Effects of resistance training with hydrolysed collagen supplementation on changes in muscle-tendon properties in young male and female athletes

Joonsung Lee

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COVID-19 preamble – Lead Supervisor Statement

The COVID-19 pandemic has had a significant impact on many people's lives. Academia and research have not been exempt from this pandemic, and for many research students, this period of time has affected the progress and outputs of their research programme.

Joon has experienced negative impacts on his research progress and outputs from:

- Closure of university research laboratories and offices, limiting the collection of complete data sets and the analysis of data requiring specialist software only available on specific university equipment;
- Limited or no access to research populations due to UK Government-enforced national lockdowns preventing individuals from completing study requirements, either at the University or their place of work;
- Travel restrictions affecting research progress;
- Financial restraints to funding research costs;
- Closure of on-campus offices for staff and students;
- Effect of repeated lockdowns and social isolation on mental health;
- Any of the above issues affecting access to timely feedback from or engagement with supervisors.

Due to the nature of Joon's PhD project, he was unable to shift to 'online and remote data collection' when COVID-19 restrictions were initiated by the UK Government. His progress was particularly affected during his final three studies (Chapters 5-7), all of which were exercise training-nutritional intervention studies. *The timing of these studies is not reflected in the order of the chapters within this thesis.* For example, the study described in the final empirical chapter (Chapter 7) was the first to be severely impacted by the

pandemic. This study started in January 2020 and the training duration was due to last 12 weeks. However, due to the sudden closure of the University in March 2020 (linked to the UK's first full national lockdown), Joon was forced to bring the study to an abrupt end after the participants had completed just six weeks' training. He managed to perform as many follow-up tests as possible in the few days prior to University closure but was unable to test all of his participants. This inevitably led to a smaller sample size than planned and, although Joon tried to repeat this training study on subsequent occasions to increase sample size, numerous ensuing national lockdowns made this impossible. Therefore, after discussion with his supervisory team, Joon decided to report the data he collected in the original six-week study, being mindful and transparent about the probable lack of statistical power, and to place more emphasis on effect sizes than p-values.

Chapter 5 took place February to May 2021 (also during a COVID-19 UK national lockdown), during which time only professional athletes were legally permitted to attend their place of work (and the University for research purposes). The general public and university students were not permitted to attend either the University laboratories or their places of work. Fortunately, for this study we were able to recruit participants from a Football Association Women's Super League football club. Joon was given special dispensation from the UK Government via the University's Senior Management Team to undertake this research study at the University. The fact that no other university staff or students were available to help at the time affected the design of this study. For example, under normal conditions, this would have been a double-blind nutritional intervention study but, as Joon needed to prepare the nutritional supplements himself, he could not be blinded to participant group allocation.

Chapter 6 was performed between July and September 2021, also a time of heavy restrictions associated with the COVID-19 pandemic. For the reasons stated above, this study was also a single-blind design. Furthermore, this study was hampered by some participants contracting COVID-19 and some developing injuries (unrelated to the study), and therefore being unavailable for testing, which limited the sample size.

Considering all of the above, when reviewing this thesis, please be mindful of the extraordinary challenges Joon has had to overcome. Many thanks for your understanding.

Yours sincerely,

Rob Erskine

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Abstract

Human tendon adapts to mechanical loading (i.e. resistance exercise (RE)) by changing its morphological, mechanical, and material properties. Type I collagen is the most abundant protein in tendon, and an exercise-induced increase in the rate of type I collagen synthesis has been proposed as one of the key factors that drives human tendon adaptation.

In addition to the role of exercise on tendon adaptation, a recent study has found that jump-rope exercise with gelatine ingestion increases whole body type I collagen synthesis in a dose-response manner, with 15 g being superior to 5 g and 0 g gelatine in the same group of young men. In a separate study, however, moderate intensity resistance exercise with 30 g hydrolysed collagen (HC) ingestion did not appear to increase muscle connective tissue protein synthesis in separate groups of mixed-sex cohorts. However, there are numerous limitations with these studies that preclude a definitive conclusion on the effects of RE with collagen supplementation on collagen synthesis. Accordingly, the first aim of this thesis was to investigate the effects of *high-intensity* RE combined with different doses of HC on a systemic marker of collagen synthesis, and to investigate a dose-response relationship between HC ingestion with an acute bout of RE and markers of collagen turnover in resistance-trained men (Chapter Three). For this study, 10 healthy, resistance-trained, young men were recruited, the findings demonstrated the dose × time area under the curve (AUC) of a biomarker of type I collagen synthesis (serum procollagen type I amino-terminal propeptide, PINP) was higher in the 30 g HC intervention compared to the 15 g (P = 0.039) and 0 g (P = 0.005) HC interventions but there was no difference between 15 g and 0 g (P = 0.675) HC. Similarly, the dose × time AUCs for glycine and proline (the most abundant amino acids in collagen) were greater for 30 g

than for 15 g and 0 g HC ($P < 0.05$). However, a biomarker of type I collagen breakdown (plasma beta-isomerized C-terminal telopeptide, β-CTX) decreased after 6 h post-exercise ($P < 0.05$) regardless of HC dose, suggesting RE suppressed collagen breakdown regardless of collagen ingestion.

The first aim of the second experimental study (Chapter Four) was to investigate whether the collagen synthetic response to high-intensity RE could be increased following 30 g HC ingestion in a eumenorrheic, resistance-trained, young woman. The second aim of this study was to determine if this effect was associated with circulating endogenous oestrogen concentration. In this case study ($n = 1$), RE with and without 30 g HC was performed when circulating oestrogen concentration was low (onset of menses, OM); and when it was high (late follicular phase, LF) during two consecutive menstrual cycles. Serum 17β-oestradiol concentration was 5-fold greater at LF (891 ± 116 $pmol \cdot L^{-1}$) than at OM (180 ± 13 $pmol \cdot L^{-1}$). The PINP AUC was higher in the 30 g OM intervention (201 $\mu g \cdot L^{-1} \cdot h$) than the 30 g LF (144 $\mu g \cdot L^{-1} \cdot h$), 0 g OM (151 $\mu g \cdot L^{-1} \cdot h$), and 0 g LF (122 $\mu g \cdot L^{-1} \cdot h$) interventions. Plasma β-CTX concentration decreased 1.4-fold after 6 h post-RE, regardless of HC dose or menstrual cycle phase. Thus, high endogenous oestrogen concentration was associated with lower collagen synthesis following RE in a eumenorrheic, resistance-trained woman but ingestion of 30 g HC with RE augmented the collagen synthetic response at both LF and OM (having its greatest effect at OM).

Based on findings from Chapters Three and Four, in which 30 g HC with RE showed greater collagen synthesis response compared to 15 g and 0 g HC, the third (Chapter Five) and fourth (Chapter Six) experimental studies aimed to investigate the effect of 30 g HC with 10 weeks' soccer training (incorporating resistance/plyometric exercise) on changes

in patellar tendon (PT) properties in female soccer players. In Chapter Five, 17 female soccer players from the Under 21s squad of a Football Association (FA) Women's Super League Football Club were allocated into two groups: HC (COL, $n = 8$) and placebo (PLA, $n = 9$). Participants performed three training sessions per week, which comprised body-weight resistance/plyometric exercise and pitch-based exercise for 10 weeks in-season. COL and PLA consumed 30 g HC and energy matched maltodextrin, respectively, immediately after each training session. COL increased PT stiffness (+18.0 ± 12.2% *vs*. +5.1 ± 10.4%, $P = 0.049$) and Young's modulus (+17.3 ± 11.9% *vs*. +4.8 ± 10.3% $P = 0.035$) more than PLA. However, maximum isometric knee extension (KE) torque, vastus lateralis (VL) muscle thickness, the mean PT cross-sectional area (CSA) did not change in either group. The lack of change in PT CSA might have been caused by not enough RE intensity and thus, the aim of Chapter Six was to investigate the effect of including *high-intensity* RE into soccer training combined with HC or PLA within a FA Women's Championship first team soccer squad during pre-season. Eleven professional soccer players were allocated into COL ($n = 6$) and PLA ($n = 5$). Using the same method as used in Chapter Five, participants consumed 30 g HC or energy matched maltodextrin (PLA) immediately before each training session, which comprised externally loaded resistance exercise (75% – 90% of one-repetition maximum), plus plyometric- and pitch-based exercise three days/week for 10 weeks in the pre-season period. The results from Chapter Six showed that PT stiffness (COL, +15.4 ± 3.1% vs. PLA, +4.6 ± 3.0%, $P = 0.002$) and Young's modulus (COL, +14.2 ± 4.0%vs. PLA, +3.4 ± 2.8%, $P = 0.004$) increased more in COL than in PLA. Although PT CSA increased in both groups ($P < 0.05$), there was no difference in the percentage change between groups ($P > 0.05$). So, although the *high-*

intensity RE led to PT hypertrophy, the relatively low frequency of (once a week) was probably insufficient to enable to HC to further augment this hypertrophic effect.

The aim of the final experimental study (Chapter Seven) was to investigate the effects of 30 g HC ingestion with a shorter (six weeks) period of high-intensity RE on muscle-tendon adaptations in resistance-trained healthy young men. COL (n = 5) and PLA (n = 7) consumed 30 g HC or energy-matched maltodextrin (PLA), respectively, immediately before each training session, which was performed twice a week for six weeks. There were no group differences in the changes in PT properties following six weeks' resistance training ($P > 0.05$). However, changes in PT stiffness (+10.1 ± 6.8%; $P = 0.019$; $d = 1.7$ *vs.* +4.5 ± 5.0%; $P = 0.061$; $d = 0.9$) and Young's modulus (+8.7 ± 7.4%; P = 0.085; d = 1.0 *vs.* +3.3 ± 5.2%; P = 0.151; $d = 0.6$) were approximately 2.2 times greater in COL than in PLA with large effect sizes for changes in PT stiffness and Young's modulus in COL compared to PLA, suggesting that the outcome may have been different with a larger sample size and/or a longer training duration. Although both groups increased mean PT CSA, VL muscle thickness, anatomical-cross sectional area and volume ($P < 0.05$), these changes did not differ between groups ($P > 0.05$).

In summary, this thesis demonstrates a dose-response effect of HC ingestion with a single bout of high-intensity RE on whole body collagen synthesis, and a chronic effect of HC supplementation with exercise training (incorporating muscle-tendon overload) on changes in tendon properties. For resistance-trained healthy young men, 30 g HC with RE conferred a greater marker of collagen synthesis compared to 15 g and 0 g HC. Further, for a eumenorrheic, resistance-trained woman, the collagen synthetic response was

greater when 30 g HC was ingested compared to RE alone, and a lower endogenous oestrogen concentration was associated with a higher whole body collagen synthesis response to RE. Regarding the chronic effect of HC ingestion with RE on muscle-tendon adaptation, 30 g HC ingestion with 10 weeks' soccer training (incorporating resistance/plyometric exercise) increased tendon stiffness and Young's modulus in female soccer players. Similarly (although non-significant), 30 g HC ingestion with six weeks' RE had a positive effect on changes in PT stiffness and Young's modulus in resistance-trained healthy young men. Future research should investigate if these novel findings might translate to improved athletic performance and/or reduced risk of soft tissue injury in athletic populations.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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The participants who took the time to contribute to the experimental part of my studies, and without whom nothing would have been feasible. Further thanks go to the technicians who kindly assisted me during my sessions at the Tom Reilly Building and everyone who has helped with the data collection in some way including Joe Page, Josh Bridge, Dr. David Robshaw, Dr. Stephen McQuilliam and Danielle Williams. Special thanks to Chris Nulty for helping me with my experiment and it was always enjoyable talking about projects and preparing the conference as well as my colleagues, Changki Park and Seokwon Lee, for helping me improve my research skills and competencies.

