comparative effects, with awareness that specific regenerative effects are dependent upon the host tissue cells.

A number of studies describe effects on anti-oxidant capacity of the supplements as an indirect influence on tendon healing. Two studies (Hung et al., 2013; Ömeroğlu et al., 2009) examined vitamin C and its role as a free radical scavenger (Buettner & Moseley, 1993). In the presence of reactive oxygen species, vitamin C donates two electrons to reduce the free radical, and a less reactive ascorbyl radical is released. Ömeroğlu et al. (2009) examined a rodent model of Achilles tendon rupture and provided injections of 150mg vitamin C every other day for 21 days. In comparison to saline injections the study found that vitamin C increased tissue perfusion in the early proliferative phase of healing alongside increased fibroblast proliferation (3 days post). At 10 and 21 days, collagen proliferation and diameter is elevated, and at 43 days tendon swelling is less. This may indicate an early healing response, leading to elevated collagen development and longer term benefits. Hung et al.

(2013) demonstrated reductions in fibroblast proliferation at 10 and 43 days (corresponding with the findings of greater balance between collagen formation and fibrosis) and increased glutathione at 14 days, indicating enhanced anti-oxidant action. These changes contributed to reduced fibrotic size and resistance to tendon gliding at 43 days. The influence of a mixed anti-oxidant supplementation (including vitamin C) was measured in a double-blind, randomised, placebo controlled trial of trauma patients with non-musculoskeletal wounds (Blass et al., 2012). The results indicated that anti-oxidants (alongside glutamine) increased rate of healing by faster wound closure time in the supplement group versus placebo (35 days ± 22 vs. 70 days ± 35, $p<0.01$). Examination of topical vitamin C showed similar results, alongside increased collagen fiber density ($p<0.05$) (Lima et al., 2009). These results, alongside the findings of this review, demonstrate an apparent anti-oxidant mechanism to promote similar enhancement of healing in tendon.

Another anti-oxidant is taurine; an amino acid which has been found to regulate collagen production and inhibit fibrosis (Gordon et al., 1986). A controlled animal study examined taurine's effect on skin wound tensile strength and malondialdehyde (MDA) content- a measure of lipid peroxidation and oxidative stress following injury (Abuja and Albertini, 2001; Dinçer et al., 1996). The results demonstrate taurine enhanced wound content and strength relative to histological evidence of enhanced collagen production and greater force displacement ($p<0.01$), and that topical application reduced MDA content more than injection. It could be suggested that increases in wound strength are secondary to increased anti-oxidant capacity and collagen synthesis, and assisted by taurine's ability to aid vitamin C metabolism (Kaplan et al., 2004). Akdemir et al. (2015) examined the effect of 200mg taurine injections post rodent Achilles tendon repair, measuring UTS and histopathological

assessment after 42 days. Their results found reductions in immune cell activity, reduced fibroblast proliferation/ increased fibroblast differentiation, and increased UTS compared to saline control. The authors suggest taurine may reduce fibrosis and elevate repair integrity, and postulate this is due to both anti-oxidant and anti-inflammatory effects, and inhibition of fibronectin expression and fibrin formation during healing. This suggests that the influence of taurine on tendon wounds, is at least in part, in concurrence to the findings in skin wounds.

Other supplements included in this review demonstrate more direct influences on tendon healing. Previous work by Ehrlich et al. (1973) examined the effect of vitamin A on the restoration of hydroxyproline content and histological tissue grading after provision of gluticorticoid which inhibits collagen synthesis and connective tissue repair. A rodent model of implanted polyvinyl sponge granuloma was used with sub-grouping into control, corticoid alone, vitamin A alone and combined corticoid and vitamin A. Injection was the route of administration for all groups. The results indicated vitamin A was able to partial reverse declines in fibroblast, collagen and hydroxyproline content diminished by gluticorticoid injection. Previous work by the same authors show that healing rate of skin wounds, not inhibited by cortisone, is not enhanced by vitamin A provided topically or via intramuscular systemic administration (Hunt et al., 1969). There is therefore some confusion as to whether vitamin A can influence collagen accumulation. One study in this review examined the effects of vitamins A (150,00 IU/kg) and E (1000 IU/kg) in a model of tendon repair healing in chickens (Greenwald et al., 1990). The animals were given daily oral doses and ultimate tensile stress (UTS) was evaluated at 7 and 45 days post repair. The results show an increase in UTS at both 7 days (897g +/- 164 vs. 356g +/- 68, $p<0.007$) and 45 days (1972g +/- 255 vs.