My biggest thanks to my Mum and Dad, the never-ending support you have shown me all these years and the unconditional love that constantly inspires me to do better and improve myself. Lastly, I would like to thank my partner, Jennie who always supported me throughout the whole process.

List of Abbreviations

ACSA; Anatomical cross-sectional area

AT; Achilles tendon

β-CTX; Beta-isomerized C-terminal telopeptide

BFlh; Biceps femoris long head

BWSE; Bodyweight strength exercise

COL, Collagen group

CPS, Collagen protein synthesis

CSA; Cross-sectional area

CTX-I; Carboxy-terminal cross-linking telopeptide of type I collagen

ECM; Extracellular matrix

EMG; Electromyography

L_f; Fascicle length

θ_p; Fascicle pennation angle

FFM; Fat-free mass

FSR; Fractional synthesis rate

FPS; Fractional protein synthesis

HC; Hydrolysed collagen

HRT; Hormone replacement therapy

IKD; Isokinetic dynamometer

IL; Interleukin

KE; Knee extension

KF; Knee flexion

MC; Menstrual cycle

MIVC; Maximum isometric voluntary contraction

MMPs; Matrix metalloproteinases

Mammalian target of rapamycin (mTOR)

Mammalian target of rapamycin complex 1 (mTOR1)

MTU; Muscle-tendon unit

V_m; Muscle volume

MVC; Maximum voluntary contraction

1-RM; One-repetition maximum

OCP; Oral contraceptive pill

PBS; Pitch-based session

PCSA; Physiological cross-sectional area

PINP; Procollagen type I amino-terminal propeptide

PICP; Procollagen type I carboxy-terminal propeptide

PLA; Placebo group

PLY; Plyometric exercise

PT; Patellar tendon

RE; Resistance exercise

RF; Rectus femoris muscle

RFD; Rate of force development

RMS; Root mean square

RMVC; Ramped isometric maximal voluntary contraction

RT; Resistance training

SAT; Subcutaneous adipose tissue

VL; Vastus lateralis muscle

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Table 6. Patellar tendon morphological, mechanical, and material properties in PLA and COL groups before (PRE) and after (POST) training. Data are mean ± SD.

Chapter Seven

Chapter One

General Introduction

1.1 Introduction

Force produced by skeletal muscle contraction is transmitted via tendon to bone to maintain posture and initiate movement (Trotter, 2002). Thus, a tendon's load-bearing capacity is essential for the maintenance of a healthy musculoskeletal system in order to complete athletic and daily life tasks. Tendons bear shear and compressive force (Khan and Scott, 2009) and joint loads during walking, running, and jumping are approximately four to eight times greater than body weight (Finni et al., 2000, Giddings et al., 2000). However, excessive repetitive stretching of tendon can cause a non-rupture injury in tendon, termed tendinopathy (Scott et al., 2015). Incidence of tendinopathy is common in professional and recreational athletes (Ferretti, 1986, Frost et al., 2002, Woods et al., 2002, Zwerver et al., 2011) and may require surgery and prolonged rehabilitation.

However, regular controlled mechanical loading, e.g. in the form of chronic resistance exercise (RE), or resistance training (RT), can improve tendon mechanical properties in both young (Seynnes et al., 2009) and older (Reeves et al., 2003a) individuals. These increases in tendon properties are likely due to changes in the constitution of the tendon. For example, human tendon comprises 60 – 85% collagen, with type I collagen being the most abundant fibril-forming collagen (Kjaer, 2004). An acute bout of RE can increase type I collagen synthesis in the patellar tendon (Miller et al., 2005) and the peritendinous space (Olesen et al., 2007). Further, Kubo et al. (2012) found that a marker of collagen synthesis (procollagen type I carboxy-terminal propertied) and Achilles tendon stiffness were increased after three months' RT in healthy young men, which suggests that an RE-induced increase in collagen synthesis leads to an increase in tendon stiffness. Further,

chronic mechanical loading induces a greater density of tendon collagen fibrils and collagen cross-linking, which is associated with greater tendon size and stiffness (Couppé et al., 2014, Couppé et al., 2021).

Exercise-induced human tendon adaptation is well addressed, and recent research has investigated the effect of exercise with collagen supplementation on changes in collagen synthesis. Hydrolysed collagen (HC) and gelatine are collagen-derived supplements that are commonly available (Daneault et al., 2017), with glycine, proline and hydroxyproline being the most abundant amino acids in these supplements (Alcock et al., 2019). Fifteen grams' gelatine intake with jump-rope exercise increased a marker of collagen synthesis (procollagen type I amino-terminal tripeptide, PINP) approximately two-fold more than 5 g gelatine intake in healthy young men (Shaw et al., 2017). Also, collagen content in engineered ligament (treated with the serum obtained from the male participants after consuming 15 g gelatine and performing the exercise) was higher than the control engineered ligament (Shaw et al., 2017). This suggests that consumption of HC with RE may confer greater tendon adaptation compared to exercise alone. However, a recent study found that 30 g HC intake with an acute bout of moderate-intensity RE neither increased serum PINP concentration nor muscle connective protein synthesis compared to RE with 30 g whey protein or RE alone in healthy young men and women (Aussieker et al., 2023). It should, however, be noted that this study implemented a between-group design and RE (barbell back squat exercise) intensity of 60% of one-repetition maximum (1-RM). Thus, between group variability may have confounded any effect of HC and, as exercise intensity is one of the key variables to induce exercise-induced tendon adaptation (Bohm et al., 2015), it is unknown whether HC intake with high-intensity RE would further increase collagen synthesis or not.

Although a very limited number of studies had focused on the acute effect of HC on collagen synthesis when this PhD project began in 2018, no studies had investigated the effect of RT with collagen supplementation on human tendon adaptation. Thus, it was unknown whether HC intake with high-intensity RE would further increase collagen synthesis in young, resistance-trained athletes, or not. It was also unknown whether there would be a dose-response relationship between HC dose (e.g. 30 g *vs.* 15 g HC) with high-intensity RE and collagen synthesis, as Shaw et al. (2017) found a dose-response relationship between gelatine intake with an impact-type exercise (skipping) and collagen synthesis in healthy young men. Secondly, previous research had focused on changes on body composition (Kirmse et al., 2019), muscle size (Balshaw et al., 2023a), or rate of force development (Lis et al., 2021) following different doses of HC (15 g – 20 g) with RT in young population. Although very recent studies have found evidence for an effect of RT and HC supplementation on changes in human tendon properties in previously untrained male participants (Jerger et al., 2022, Balshaw et al., 2023b, Jerger et al., 2023), the effect of HC intake on tendon adaptation in young female athletes is unknown.

1.2 Aims and Objectives

The overarching aims of this PhD thesis are to investigate (i) the acute effect of HC intake with a single bout of RE on markers of collagen turnover; and (ii) the chronic effect of HC supplementation with 6 – 10 weeks' RT on changes in muscle-tendon properties in young, resistance-trained male and female athletes.

The PhD project has the following objectives:

1. To investigate a dose-response relationship between HC ingestion and markers of collagen turnover following an acute bout of RE in resistance-trained, young men. This

is addressed in the work described in Chapter Three.

2. To ascertain the individual and combined effects of resistance exercise, collagen ingestion and circulating oestrogen concentration on markers of collagen turnover in a eumenorrheic, resistance-trained, young woman. This is addressed in the work described in Chapter Four.
3. To establish the effect of chronic HC intake (three times a week) with 10 weeks' *in-season* soccer training (incorporating bodyweight plyometric and strength exercises, as well as pitch-based exercise four times a week) on changes in muscle-tendon properties in female *academy* soccer players. This is addressed in the work described in Chapter Five.
4. To determine the effect of 10 weeks' *pre-season* soccer training (incorporating *high-intensity* resistance training once a week, plus plyometric and pitch-based exercise three times a week) supplemented with HC intake (three times a week) on changes in muscle-tendon properties in *professional* female soccer players. This is addressed in the work described in Chapter Six.
5. To investigate the effect of six weeks' *high-intensity* resistance training (performed twice a week) with HC supplementation on changes in muscle-tendon properties in resistance-trained, young men. This is addressed in the work described in Chapter Seven.

Chapter Two

The effect of exercise and nutritional supplementation on changes in muscle-tendon unit properties in young individuals: A narrative review of the literature

2.1 Introduction

It is well established that human tendon undergoes morphological and mechanical adaptation in response to chronic resistance exercise (RE) (Arampatzis et al., 2007, Kongsgaard et al., 2007, Seynnes et al., 2009), plyometric exercise (Foure et al., 2010) and habitual loading (Couppé et al., 2008, Couppé et al., 2021). An acute bout of resistance-type exercise increases collagen synthesis in the patellar tendon (PT) of healthy young men (Miller et al., 2005) and chronic resistance training (RT) concomitantly increases a biomarker of collagen synthesis (procollagen type I carboxy-terminal propeptide (PICP)) and tendon stiffness in young healthy males' Achilles tendon (AT) (Kubo et al., 2012). Thus, an increase in collagen synthesis is likely one of key factors that drives remodelling of human tendon in response to mechanical loading.

In addition to the role of RT on tendon adaptation, nutritional supplements also have beneficial effects on soft tissue, such as skeletal muscle and tendon, when they are combined with exercise. A recent study found that another biomarker of collagen synthesis, procollagen type I amino-terminal propeptide (PINP), was increased in a dose-response manner following consumption of different amounts of gelatine (0 g, 5 g and 15 g) with an acute bout of impact-type exercise (skipping) in healthy, young men (Shaw et al., 2017). It is therefore possible that, by combining collagen supplementation with regular exercise, such a strategy may help to improve tendon health and physical performance in both the general public and athletes alike (Baar, 2017, Baar, 2019).

However, there is limited evidence of the effect of long-term hydrolysed collagen (HC) ingestion with exercise on tendon adaptation, and the evidence that is available is equivocal. Previous studies in this area have mainly investigated the effect of HC with chronic

resistance training on body composition (Kirmse et al., 2019, Zdzieblik et al., 2021), muscle size (Balshaw et al., 2023a), rate of force development (Lis et al., 2021) and pain and function in tendinopathy (Praet et al., 2019). A recent study showed 5 g HC ingestion with 14 weeks' high-intensity RT increased AT (Jerger et al., 2022) and PT (Jerger et al., 2023) cross-sectional area (CSA) compared to RT alone, with no difference in stiffness or modulus changes between groups. The fact that serum PINP concentrations following ingestion of 0 g gelatine was not significantly different to 5 g gelatine (Shaw et al., 2017), suggests that a greater amount of HC may be required to change tendon mechanical properties, although the optimal dose of HC is not yet known.

This review will identify knowledge gaps in the current literature with regards to exercise-nutritional interventions designed to increase collagen synthesis after a single bout of exercise, or tendon adaptation following a period of RT. The content is presented in four sections. Firstly, the structural and mechanical properties of human tendon will be briefly covered. Secondly, an overview of collagen turnover and methods used to measure turnover will be provided. Thirdly, the impact of exercise training protocols on morphological and mechanical properties of human tendon and how oestrogen influences collagen synthesis in women will be presented. The fourth and final section will discuss roles for nutritional supplementation on collagen synthesis and tendon adaptation.

2.2 Basic tendon structure and function

Tendon composition and structure

Tendon is a fibrous connective tissue, which consists of 55 – 70% water and 30 – 45% extracellular matrix (ECM) proteins, including collagens, proteoglycans, glycosaminoglycans, elastin, fibronectin, laminins, and glycoproteins (Elliott, 1965, Kjaer, 2004).

Among the ECM proteins, collagen is the most abundant, with type I collagen comprising 60 – 80% tendon dry mass (Kjaer, 2004), together with other fibril-forming (i.e. type III and V) and fibril-associated collagens with interrupted triple helices (i.e. type XII and XIV) (Amiel et al., 1983, Zhang et al., 2005).

The structure of the collagen molecule is a triple helix comprising three polypeptide chains called α chains. For example, type I collagen has two identical α1 chains, while the third chain is α2 (i.e. $[\alpha1(I)]_2$ and α2(I)). Each polypeptide has a repeated amino acid sequence (glycine–X–Y), where proline and hydroxyproline are frequently occupied in X and Y positions, respectively (Kadler et al., 1996).

Tendon structure is hierarchical, having distinct connective tissue compartments. Tropocollagen is the basic unit of collagen fibrils, which are packed in parallel bundles, constituting a collagen fibre (Benjamin et al., 2008). A collagen fascicle (a bundle of collagen fibres) is surrounded by the endotenon, which is continuous with a further sheet of connective tissue, called the epitenon (Benjamin et al., 2008).

Function of tendon

As tendon attaches muscle to bone, its main role is to transmit the force produced by skeletal muscle to induce joint movement and/or stability. When tendon is passively elongated during a muscle contraction, three different regions in the tendon stress-strain curve are observed. The initial region is known as the toe region, where collagen fibres are initially 'stretched' from their crimp formation under low load, leading to a nonlinear stress-strain relationship. As the load increases, the collagen fibres are stretched beyond their normal resting (uncrimped) length, leading to a linear stress-strain relationship be stretched. This region is called the linear region and, if the tendon is further strained, the

tendon eventually fails in its load-bearing capacity, causing either micro-tears in the fascicles or complete tendon rupture (Butler et al., 1978).

2.3 Collagen turnover and methods for measuring collagen turnover in humans

Collagen synthesis is a complex chain of events, including co-translational and post-translational modifications. In brief, the first step of collagen fibrillogenesis is the synthesis of collagen's precursor in the endoplasmic reticulum in fibroblast, called procollagen, which has globular extensions at both ends, called the amino (N)- and carboxy (C)-terminals (Canty and Kadler, 2005). The cleavage of N- and C-terminal propeptides by two specific enzymes (i.e. procollagen N- and C-proteases) in the ECM is a prerequisite for the assembly of the collagen fibril (Canty and Kadler, 2005). Therefore, measuring the concentration of either of these two propeptides in the blood, i.e. procollagen type I amino-terminal propeptide (PINP) or procollagen type I carboxy-terminal propeptide (PICP), provides a biomarker of collagen synthesis. Previous studies have measured these biomarkers of collagen synthesis in the blood and ECM following an acute bout of exercise in humans (Langberg et al., 1999, Langberg et al., 2000, Langberg et al., 2001, Hansen et al., 2009a, Moerch et al., 2013). In the latter case, using a microdialysis technique, a catheter is inserted into the peritendinous space of the tendon, allowing the measurement of PINP or PICP concentration (Heinemeier et al., 2016). Although measuring circulating concentrations of PINP or PICP has been widely used, they measure collagen synthesis indirectly, as they are not tissue specific, and usually reflect bone collagen synthesis due to the faster collagen turnover in bone than in soft tissues (Koivula et al., 2012).

On the other hand, the use of stable isotope analysis enables a direct measurement of collagen synthesis in human skeletal muscle or tendon. Intravenous administration of labelled amino acids can be traced in a target tissue and greater changes in isotopic enrichment (the ratio of tracer/traces) indicates a protein synthesis rate (Kim et al., 2016, Heinemeier et al., 2016).

Similarly, collagen degradation is also an essential process for soft tissue remodelling following mechanical loading and wound healing (Visse and Nagase, 2003, Kjaer, 2004). Extracellular degradation of collagen is initiated by members of the matrix metalloproteinases (MMPs) family, such as MMP-1, MMP-8 and MMP-13 (Visse and Nagase, 2003). Additionally, cathepsin K is one of cysteine proteinase family, whose role is to preferentially degrade collagen in bone and tendon (Delaissé et al., 2003, Panwar et al., 2015). Type I collagen has two sites of cross-links at its ends called non-helical telopeptide regions (N- and C-terminal telopeptides) (Orgel et al., 2000). In mature collagen fibrils, N- and C-terminal telopeptides are crosslinked by pyridinium at a helical residue 930 and 87 respectively (Calvo et al., 1996, Eyre et al., 1984). Cathepsin K degrades the C-terminal crosslinked telopeptide β-isomerized Asp-Gly sequence (Fledelius et al., 1997, Borel et al., 2012), and thus, an immunoassay has been developed to measure beta cross-linked C-terminal telopeptide of type I collagen (β-CTX) , as a marker of collagen degradation in blood or urine samples (Chubb, 2012).

2.4 Collagen cross-linking

As stated above, Type I collagen in tendon plays an important role in transmitting force between muscle and bone and is, therefore, one of main contributors of tendon mechanics. In addition, cross-linking between collagen molecules within the collagen fibrils plays a

key role in influencing tendon mechanical properties (Puxkandl et al., 2002), and enzymatic and non-enzymatic cross-linking are the two primary mechanisms of intermolecular cross-links for the stabilization of collagen fibres (Avery and Bailey, 2005). Although there is a lack of evidence regarding the effect of mechanical loading on collagen cross-linking in humans, some studies have found that, while long-term habitual loading increases the concentration of hydroxylysyl pyridinoline (HP), which is formed by lysyl oxidase (LOX)-mediated enzymatic process in endurance-trained young men (Couppé et al., 2014), 10 week's RT in healthy young women (Dalgaard et al., 2019) and 12 month's RT in healthy older men and women (Eriksen et al., 2019) did not change HP concentration. In addition, higher serum concentration of oestrogen may affect collagen cross-linking. For example, Lee et al. (2015) found that when engineered ligaments were treated with higher 17β-oestrogen concentrations, LOX mRNA activity decreased by approximately 62%, which was associated with a decrease in Young's modulus and ultimate tensile stress. This indicates that higher oestrogen concentrations may affect the mechanical strength of connective tissue such as tendon and ligament, and more details of how oestrogen may influence tendon metabolism will be discussed in following section.

2.5 The effects of oestrogen on collagen synthesis in women

In eumenorrheic women, the endogenous production of the female sex hormone, oestrogen, fluctuates during the course of each menstrual cycle, while production starts to decrease dramatically during and postmenopause. Over the past decades, the effect of oestrogen on skeletal muscle and tendon metabolism has been extensively investigated (Miller et al., 2007, Hansen et al., 2008, Hansen et al., 2009a, Hansen et al., 2009c). Furthermore, in rabbit anterior cruciate ligament (ACL) fibroblasts, an inverse relationship between the rate collagen synthesis and 17β-oestradiol concentration was found, where

higher collagen synthesis was associated with lower 17β-oestradiol concentration (Liu et al., 1997). Similarly, when cyclic mechanical loading was applied, higher mRNA expression of type I collagen in the porcine ACL fibroblasts was associated with lower 17β-oestradiol concentration (Lee et al., 2004). However, some previous studies found contradictory findings regarding the effect of oestrogen on collagen synthesis. Different 17β-oestradiol concentrations did not affect collagen synthesis in bovine ACL fibroblasts (Seneviratne et al., 2004) or human ligamentum flavum cells (Chen et al., 2014). Furthermore, PT collagen fractional synthesis rate (FSR) was higher in postmenopausal women using hormone replacement therapy compared to aged-matched postmenopausal women, suggesting that higher oestrogen concentration may augment collagen synthesis (Hansen et al., 2009a).

Oestrogen receptors are present in human connective tissue, such as tendon and ligament (Liu et al., 1996, Faryniarz et al., 2006, Bridgeman et al., 2010), thus enabling circulating oestrogen to affect the metabolism of these tissues. Previous studies investigating collagen synthesis in response to exercise in women found that PINP concentration was increased following a bout of RE in young healthy women, who did not use the combined (containing both oestrogen and progesterone) oral contraceptive pill (OCP), while PINP concentration was unchanged in age-matched OCP users, who had used OCP for 7 ± 2 years (Hansen et al., 2008).The same research group found that PT collagen FSR at rest and 24 h post-RE was higher in non-OCP users compared to OCP users (Hansen et al., 2009c). On the other hand, Miller et al. (2007) measured PINP concentration and PT collagen FSR at two different phases of the menstrual cycle (follicular and luteal), and changes in both PINP concentration and PT collagen FSR following a bout of RE were unaffected by these different menstrual cycle phases in young, healthy, eumenorrheic

women. It should be noted, however, that endogenous oestrogen concentrations in these two phases tested in the study were overlapped (0.07 – 0.42 nmol·L^{-1} in the follicular phase and 0.24 – 0.79 nmol·L^{-1} in the luteal phase) (Miller et al., 2007, Hansen et al., 2008). This might have influenced the lack of difference in the collagen synthetic response following RE between the different menstrual cycle phases.

Although previous studies have investigated the effect of HC ingestion with exercise on collagen synthesis, all the participants have been healthy young men (or a mix of men and women). Thus, considering the impact of oestrogen on tendon metabolism in women, and how oestrogen influences collagen synthesis in response to HC ingestion with RE in young, healthy, eumenorrheic women is currently unknown.