915g +/- 113, p<0.001) which supports the findings of Ehrlich et al. (1973), however without concurrent histopathological evidence of collagen change, this is speculative.

A systematic review examining the effectiveness of vitamin E on measures on cell proliferation, infection and wound healing found a dearth of robust studies in this area (Hobson, 2016). This is despite the search finding a high number of primary research studies (n=31) and representing a reasonable level of quality (level 2b). Greenwald et al. (1990) found a reduction in UTS compared to control at 7 (163g +/- 81 vs. 356 +/- 68, P<0.004) and 45 days (445g +/- 125 vs. 915g +/- 113, p<0.001) indicating vitamin E has a deleterious influence on healing tendon. Vitamin E has been shown to act as a lysosomal membrane stabiliser (Ehrlich et al., 1972). Lysosomes- organelles responsible for the enzymatic responses to injury to balance matrix regeneration (Stromberg et al., 1977)- have been found to inhibit tendon healing (Ehrlich et al., 1972) leading to a reduction in collagen cell number and decreased UTS. It is possible that similar mechanisms may explain these results.

Dietary arginine above recommended daily allowance has been shown to increase collagen accumulation in healthy animals (Barbul et al., 1990; Kirk et al., 1993). This review found no studies investigating the effect of this amino acid in animal models. Two human studies examined the effects of arginine L-α-ketglutarate within a combined integrator. The studies examined the effect of the supplement on rotator cuff repair integrity (Gumina et al., 2012); and tissue oximetry in Achilles tendinopathy (Notarnicola et al., 2012). Gumina et al. (2012) combined arginine with methylsulfonylmethane, hydrolysed collagen I and bromelain, for a dosing period of 12 weeks. Subsequently, they evaluated repair integrity in the intervention group against a no-supplement control and found a higher incidence of more favourable

Sugaya classification type I-II repair intensity (Sugaya et al., 2007); and a lower rate of re-rupture. Unfortunately, whilst the trial randomised and concealed allocation, they failed to report the dosage of supplements provided in the integrator. Additionally, the trial did not incorporate drop outs to the final analysis and failed to utilise a placebo control, and subsequently demonstrates a high risk of bias.

Polytetrafluoroethylene implants allow researchers to evaluate deposition of fibroblasts and extracellular matrix tissue. A double-blind trial (Williams et al., 2002) randomised healthy adults (n=18) to receive daily amino acids (14g arginine, 3g HMB, 14g glutamine) or an isonitrogenous, isocaloric control supplement of non-essential amino acids. Implants removed at 7 or 14 days were analysed for hydroxyproline and amino-nitrogen. Whilst no significant differences in collagen accumulation (measured by hydroxyproline) were evident at 7 days, at 14 days there were significant increases in hydroxyproline content accumulated (+67%, $p<0.03$); occurring independent of an increase in total protein deposition. Evidence from elderly humans (Kirk, 1993) supports these results, demonstrating a 52% collagen increase, via a pro-inflammatory mechanism of action and enhancement of fibroblastic synthesis. During healing fibroblasts stimulate collagen synthesis deposition within the ECM (Stechmiller et al., 2005); and the production of growth factors (e.g. insulin-like growth factor 1, transforming growth factor β) promotes proliferation, angiogenesis and protein synthesis (Schultz & Mast, 1998). During the remodelling phase fibroblasts produce ECM components (collagen, gelatin and proteoglycans), and release metalloproteinases and tissue inhibitors of metalloproteinases to orchestrate tissue remodelling (Bryant, 2000; Schultz & Mast, 1998; Tarnuzzer & Schultz, 1996).