2.6 Exercise-induced tendon adaptation

It is well documented that connective tissue undergoes mechanotransduction; transduction of a mechanical load into chemical signals, leading to alterations in cellular function and structure (Wang and Thampatty, 2006, Chiquet et al., 2009). An increase in type I collagen gene expression following cyclic stretching in human PT fibroblasts (Yang et al., 2004) and in the rate of type I collagen synthesis following an acute bout of exercise in human PT (Miller et al., 2005) indicate the human tendon's ability to respond to acute mechanical loading. Repetitive loading over a prolonged period of time, e.g. RT, is likely to lead to chronically elevated collagen synthesis, which may be required to elicit changes in the structure and composition on tendon, leading to changes in its mechanical properties (Kubo et al., 2012). This section will only examine PT and AT adaptations following chronic RT, as both tendons are located superficially, which allows the measurement of changes in the morphological and mechanical properties following RT.

There have been various RT protocols used in different populations of varying age (younger or older), sex (men or women), varying endogenous oestrogen concentrations in young women (OCP users or non-users), varying modes of muscle contraction (concentric, isometric, or eccentric contractions), training durations (8 weeks to 12 months) or training status (previously trained or untrained). **Table 1** illustrates the studies that have investigated the effect of RT on changes in human tendon properties. Among the parameters of morphological and mechanical properties, changes in tendon CSA, stiffness and Young's modulus can be regarded as representative indications of exercise-induced tendon adaptation. It has been hypothesised that a larger tendon CSA would reduce stress, as stress is defined as force per tendon CSA (Couppé et al., 2014). Tendon stiffness is a measure of resistance to deformation against tendon force. Young's modulus is also a measure of tendon stiffness when tendon size is taken into account, and a tendon with high Young's modulus can bear a higher force (LaCroix et al., 2013). Following chronic RT, there is a concomitant increase in maximal tendon force production and tendon stiffness, regardless of age, sex, and mode of contraction. In the AT, stiffness and Young's modulus were increased by 19 – 57% and ~51%, respectively (Kubo et al., 2002, Arampatzis et al., 2007, Kubo et al., 2007, Arampatzis et al., 2010, Bohm et al., 2014). In the PT, 6 – 65% increases in stiffness and 14 – 69% increases in Young's modulus (Kubo et al., 2001, Reeves et al., 2003a, Kubo et al., 2006a, Kongsgaard et al., 2007, Kubo et al., 2009, Seynnes et al., 2009, Kubo and Yata, 2017, Massey et al., 2018) have been reported following chronic RT. Regarding exercise-induced tendon hypertrophy, it should be noted that only a few studies have reported an increase in tendon CSA (Arampatzis et al., 2007; Kongsgaard et al., 2007; Bohm et al., 2014; Seynnes et al., 2009; Dalgaard et al., 2019; Eriksen et al., 2019). This may be due to the general consensus that the PT tends

to hypertrophy only at the proximal and distal ends and not in the centre, and those studies reporting no PT hypertrophy following RT measured CSA at 50% of the tendon length (Kubo et al., 2006b, Kubo and Yata, 2017). A recent meta-analysis of the RT for human tendon adaptation has revealed that there is no superior mode of contraction for changes in tendon properties but a training period of at least 12 weeks is recommended (Bohm et al., 2015). In addition to the training duration, training intensity is another key factor for tendon adaptation, as high-intensity RT conferred a greater effect on increasing tendon CSA and stiffness in young men compared to moderate intensity RT (Kongsgaard et al., 2007).

In the study of Kubo et al. (2006b), tendon CSA and stiffness were not changed following isometric squat exercise for 12 weeks. Similarly, barbell back squat RT at different depths did not lead to differences in either tendon hypertrophy or changes in serum PINP concentration (PT mechanical properties were not measured) (Bloomquist et al., 2013). It should be noted, however, that PT protein FSR increased following a 4-week eccentric or concentric contraction only leg press exercise (4 sets of 12 – 15 repetitions) at 60% 1-RM in healthy young and older men but PT FSR did not further increase after a further 4-week RT period (Crossland et al., 2023). Theoretically, back squat exercise should be one of best exercises to induce mechanodransduction in the PT, to elicit changes in its structure and function, as compressive force at the knee joints during squatting at moderate loads (65 – 75% 1-RM) is 4 – 7 times greater than bodyweight (Escamilla, 2001). Therefore, no change in serum PINP concentration following 12 weeks' barbell-back squat exercise in the study by Bloomquist et al. (2013) may have been caused by the choice of inappropriate time points for measuring a chronic change in the collagen synthetic response to RT.

In summary, there is strong evidence that chronic RT improves morphological and mechanical properties *in vivo*. The training period and intensity rather than mode of contraction are key factors for human tendon adaptation to RT.

Table 1. Summary of the resistance training methods in previous literature investigating tendon adaptation**.**

Author	Participants	Tendon	Training frequency and duration		Training intensity	Exercise	Stiffness	CSA
(Kubo et al., 2002)	8 M	UT (21.0 ± 2.0 yr)	AT	8 wk^{-1}	4 d· wk^{-1}	70% 1RM	Isokinetic plantar flexion	↑	–
(Arampatzis et al., 2007)	8 W 3 M	UT (29.5 ± 5.0 yr)	AT	14 wk^{-1}	4 d· wk^{-1}	90% MVC	3-s isometric plantar flex-ion	↑	↑
(Kubo et al., 2007)	10 M	UT (22.0 ± 2.0 yr)	AT	12 wk^{-1}	4 d· wk^{-1}	80% 1RM	3 s-ECC and 1 s-CON an-kle flexion (fully ROM)	↑	–

(Arampatzis et al., 2010)	11 M	UT (23.9 ± 2.2 yr)	AT	14 wk^{-1}	4 d· wk^{-1}	90% MVC	1 s- isometric planar flex-ion	↑	–
(Bohm et al., 2014)	12 M	UT (29.5 ± 3.0 yr)	AT	14 wk^{-1}	4 d· wk^{-1}	90% MVC	3-s isometric planar flex-ion	↑	↑
(Ishigaki and Kubo, 2018)	10 M	UT (20.9 ± 3.1 yr)	AT	12 wk^{-1}	3 d· wk^{-1} and 6 d· wk^{-1}	50% 1-RM (3 d· wk^{-1}) BW (6 d· wk^{-1})	Unilateral eccentric plan-tar flexion	–	–
(Kubo et al., 2001)	8 M	UT (22.6 ± 2.8 yr)	PT	14 wk^{-1}	4 d· wk^{-1}	70% MVC	20 s-isometric knee exten-sion	↑	–

(Reeves et al., 2003a)	5 W 4 M	UT (74.3 ± 3.5 yr)	PT	14 wk^{-1}	3 d· wk^{-1}	80% 5 RM load	Isokinetic knee extension and leg press	↑	–
(Kubo et al., 2006a)	9 M	UT (24.0 ± 1.0 yr)	PT	12 wk^{-1}	4 d· wk^{-1}	70% MVC	15 s-isometric knee extension	↑	–
(Kubo et al., 2006b)	8 M	UT (20.0 ± 1.0 yr)	PT	12 wk^{-1}	4 d· wk^{-1}	70% MVC	15 s-isometric squat	–	–
(Kongsgaard et al., 2007)	12 M	UT (24.6 ±1.0 yr)	PT	12 wk^{-1}	3 d· wk^{-1}	70% 1RM load	Knee extension	↑	↑

(Kubo et al., 2009)	10 M	UT (22.3 ± 1.1 yr)	PT	12 wk^{-1}	4 d· wk^{-1}	70% MVC 80% 1RM	15 s-unilateral isometric knee extensions (70% MVC) Unilateral isokinetic knee extensions (1 s-CON and 3 s-ECC at 80% 1RM)	↑	–
(Seynnes et al., 2009)	15 M	UT (20.4 ± 2.2 yr)	PT	9 wk^{-1}	3 d· wk^{-1}	80% 1RM	Knee extension	↑	↑
(Kubo et al., 2010)	8 M	UT (22.0 ± 0.8 yr)	PT	3 mo^{-1}	4 d· wk^{-1}	70% MVC	15 s-isometric knee exten-sion	↑	–
(Bloomquist et al., 2013)	17 M	UT	PT	12 wk^{-1}	3 d· wk^{-1}	3 – 10 RM load	Barbell back squat (SS at 60 ° and DS at 120 ° knee	Not measured	–

	(SS = 9 and DS = 8)	(SS 23.0 ± 3.0 yr DS 25.0 ± 6.0 yr)					flexion, 0 ° = full knee extension)		
(Kubo and Yata, 2017)	9 M	UT (20.8 ± 0.5 yr)	PT	12 wk^{-1}	3 $d \cdot wk^{-1}$	80% 1RM	1 s-concentric knee extension	↑	–
(Massey et al., 2018)	29 M (14 ECT and 15 SCT)	UT (ECT 25.0 yr ± 2.0 SCT 25.0 ± 2.0 yr)	PT	12 wk^{-1}	3 $d \cdot wk^{-1}$	75 – 80% MVT	3 s-isometric knee extension at 75% MVT Brief isometric knee extension at 80% MVT	↑	–

(Dalgaard et al., 2019)	14 OCP 14 Non-OCP	UT (24.0 ± 1.0 yr)	PT	10 wk^{-1}	3 d· wk^{-1}	10 – 15 RM load	Knee extension and leg press) OCP users	Not measured	↑
(Eriksen et al., 2019)	17 W 19 M	UT (67.0 ± 2.0 yr)	PT	12 mo^{-1}	3 d· wk^{-1}	70% − 85% 1RM	Leg press and leg exten-sion	−	−
(Walker et al., 2020)	28 M (10 AEL and 10 TRAD)	T (AEL 21.0 ± 2.0 yr TRAD	PT	10 wk^{-1}	2 d· wk^{-1}	6 RM load	Isokinetic or accentuated ECC knee extension and leg press	−	Not measured

	21.0 ± 2.0 yr)

CSA, cross-sectional area; UT, untrained; AT, Achilles tendon; RM, repetition maximum; MVC, maximal voluntary contraction; ECC, eccentric; CON, concentric; ROM, range of motion; BW, body weight; PT, patellar tendon; SS, shallow squat; DS, deep squat; MVT, maximal voluntary torque; OCP, oral contraceptive pill, AEL; accentuated eccentric loading; TRAD; traditional; T, trained.

2.7 Vitamin C and hydrolysed collagen: effect of nutrition on collagen synthesis with exercise

2.7.1 Vitamin C

Vitamin C (otherwise known as ascorbic acid) is essential for collagen synthesis, as it acts as a cofactor for the hydroxylation of proline and lysine residues on procollagen (Kivirikko and Prockop, 1967). Insufficient ascorbic acid causes the absence of an enzyme called proly-4-hydroxylase, preventing the synthesis of new collagen fibrils (Canty and Kadler, 2005). This condition is called scurvy, in which connective tissue renewal does not occur. A previous study found that vitamin C stimulated improved tendon repair by increasing the production of type I collagen, collagen fibre diameter and the number of fibroblasts in ruptured rat AT (Omeroglu et al., 2009). However, the effect of vitamin C supplementation on changes in human muscle-tendon unit (MTU) properties is trivial. A relatively high daily dose (1,000 mg) of vitamin C has been shown to have no effect on muscle hypertrophy or strength gains with RT in humans (Bobeuf et al., 2011, Theodorou et al., 2011, Paulsen et al., 2014). Further, with regard to collagen synthesis, although 12 weeks' RT with vitamin C and E intake induced an increase in serum PINP concentration in healthy elderly men, RT-induced changes in serum PINP concentration did not differ from the control group (Stunes et al., 2017).

2.7.2 Amino acid composition in collagen and collagen supplements

Gauza-Włodarczyk et al. (2017) investigated the amino acid composition in fish skin, bovine Achilles tendon and bovine femur. A total of 18 amino acids constitutes skin, tendon, and bone collagen: asparagine, threonine, serine, glutamic acid, proline, cysteine,

glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine, hydroxyproline. Among these 18 amino acids, glycine, proline, hydroxyproline, glutamic acids and alanine were the major components in the investigated collagen-enriched tissues (Gauza-Włodarczyk et al., 2017). In human PT, glycine (41.7 ± 0.9%), proline (14.2 ± 0.4%) and alanine (13.7 ± 0.3%) were the most abundant amino acids in a study by Smeets et al. (2019), although hydroxyproline was not measured.

Previous studies measured concentrations of several amino acids in the blood after consumption of different collagen supplements. Regardless of dose (15 g and 20 g) and type (HC or gelatine) of collagen supplement, the peak concentrations of glycine, proline and hydroxyproline in blood occurred at around an hour after ingestion in healthy young men (Shaw et al., 2017, Lis and Baar, 2019, Hilkens et al., 2023). Gelatine and HC are both collagen-derived products, which have been commonly adopted in the food industry as nutritional supplements. The former is produced through a partial thermal hydrolysis of collagen derived from mammal or fish connective tissue (Daneault et al., 2017), and both products have a similar amino acid profile, whereby glycine, proline and hydroxyproline are the most abundant amino acids (Alcock et al., 2019).

2.7.3 The effects of key amino acids on collagen synthesis in vitro

Several investigations have revealed that the administration of key amino acids highly abundant in collagen has a positive effect on collagen synthesis *in vitro*. For example, chondrocytes from bovine cartilage were treated with 1.5 mM glycine, proline, or lysine for 15 days and type II collagen synthesis was found to gradually increase, with peak type II collagen synthesis occurring on day 13 (de Paz-Lugo et al., 2018). At this point, the

administration of glycine caused a 1.3-, 1.6- and 3.2-fold greater increase in type II collagen synthesis compared to when proline, lysine and no amino acid was administered, respectively (de Paz-Lugo et al., 2018). Further, when human skin fibroblasts were treated with 5 mM proline, collagen type I α1 expression was increased (Szoka et al., 2017).

2.7.4 Collagen turnover in humans following consumption of collagen supplements with an acute bout of exercise in vivo

A recently published study by Shaw et al. (2017) found that 15 g gelatine ingested prior to an impact exercise (i.e. skipping) led to a two-fold serum PINP concentration compared to when 5 g or 0 g gelatine was ingested in healthy, young men. Furthermore, Shaw et al. (2017) treated engineered ligaments with serum obtained from participants, who consumed these different doses of gelatine. The results showed that the collagen content was greater in the ligaments treated with the serum from the 15 g gelatine intervention compared to the 0 g intervention (Shaw et al., 2017). However, in a separate study, the same research group could not replicate the increase in PINP concentrations using the same methodology of Shaw et al. (2017), and the authors suggested large variability in PINP concentration (caused by the vitamin C within the supplements) reduced the ability to detect a difference in serum PINP concentration (Lis and Baar, 2019). Furthermore, a recent study found that 30 g HC intake with an acute bout of moderate-intensity RE neither increased serum PINP concentration nor muscle connective protein synthesis compared to RE with 30 g whey protein or RE alone in healthy, young men and women (Aussieker et al., 2023). It should be noted, however, that this study implemented a between-group design and RE (barbell back squat exercise) intensity of 60% 1-RM. Thus, between group variability may have confounded any effect of HC and, as exercise intensity is one of the key variables to induce exercise-induced tendon adaptation (Bohm et al., 2015), it

is unknown whether HC intake with high-intensity RE would further increase collagen synthesis or not.

Another study investigated a single bout of drop jumps with 20 g HC intake in healthy, young men and measured markers of collagen turnover (Clifford et al., 2019). Serum PINP concentration in the 20 g HC intervention was increased following exercise but this did not differ from the placebo (0 g HC) group. They also measured serum beta-isomerized C-terminal telopeptide (β-CTX) as a biomarker of collagen breakdown and β-CTX concentrations decreased after exercise but, once again, there was no difference between HC and placebo groups (Clifford et al., 2019). Crucially, however, physical performance (countermovement jump height) did recover quicker in the HC group, suggesting a beneficial effect of HC on recovery following exercise-induced muscle damage/fatigue. Hilkens et al. (2023) used a 5-min high-impact exercise (jumping) protocol with and without 20 g HC in healthy, young men to measure changes in collagen turnover. Serum PINP concentration did not change over time and were not different to the placebo (0 g HC) plus exercise intervention. Similarly, the serum concentration of carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I) was not affected by 20 g HC, although it did decrease by ~50% from immediately post-exercise to 4 h post-exercise (Hilkens et al., 2023). These studies suggest that ingestion of 20 g HC with impact exercise does not augment whole body type I collagen synthesis in healthy young men, which is inconsistent with the study by Shaw et al. (2017). However, it is unknown whether HC ingestion with a different exercise modality (i.e. high-intensity RE) would augment collagen synthesis, as chronic HC ingestion with high-intensity RT led to greater AT and PT hypertrophy in healthy, young men (Jerger et al., 2022, Jerger et al., 2023).

2.7.5 The effect of long-term HC consumption with exercise on muscle-tendon adaptations

Although a few studies have investigated the chronic effect of RT with HC intake, the main measurements were changes in body composition rather than direct measurements of muscle-tendon adaptation. To date, only three studies have measured the effect of RT with HC supplementation on changes in tendon properties. After a 14-week high-intensity RT intervention targeting the calf muscle and AT with a daily consumption of 5 g HC in healthy young men, Jerger et al. (2022) found that AT CSA at the distal 25% increased (+11%) more than RT with no HC supplementation (+5%). However, despite a main effect of RT on AT stiffness, HC supplementation did not further increase AT stiffness compared to RT alone (Jerger et al., 2022). The same research group then investigated changes in PT properties and *rectus femoris* (RF) muscle size following 5 g HC daily ingestion with 14 weeks' high-intensity RT in healthy young men (Jerger et al., 2023). Similarly, the results showed that the increase in PT CSA at 60% and 70% PT length was greater in the HC group compared to the placebo group but there was no group differences regarding changes in PT stiffness or RF CSA (Jerger et al., 2023). The lack of HC effect on tendon stiffness following 5 g HC ingestion with RT (Jerger et al., 2022, Jerger et al., 2023) may be associated with the low HC dose, as a previous study has shown no difference in the increase in collagen synthesis following 5 g or 0 g gelatine ingestion prior to exercise (Shaw et al., 2017). Considering an increase in collagen synthesis is associated with a concomitant increase in human tendon stiffness (Kubo et al., 2012), a higher HC dose may have led to between group differences in tendon stiffness changes in the studies by Jerger et al. (2022, 2023). However, another recent study investigated the effect of a 15-week RT programme with daily ingestion of 15 g HC on changes in PT properties in

healthy young men (Balshaw et al., 2023b). Despite previously showing an effect of daily 15 g HC intake and 15 weeks' RT on changes in muscle size (Balshaw et al., 2023a), this study found that 15 g HC did not further augment PT CSA, tendon stiffness or Young's modulus more than RT alone (Balshaw et al., 2023b). It remains to be seen, however, whether an even higher dose of HC is required to elicit changes in tendon stiffness and Young's modulus, when combined with a prolonged period of high-intensity RT. Furthermore, these studies were all performed in young men only. Thus, more work is required to investigate the effect of greater doses of HC (e.g. ~30 g) with RT, and particularly in female athletes.

On the other hand, previous studies have reported that 12 weeks' whole body RT with a daily intake of 15 g HC supplementation positively influenced body composition by further augmenting the increase in fat-free mass (FFM) (measured by a bioelectric impedance analysis) compared to RT alone in healthy, recreationally active, young men (Kirmse et al., 2019) and in untrained premenopausal women (Jendricke et al., 2019). Similarly, daily intake of 15 g HC with whole-body RT for 12 weeks had greater effects on the increase in FFM and the reduction in fat mass (kg) measured by dual energy x-ray absorptiometry compared to RT alone in older, sarcopenic men (Zdzieblik et al., 2015) and overweight, middle-aged men (Zdzieblik et al., 2021).

Regarding changes in muscle size induced by RT with HC supplementation, Balshaw et al. (2023a) investigated daily ingestion of 15 g HC with a 15-week RT programme in healthy, young men. Using magnetic resonance imaging, the muscle size and volume of three muscle groups (quadriceps, hamstrings and gluteus maximus) were measured before and after RT in HC and placebo groups. 15 g HC with RT induced greater regional muscle

hypertrophy, in which the volume of the vastus medialis muscle was greater in HC group (+16%) compared to PLA group (+10%), although there were no other group differences in the other muscle groups (Balshaw et al., 2023a). The authors stated that the greater increase in muscle size following HC ingestion with RT may be associated with the mammalian target of rapamycin (mTOR) signalling pathway that regulates protein synthesis, since myotube size and phosphorylation of the PI3K/Akt/mTOR signalling pathway in murine myoblasts treated with hydroxyprolyl-glycine (derived from HC) increased more than control (Kitakaze et al., 2016). In line with this finding, changes in gastrocnemius muscle thickness were greater following daily ingestion of 5 g HC with 14 weeks' RT (+0.16 cm) than RT alone (+0.05 cm) in healthy, young men (Jerger et al., 2022). In addition to the effect of HC on muscle size, Lis et al. (2021) found that daily consumption of 20 g HC with 50 mg vitamin C, together with a 3-week resistance/power training programme in male athletes increased the eccentric rate of force development (RFD) during a countermovement jump. This may imply that HC intake with exercise may be beneficial for athletic performance, as an increase in tendon stiffness is positively related to an increase in RFD (Bojsen-Møller et al., 2005).

In summary, HC and gelatine supplements containing vitamin C have been shown to increase collagen synthesis. Although there are a number of studies showing a beneficial effect of RT with 15 g HC supplementation on changes in body composition, the evidence for an effect on changes in tendon properties is limited and equivocal, and more research is required with greater doses of HC and in female athletes rather than solely male participants, as per the limited studies published to date.