The other human study in this review (Notarnicola et al., 2012) used the same combined integrator, however also added 60mg vitamin C. Subjects (n=64) were randomised and matched for age and gender and the integrator (or placebo) was provided orally for 60 days with or without extra-corporeal shockwave therapy (ESWT). The findings show that the supplement/ ESWT group had a reduced oximetry value at 6 months indicating a reduction in tendon micro-circulation and neovessel development; a component of the pathophysiology of tendinopathy (Knobloch, 2008). This study demonstrates a low risk of bias as it appropriately used randomisation, blinding, allocation concealment, intention-to-treat analysis and group matching for homogeneity. However, for both studies using the combined integrator, effects are confounded by the use of multiple ingredients and caution is needed in extrapolating these results.

The final supplement which evaluated an effect on tendon tissue is glycine; an amino acid synthesised by other amino acids, including hydroxyproline. Experiments demonstrate glycine's capacity to prevent inflammatory cell infiltration and reduce joint oedema following injuries of experimental arthritis (Li et al., 2001). It is proposed that this anti-inflammatory effect involves glycine receptor activation in leukocytes and suppression of immunocytes (Li et al., 2007). Two studies (Vieira, De Oliveira et al., 2015; Vieira, Guerra et al., 2015) assessed the effect of glycine (with or without green tea) in a rodent collagenase-induced Achilles' tendon injury model. Each provided a 5% glycine diet and showed increases in collagen proliferation, alongside elevated hydroxyproline, non-collagenous protein content and glycosaminoglycan content at 7 days. Hydroxyproline, glycosaminoglycans (GAGs) and non-collagenous proteins (NCPs) are components of the extracellular matrix. Hydroxyproline is a dominant protein of the ECM, comprising 20% of

fibrillary collagen structure; contributing to its molecular stability (Mouw et al., 2014). The non-collagenous matrix consists of GAGs (and other molecules including glycoproteins, proteoglycans) which surround collagen fibrils and bind water to assist mechanical tolerance to stress (Kannus, 2000). Increased tendon content of these ECM constituents, through glycine supplementation, may enhance collagen synthesis during the early proliferation phase, augmenting tensile strength. The addition of green tea to the glycine diet seems to enhance collagen organisation at 7 days leading to further fibre stability and load tolerance.

In vivo and *in vitro* experiments indicate that the branched chain amino acid leucine acts as a signalling molecule to regulate protein synthesis in skeletal muscle (Anthony, Anthony et al., 2000; Kimball & Jefferson, 2001; Norton & Layman, 2006). Leucine effects at a posttranscriptional level as a critical regulator of mRNA translation initiation (Anthony et al., 2001) which facilitates protein synthesis (Svanberg et al. 1997, Yoshizawa et al., 1998). The role of mammalian target of rapamycin (mTOR) signalling is essential for translation initiation (Anthony, Yoshizawa et al., 2000). Leucine facilitates an initial release of insulin (or increased insulin sensitivity in muscle); alongside a signalling cascade independent of phosphoinositol 3-kinase (PI3-K), protein kinase B (PKB/ Akt) or 3-phosphoinositide dependent protein kinase 1 (PDK1) activation (Anthony et al. 2001). At mTOR these processes facilitate optimal activation of translation initiation, and are fine-tuned by other unidentified pathways (Anthony et al., 2001). Four studies found in this review evaluated the effect of leucine on healing muscle tissue (Anthony et al., 1999; Kato et al., 2016; Pereira, Baptista et al., 2014; Pereira, Silva et al., 2014). The studies outline a process of an immediate response in the bleeding phase to attenuate inflammation and increase protein synthesis, starting with increased serum insulin and glycogen within one hour and elevated