2.8 Summary

Type I collagen is the most abundant protein in tendon and mechanical loading increases type I collagen synthesis (Miller et al., 2005), while chronic mechanical loading (e.g. RT) induces changes in morphological, mechanical, and material properties of human tendon (Reeves et al., 2003a, Kongsgaard et al., 2007, Seynnes et al., 2009). In addition to the role of exercise on tendon adaptation, recent literature has demonstrated the effects of HC intake with exercise on collagen synthesis. For example, 15 g gelatine intake with impact-type exercise (i.e. skipping) augments a marker of collagen synthesis (serum PINP) more than 5 g gelatine in healthy, young men (Shaw et al., 2017). Further, daily intake of 5 g HC with 14 weeks' RT has been shown to induce a greater increase in AT (Jerger et al., 2022) and PT (Jerger et al., 2023) size but not tendon stiffness in healthy, young men.

It should be noted, however, that evidence for an effect of RT with HC supplementation on tendon adaptation in female athletes is lacking. There is evidence that circulating oestrogen concentration can affect collagen synthesis (Hansen et al., 2008, Hansen et al., 2009a) so we cannot assume that findings from studies in men can be transferred directly to females. Furthermore, previous studies investigating changes in serum PINP concentration following HC ingestion prior to exercise have focused on 15 – 20 g HC intake with impact-type exercise (Shaw et al., 2017, Clifford et al., 2019, Hilkens et al., 2023). As high-intensity RT is known to increase maximum strength, muscle size and alterations tendon properties (Reeves et al., 2003a, Kongsgaard et al., 2007, Seynnes et al., 2009, Erskine et al., 2010), it is likely that a combination of high-intensity RE with a high dose (e.g. 30 g) of HC would confer greater increases in collagen synthesis after an acute bout of RE, while combining these factors over a prolonged period of RT, it is likely to elicit greater gains in tendon CSA, stiffness and young's modulus than RT alone.

The subsequent empirical studies in this thesis will attempt to (i) elucidate the dose-response of HC supplementation on a marker of collagen synthesis in young, resistance-trained men; (ii) determine the individual and combined effects of circulating oestrogen and HC ingestion on collagen synthesis in a young, resistance-trained woman; (iii) explore the effect of chronic exercise and HC supplementation on changes in tendon adaptation in young female athletes; and (iv) examine the effect of RT and HC supplementation on changes in muscle-tendon properties in young, resistance-trained men.

Chapter Three

The circulating procollagen type I N-terminal propeptide response to an acute bout of resistance exercise is greater when ingesting 30 g hydrolysed collagen compared with 15 g and 0 g in resistance-trained young men

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Prelude

It is currently not known what the dose-response relationship is between hydrolysed collagen (HC) ingestion prior to an acute bout of high-intensity resistance exercise and collagen turnover in humans. Therefore, this chapter will investigate the effect of 30 g *vs.* 15 g *vs.* 0 g HC ingested prior to high-intensity, high-volume back squat resistance exercise on biomarkers of collagen turnover in resistance-trained healthy young men. This study was performed between January and August 2019.

Abstract

Resistance exercise (RE) stimulates collagen synthesis in skeletal muscle and tendon but there is limited and equivocal evidence regarding an effect of collagen supplementation and exercise on collagen synthesis. Furthermore, it is not known if a dose-response exists regarding the effect of hydrolysed collagen (HC) ingestion and RE on collagen synthesis. We aimed to determine the HC dose-response effect on markers of collagen turnover following high-intensity RE in resistance-trained young men. Using a double-blind, randomized cross-over design, 10 resistance-trained men (age: 26±3 years; height: 1.77±0.04 m; mass: 79.7±7.0 kg) ingested 0g, 15g or 30g HC with 50mg vitamin C 1h prior to performing four sets' barbell back-squat RE at 10-repetition maximum load, after which they rested for six hours. Blood samples were collected throughout each of the three interventions to analyse procollagen type I amino-terminal propeptide (PINP) and β-isomerized C-terminal telopeptide of type I collagen (β-CTX) concentration, and the concentration of 18 collagen amino acids. The serum PINP concentration×time area-under-the-curve (AUC) was greater for 30g (267±79 $\mu g \cdot L^{-1} \cdot h$) than 15g (235±70 $\mu g \cdot L^{-1} \cdot h$, P=0.013) and 0g HC (219±88 $\mu g \cdot L^{-1} \cdot h$, P=0.002) but there was no difference between 0g and 15g HC (P=0.225). The AUCs of glycine and proline were greater for 30g than for 15g and 0g HC (P<0.05). Plasma β-CTX concentration decreased from -1h to +6h (P<0.05), with no differences between interventions. In conclusion, the greater PINP AUC suggests 30g HC ingested prior to high-intensity RE augments whole body collagen synthesis more than 15g and 0g HC in resistance-trained young men.

3.1 Introduction

Musculoskeletal tissues, such as skeletal muscle and tendon are crucial for generating and transmitting force to the bone, enabling movement. The structure and function of these tissues are therefore essential for musculoskeletal health and physical performance. Unfortunately, however, injuries to these tissues are common in athletes, with soft-tissue injuries making up the majority of all injuries in male athletes (Ekstrand et al., 2011). One of the biggest risk factors for soft-tissue injury is muscle weakness (Keller et al., 1987, Mendiguchia et al., 2012) and one of the most common methods used by athletes to mitigate this risk factor is to perform chronic resistance exercise (RE) (Lauersen et al., 2018). Chronically overloading the muscle-tendon unit in this way causes the muscle to adapt by hypertrophying and getting stronger (Erskine et al., 2010), while the tendon also adapts by hypertrophying and increasing its stiffness and elastic modulus (Kongsgaard et al., 2007, Seynnes et al., 2009).

A stiffer tendon has a higher loading capacity, as there is a linear relationship between Young's modulus and ultimate tensile stress (LaCroix et al., 2013). Given that collagen (mainly type I) makes up 60–85% tendon dry weight (Kjaer, 2004), it is considered a crucial component in the tendon's adaptation to RE, particularly as gains in tendon stiffness are thought to be influenced by both tendon hypertrophy and an increase in collagen fibril density (Heinemeier and Kjaer, 2011, Couppé et al., 2021). An increase in type I collagen fibril content over time is likely the product of an overload-induced increase in collagen synthesis after each bout of RE.

Collagen synthesis can be assessed either directly from the overloaded tissue, e.g. by measuring skeletal muscle or tendon collagen fractional synthetic rate (FSR), or indirectly

from serum concentration of procollagen type I carboxy-terminal propeptide (PICP) or procollagen type I amino-terminal propeptide (PINP), which are both cleaved off during the maturation of procollagen to collagen. Indeed, an acute bout of RE in young men has been shown to increase patellar tendon collagen FSR (Miller et al., 2005) and serum PINP concentration (Huang et al., 2022). This response is likely due to RE initiating mechanotransduction (i.e. mechanical stress initiating fibroblast intracellular signalling) (Chiquet et al., 2009), and the secretion of growth factors [e.g. transforming growth factor beta (TGF-β) and insulin-like growth factor-1 (IGF-I)], with these growth factors being crucial for procollagen formation in tendon (Heinemeier et al., 2003, Olesen et al., 2006). Furthermore, these newly synthesized procollagen molecules undergo post-translational modifications, for which the presence of vitamin C is an essential co-factor during collagen synthesis (Murad et al., 1981), transport and assembly into tendon (Canty and Kadler, 2005).

Thus, RE appears crucial for inducing increases in serum PICP/PINP concentration and muscle-tendon collagen FSR, which may lead to changes in connective tissue properties in the longer term. Indeed, concomitant increases in serum PICP concentration and human Achilles tendon collagen content after two months' chronic RE, followed by an increase in Achilles tendon stiffness with a further month's RE training (Kubo et al., 2012), suggest that augmented tendon collagen synthesis and content are necessary to cause an increase tendon stiffness. Not only is tendon stiffness important for mitigating soft-tissue injury risk but it can also influence performance during 'explosive' actions, as a stiffer tendon can transmit muscle force more effectively to the bone, thus increasing the rate of force development (Bojsen-Møller et al., 2005).

In addition to the role of exercise on collagen synthesis, ingestion of vitamin C-enriched collagen appears to further augment an exercise-induced increase in collagen synthesis in a dose-response manner, i.e. 15 g gelatine increased serum PINP concentration by more than two-fold compared to 5 g and 0 g gelatine (Shaw et al., 2017). This is currently the only study to investigate a dose-response effect of collagen ingestion on changes in collagen synthesis following exercise, albeit in jump-rope exercise not RE. Although no study has examined a collagen dose-response relationship without exercise, chronic collagen supplementation alone has been shown to induce improvements in bone mineral density (König et al., 2018) and cartilage health (McAlindon et al., 2011), suggesting collagen ingestion might stimulate human connective tissue collagen synthesis independently of exercise. Thus, just as ingestion of 40 g whey protein has been shown to augment the muscle protein synthesis response to RE more than 20 g (Macnaughton et al., 2016), it is possible that collagen ingestion may further augment the RE-induced rise in collagen synthesis (Miller et al., 2005, Huang et al., 2022) in a dose-response manner.

The aim of this study was therefore to investigate the effect of 30 g *vs*. 15 g *vs*. 0 g HC ingested prior to high-intensity back squat RE on whole body collagen synthesis. We hypothesized that 30 g HC would elicit a greater serum PINP response than 15 g HC, which would induce a greater response than 0 g HC. We also hypothesized that 30 g HC would lead to a greater blood availability of the amino acids necessary for collagen synthesis to occur, e.g. glycine and proline.

3.2 Methods

Participants

Thirteen healthy young men volunteered to take part in the study. However, three were excluded prior to participation due to not meeting the inclusion criteria (n = 1) and declining to proceed with participation (n = 2) (**Figure 1**). Therefore, 10 resistance-trained, healthy young men (mean ± SD; age: 26 ± 3 years, height: 1.77 ± 0.04 m, body mass: 79.7 ± 7.0 kg, 4 ± 3 years' RE experience), who performed RE 4 ± 1 times per week, provided written informed consent before completing this study. The study was registered at https://clinicaltrials.gov/ (identifier: NCT05932771), was approved by Liverpool John Moores University Ethics Committee (approval number: 18/SPS/059) and complied with the Declaration of Helsinki. Participants were recruited from a university student population and recruitment began in January 2019 and data collection was completed in August 2019. To be eligible to participate, volunteers had to be male, have at least 12 months' resistance training experience (including barbell back squat exercise performed at least once a week) and to be free from musculoskeletal injury. Volunteers were excluded if they had a history of patellar tendon pathology, were vegan (due to the bovine source of HC), consumed nutritional supplements or medication purported to have beneficial effects on muscle-tendon properties (e.g. antioxidants, protein, etc.), had sustained a lower limb injury in the previous six months, smoked or were <18 or >30 years old.

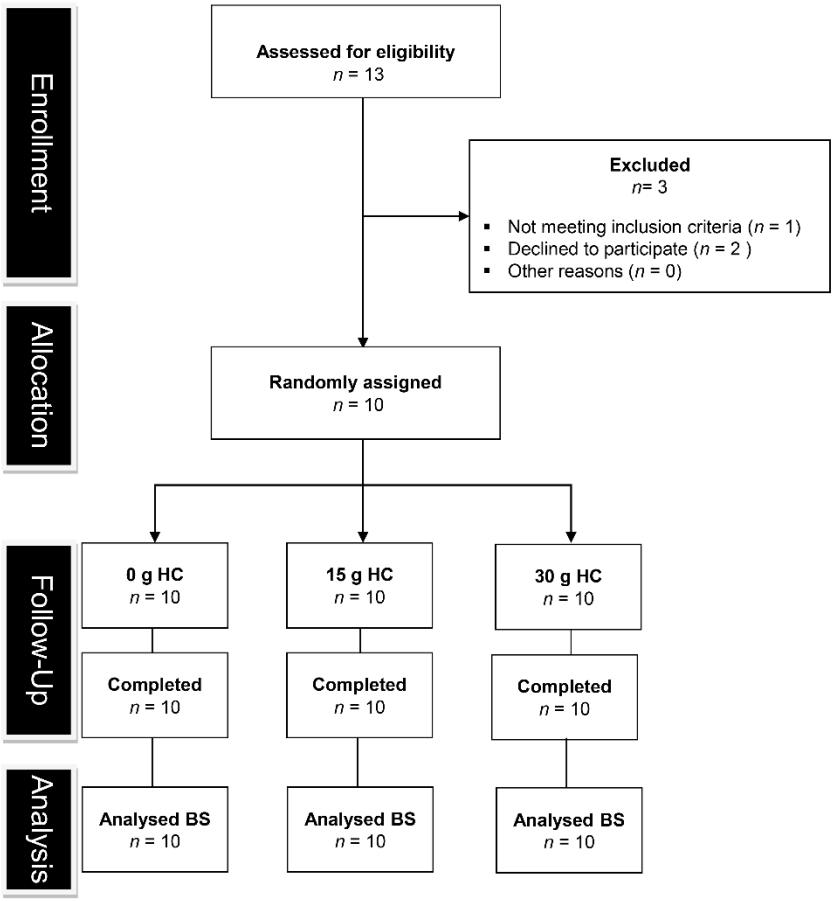

Figure 1. CONSORT flow diagram. HC, hydrolysed collagen; BS, blood samples collected for serum procollagen type I amino-terminal propeptide (PINP) concentration, plasma β-isomerized C-terminal telopeptide of type I collagen (β-CTX) concentration, and serum amino acid concentration.

Experimental design

This study was a double-blind, randomized cross-over design. All participants attended the laboratory on four occasions, each separated by a week, and no strenuous physical activity was performed 48 h prior to each visit. Visit 1 was used to assess barbell back squat 10-RM; while visits 2 – 4 began with participants consuming a drink containing 0 g, 15 g, or 30 g HC (each containing 50 mg vitamin C), followed by four sets' 10-RM

barbell back squat RE (with 2 min rest in between sets), which typically took 20 min to complete. The three interventions (with a seven-day wash-out period interspersed between each intervention) were performed at the same time of day (08:00 – 15:00), following a 10 h overnight fast. After consuming the supplement and completing the RE, participants rested for 6 h and 10 × 5-mL blood samples were collected at different times points over a 7 h period (**Figure 2**). In addition to the supplement, only water was allowed to be consumed (ad libitum) during each intervention. Participants were instructed to record their dietary intake on the day before their first intervention and to replicate that dietary behaviour on the day preceding each of the subsequent interventions.

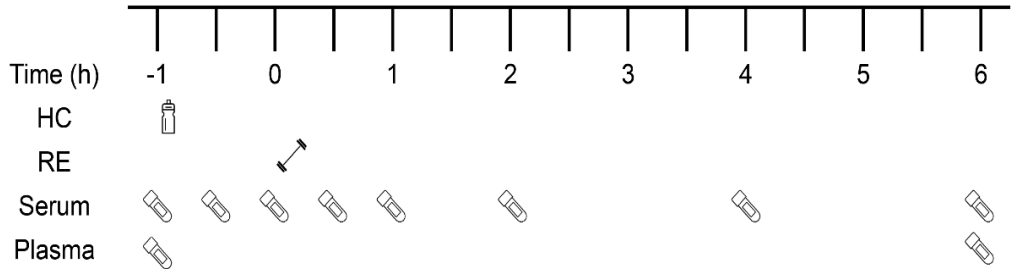

Figure 2. Schematic diagram of the experimental protocol. HC, hydrolysed collagen; RE, resistance exercise; -1, rest prior to HC intake; -0.5, 0.5 h HC-ingestion; 0, 1 h HC-ingestion; +0.5, 0.5 h post-RE; 1, 1 h post-RE; 2, 2 h post-RE; 4, 4 h post-RE; 6, 6 h post-RE.

The barbell back squat 10-repeitition maximum (RM) assessment and 10-RM bout during each intervention

The squat depth during the barbell back squat was standardized for all participants to induce the same mechanical loading on the quadriceps femoris muscle-tendon unit during all three experimental interventions. Participants were instructed to place a 20 kg Olympic barbell on their shoulders (the high bar position), place their feet shoulder-width apart

(foot location was marked on the floor for subsequent sets) and descend until their knee joint angle reached 90°, measured using a goniometer. While participants held the position at 90° knee flexion, the vertical distance from the floor to the ischial tuberosity was measured. The 10-RM assessment was performed in a squat rack and a resistance band was stretched across both sides of the squat rack to indicate the participant's 90° depth (**Figure 3**). A warm-up comprised two dynamic exercises (low lunge and squat to stand) prior to the actual 10-RM assessment, which comprised the following sets of barbell back squat: 10 repetitions with the 20 kg barbell, 8 repetitions at 50% of the estimated 10-RM, 4 repetitions at 70% and 1 repetition at 90% of the estimated 10-RM). After a 5-min rest period, participants performed 10-RM attempts separated by 5-min rest periods until 10-RM load was obtained. Two researchers observed each test procedure to provide a cue when the participant's proximal hamstrings/gluteus maximus touched the elastic band and to spot the participant. The 10-RM bout during each experimental intervention was preceded by a similar warm-up, i.e. two dynamic exercises followed by 10 repetitions' barbell back squat with the 20 kg barbell, 8 repetitions at 50% of the measured 10-RM, 4 repetitions at 70% 10-RM and 1 repetition at 90% 10-RM. The barbell back squat 10-RM load was 118 ± 21 kg during all three interventions.

Figure 3. 90° barbell back squat. A resistance band was attached to the squat rack to indicate when the participant had reached 90° knee flexion during each repetition of the back squat 10-RM.

Nutritional supplementation

Before commencing each intervention, a laboratory technician (independent to the study) made up the supplement and randomly assigned the order of HC dose (Excel 2016, Microsoft, Washington, USA) for each participant. For each intervention, the technician recorded the date, randomly allocated intervention number (1, 2 or 3) and corresponding HC dose. The study investigators and participants were blinded to HC dose until after all analyses were completed, after which time the technician provided the lead researcher with the participants' intervention numbers and corresponding HC doses. Three doses of HC (0 g, 15 g and 30 g, Myprotein, Cheshire, UK) with 50 mg vitamin C powder (Holland and Barrett Retail Limited, Warwickshire, UK) were dissolved in 300 mL water in an

opaque drinks bottle. To match the calories of 30 g HC in the other two interventions, 34.1 g and 15.4 g maltodextrin (Myprotein, Cheshire, UK) was used in the 0 g and 15 g HC interventions, respectively. Although the supplements were described by the manufacturers as "flavourless", 4 g non-caloric sweetener (Truvia®, SilverSpoon, London, UK) was added in all drinks to mask any potential taste difference between interventions. The amino acid profile of the HC supplement is shown in **Table 1**.

Table 1. Amino acid composition of the hydrolysed collagen supplement.

Amino acids	Weight (%)
Glycine	21.0
Proline	12.8
Hydroxyproline	12.2
Glutamic acid	10.3
Alanine	8.9
Arginine	7.3
Aspartic acid	6.0
Lysine	3.5
Serine	3.1
Leucine	2.7
Valine	2.4
Phenylalanine	2.1
Threonine	1.9

Hydroxylysine	1.5
Isoleucine	1.5
Histidine	1.1
Tyrosine	1.0
Methionine	0.9

Blood sampling

The BD Nexibia™ closed IV catheter system (22 G, Becton, Dickinson and Company, Franklin Lakes, USA) was inserted into a peripheral vein in the right antecubital fossa by a trained phlebotomist. A dressing band (3M™ Tegaderm™ I.V. Advanced Securement Dressing, 3M Health Care, Loughborough, UK) then covered the catheter in order to secure the catheter site and to keep it clean. Eight 5 mL venous blood samples were collected in specialized serum collection tubes (BD Vacutainer™ Serum Separation Tube (SST™) II Advance, Dickinson and Company, Franklin Lakes, USA) at the following time points: at rest immediately prior to HC ingestion, 0.5 h post HC ingestion, 1 h post HC ingestion, 0.5 h post RE, 1 h post RE, 2 h post RE, 4 h post RE and 6 h post RE for serum preparation (Figure 2). The samples were used to analyse serum PINP and amino acid concentration. Two × 5 mL venous blood samples were collected in EDTA plasma collection tubes (BD Vacutainer™ Hemogard Closure Plastic K2-Ethylenediaminetetraacetic acid (EDTA) Tubes, Dickinson and Company, Franklin Lakes, USA) at rest immediately prior to HC ingestion and 6 h post RE for plasma preparation. These samples were used to analyse plasma β-CTX. The catheter was flushed by 3 mL sterile pre-filled

flush syringes containing sodium chloride 0.9% (BD PosiFlush™ Pre Filled Saline Syringe, Dickinson and Company, Franklin Lakes, USA) every 30 min to clean and prevent blood from clotting and blocking the catheter. The SSTs were stored in a tube rack for 30 min for clotting at room temperature and the EDTA tubes were immediately placed on ice before being centrifuged at 1000 g at 4°C for 10 min. The serum and plasma samples were then aliquoted into 5 mL round-bottom polystyrene tubes (Falcon™, Thermo Fisher Scientific, Whitby Canada) and stored at -80 °C until subsequent analysis.

Blood analyses

Markers of collagen synthesis and breakdown were analysed by measuring the circulating concentration of PINP and β-CTX, respectively. Further, circulating collagen amino acid concentrations were measured throughout the entirety of each intervention. PINP analyses were performed at Liverpool John Moores University, while β-CTX and amino acid profile analyses were performed at the Bioanalytical Facility, University of East Anglia.

PINP

Six serum samples (at rest prior to HC ingestion, 0.5 h-post RE, 1-h post RE, 2 h-post RE, 4 h-post RE and 6 h-post RE) were used to measure serum PINP concentrations using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (USCN Life Sciences, Wuhan, China). The intra-assay coefficient of variation (CV) was <10% and the inter-assay CV was <12%, with a detection range of 2.47-200 $\mu g \cdot L^{-1}$, and sensitivity of <0.91 $\mu g \cdot L^{-1}$. The ELISA absorbance readings were performed at 450 nm, using a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). The concentration × time total area under the curve (AUC) for PINP and amino acids (see below)

were calculated using Prism software (version 9.4.1, GraphPad Inc., San Diego, San Diego, California USA).