fractional rate of protein (FRP) synthesis (Anthony et al., 1999). Reduction of the pro-inflammatory cytokine, interleukin-6, occurs alongside reduction in muscle fibre damage. Subsequently, cross sectional muscle area is increased, with reduction in protein ubiquination; reduction in hydroxyproline as a proxy for reduced collagen; and modification of transcription signalling pathways to attenuate cell proliferation (e.g. expression of phosphorylated TGF-beta/ reduced Smad2/3+ nucleus). Pereira, Baptista et al. (2014) identify that the reduction in mTOR expression occurs without other changes to the PI3K/ Akt/ mTOR pathways and that the change in FOXO3a expression is indicative of reduced post exercise proteolysis. Additionally, Pereira, Silva et al., (2014) also demonstrates a genetic shift towards adult Myosin Heavy Chain which assists muscle action. Alongside the leucine-assisted changes to collagen synthesis, this may suggest a fine tuning of healing response to ensure muscle contractile action is balanced with collagen formation. In summation leucine acts during bleeding/ early proliferation to enhance muscle regeneration.

Implications/ Limitations

This review summarises potential mechanisms for how selected supplements can influence tendon and muscle healing, the basis of which are limited to the outcomes used by the original studies. As no consensus is clear across the literature, minimal recommendations can be given as to the clinical utility of such supplements for musculoskeletal healing. Clinicians should however remain cognisant of nutritional practices throughout rehabilitation, particularly in respect to the regenerative influences of vitamins and amino acids.

As the predominant source of this data is from animal models it is essential to reflect on their use to guide human consumption. Animal models can approximate human physiological response to injury (Woo & Buckwalter, 1988) and act as a test for wound healing agents (Gottrup et al., 2000). However, all animal studies analysed showed a high risk of bias, indicating common methodological shortcomings. Lack of blinding, randomisation, or simply poor reporting would be assisted by adherence to the Animals in Research: Reporting in vivo experiments (ARRIVE) guidelines (Kilkenny et al., 2009) and support future developments in research. Additionally, two articles are likely influenced by confounding variables; specifically extra-corporeal shockwave therapy (Notarnicola et al. 2012) and the addition of green tea (Vierra, Guerra et al. 2015), which must be considered when evaluating supplement effects. An additional limitation of this research is that the search results did not produce evidence for effects of supplements commonly associated with recovery such as branched-chain amino acids (Negro et al., 2008; Sharp and Pearson, 2010). This is likely due to the strict inclusion criteria of randomised trial design. As such this review may not sufficiently elucidate all mechanisms of tissue response to injury related to amino acids supplementation.

Future Research

High quality animal experimentation studies investigating the effects of supplements on molecular, cellular and whole tissue levels; utilising outcomes of histological evidence of tissue synthesis and biomechanical integrity; and at time points throughout the healing process, need to be conducted to elaborate further on these findings. Human studies should

concentrate on assessing the effectiveness of supplements compared to isocaloric/ isonitrogenous placebo and be investigated with clinical and radiological outcomes to evaluate whether the proposed mechanisms translate to practice.

Conclusions

Amino acids and vitamins demonstrate both indirect (anti-oxidant) and direct (synthesis rate modifying) mechanisms of action in healing of tendon and muscle in animal models. These mechanisms act at various stages of the healing cycle, and work on all physiological levels from molecular to morphological. The translation of these mechanisms in humans is speculative, however there is potential that supplements may provide some clinical utility. Further research is required to test these hypotheses.

[Words 4484]

Authorship and Conflict of interest

All below stated authors have made contributions to this thesis.

Mr Christopher Tack- Primary investigator and author

Mrs Faye Shorthouse- Secondary data extraction and critical appraiser

Ms Lindsy Kass- Dissertation supervisor

I declare that I am the primary author of this article and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this article to any other publication.

I declare that I have no affiliations with or involvement with any entity or organisation with any financial interest or non-financial interest in the subject matter of this manuscript.

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