β-CTX

EDTA plasma concentrations of β-CTX were measured using electrochemiluminescence immunoassay on a Cobas e601 analyser (Roche Diagnostics, Germany). The inter-assay CV for β-CTX was ≤3% between 0.2 and 1.5 $\mu g \cdot L^{-1}$ with the sensitivity of 0.01 $\mu g \cdot L^{-1}$.

Amino acid profile

Eight serum samples (at rest immediately prior to HC ingestion, 0.5 h post HC ingestion, 1 h post HC ingestion, 0.5 h post RE, 1 h post RE, 2 h post RE, 4 h post RE and 6 h post RE) were used to assess the concentration of 18 amino acids associated with collagen composition (glycine, proline, hydroxyproline, glutamic acid, alanine, arginine, aspartic acid, lysine, serine, leucine, valine, phenylalanine, threonine, isoleucine, histidine, tyrosine, methionine, and glutamine, but not hydroxylysine). All 18 amino acid concentrations were measured simultaneously using anionic ion-pair reverse phase liquid chromatography tandem mass spectrometry (LC-MS/MS) system following derivatisation of the amino acid with *n*-butanol hydrogen chloride. The LC-MS/MS system used a Micromass® Quattro Ultima™ Pt (Manchester, UK) coupled to an Agilent 1100 series (Cheadle, UK) high performance liquid chromatography binary pump. Electrospray ionisation source operating in positive ion mode, mass detection for each amino acid butyl ester was achieved in multiple reaction monitoring mode. Certified amino acid standards were purchased from Wacko Chemicals GmbH (Neuss, Germany) and Sigma-Aldrich (Dorset, UK). Internal standards used were glucosaminic acid and S-(2-Aminoethyl)-L-cysteine hydrochloride (Sigma-Aldrich, Dorset, UK) and L-Citrulline-2,3,3,4,4,5,5-d7 (Isoscience,

King of Prussia, PA, USA). Three internal quality controls (QC) at low, medium and high concentrations were made from pooled human serum. For each batch of analysis, 10 µL of standards, QC and test samples were added to a microcentrifuge tubes, to which 440 µL of internal standards made up in 0.1M hydrochloride in methanol was added. The mixture was vortexed twice, each time allowed to stand for 10 min, then centrifuged at 10,800 × g for 5 mins. The supernatant was then transferred into a borosilicate tube and dried to completeness under nitrogen gas at a temperature of 60ºC. 100 µL of 3N *n*-butanol hydrogen chloride was added to the dried residue, vortex mixed, capped and incubated at 60ºC for 7 min. Following butylation, the mixture was dried completely under nitrogen gas, and then reconstituted with 250 µL of 12% acetonitrile:water containing 0.025% heptafluorobutyric acid (HFBA). After a final vortex mix, the samples were transferred to a polypropylene autosampler vial for injection into the LC-MS/MS.

Chromatographic separation was achieved using a Modus AAC 100 x 2.1mm 3µm column (Chromatography Direct Ltd, Runcorn, UK) maintained at 40ºC. Anionic ion-pair reagent HFBA was added to the mobile phases to improve analyte interaction with the stationary phase. A gradient elution profile at a flow rate of 350 $\mu L \cdot min^{-1}$ was used throughout. Initial conditions were 88% mobile phase A (0.025% HFBA in water) and 12% mobile phase B (0.025% HFBA in acetonitrile). This was held for 30 s. Mobile phase B was increased linearly to 20% at 10 min, with a further linear increase in mobile phased B to 60% at 15 min. This was held constant until 16.9 min and returned to the initial conditions at 17 min. Injection volume was 10 µL with an injection cycle time of 20 min. The assay range was 0 – 2000 $\mu mol \cdot L^{-1}$ for all 18 amino acids studied. Inter-assay precision coefficient of variation (CV) for all amino acids were between 3.3% to 10.3%.

Statistical analyses

Data were analysed using the statistical software package SPSS (Version 26, IBM Inc., Armonk, NY, USA). Sample size was estimated prior to conducting the study with G*Power software (version 3.1.9.6, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The estimation was performed using a large effect size ($\eta_p^2 = 0.22$), based on the results from Shaw et al. (2017), which demonstrated a two-fold increase in the serum PINP concentration × time area under the curve (AUC) following exercise with 15 g vs. 5 g gelatine ingestion. The results from our a priori power calculation deemed a minimum of eight participants was necessary to detect an effect of HC dose (one-way repeated measures analysis of variance (ANOVA); α: 0.05; power: 0.80). We recruited 10 participants to account for an expected 10 – 20% drop out. Using the Shapiro-Wilk test, all data were deemed to be normally distributed except for the amino acid data. The latter data were therefore log transformed prior to undergoing subsequent statistical analyses. One-way within-subject ANOVA models were performed to compare baseline (-1 h) concentrations of PINP and β-CTX in all three trails. Two-way within-subject ANOVAs (dose × time) were performed to detect changes in serum PINP and amino acid concentrations over time. To detect changes in plasma β-CTX concentration, a two-way within-subject ANOVA (dose × time) was performed. One-way repeated measures ANOVA models were performed to detect dose-dependent differences in concentration × time AUCs for PINP and each of the 18 amino acids analysed. Where Mauchly's test of sphericity had been violated, Greenhouse-Geisser ($\varepsilon < 0.75$) or Huynh-Feldt ($\varepsilon > 0.75$) corrections were applied. Where a main effect of HC dose existed, Fisher's LSD post-hoc pairwise comparisons were performed to reveal which doses differed. Partial eta squared effect sizes (η_p^2) were reported for each statistical model, and the thresholds for η_p^2 are defined as small

($\eta_p^2 = 0.01$), medium ($\eta_p^2 = 0.06$) and large ($\eta_p^2 = 0.14$) (Cohen, 1988). All data analyses matched the research design, as there were no missing data for any of the dependent variables. The level of statistical significance was set at $P < 0.05$ and all data are presented as mean ± standard deviations with 95% confidence intervals (CI, where applicable), unless stated otherwise.

3.3 Results

Serum PINP concentration and AUC

Baseline serum PINP concentrations for the 0 g, 15 g and 30 g HC interventions did not differ ($P = 0.990$, $\eta_p^2 = 0.001$; 0 g HC 32.7 ± 28.0 (95% CI: 12.7 – 52.7) $\mu g \cdot L^{-1}$; 15 g HC 32.7 ± 24.2 (95% CI: 15.4 – 49.9) $\mu g \cdot L^{-1}$; 30 g HC 32.3 ± 21.6 (95% CI: 16.9 – 47.7) $\mu g \cdot L^{-1}$. Regarding serum PINP concentration, there was a main effect of HC dose ($P = 0.004$, $\eta_p^2 = 0.462$) and time ($P = 0.013$, $\eta_p^2 = 0.458$) but no dose × time interaction effect ($P = 0.732$, $\eta_p^2 = 0.071$, **Figure 4A**). These results suggest the dose effect was not time specific. Post-hoc pairwise comparisons revealed that 30 g HC had a greater PINP response than 0 g HC ($P = 0.002$) and 15 g HC ($P = 0.020$), while 15 g HC did not different from 0 g HC ($P = 0.245$). Regarding the serum PINP concentration × time AUC, there was a main effect of HC dose ($P = 0.001$, $\eta_p^2 = 0.517$), and post-hoc pairwise comparisons revealed that 30 g HC had a greater AUC (267 ± 79 [95% CI: 211 – 323] $\mu g \cdot L^{-1} \cdot h$ than 15 g HC (235 ± 70 [95% CI: 184 – 284] $\mu g \cdot L^{-1} \cdot h$, $P = 0.013$) and 0 g HC (219 ± 88 [95% CI: 155 – 281] $\mu g \cdot L^{-1} \cdot h$, $P = 0.002$), but 15 g HC AUC did not differ from 0 g HC AUC ($P = 0.225$, **Figure 4B**).

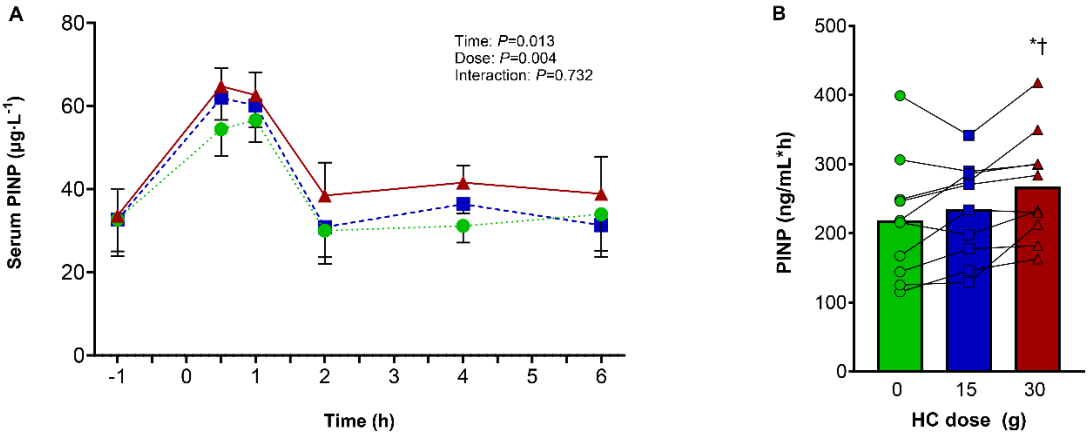

Figure 4. Collagen synthesis following hydrolysed collagen (HC) ingestion (-1 h) and performing resistance exercise. (A) serum PINP concentrations (B) serum PINP concentration × time area under the curve following 0 g HC (green circles), 15 g HC (blue squares), and 30 g HC (red triangles) ingestion. *Greater than 0 g HC (P = 0.005); †Greater than 15 g HC (P = 0.039). Values represent means ± SEM.

Plasma β-CTX

Baseline plasma β-CTX concentrations for the 0 g, 15 g and 30 g HC interventions did not differ (P = 0.311, η_p^2 = 0.122; 0 g HC 0.8 ± 0.3 (95% CI: 0.6 – 1.0) µg·L^{-1}; 15 g HC 0.8 ± 0.3 (95% CI: 0.6 – 0.9) µg·L^{-1}; 30 g HC 0.8 ± 0.2 (95% CI: 0.6 – 0.9) µg·L^{-1}. There was a main effect of time (P = 0.007, η_p^2 = 0.577) but no main effect of HC dose (P = 0.286, η_p^2 = 0.127) and no dose × time interaction (P = 0.748, η_p^2 = 0.031), i.e. plasma β-CTX concentration decreased from -1 h (prior to HC ingestion and RE) to 6 h post RE for all three interventions, with no difference between intervention (**Figure 5**).

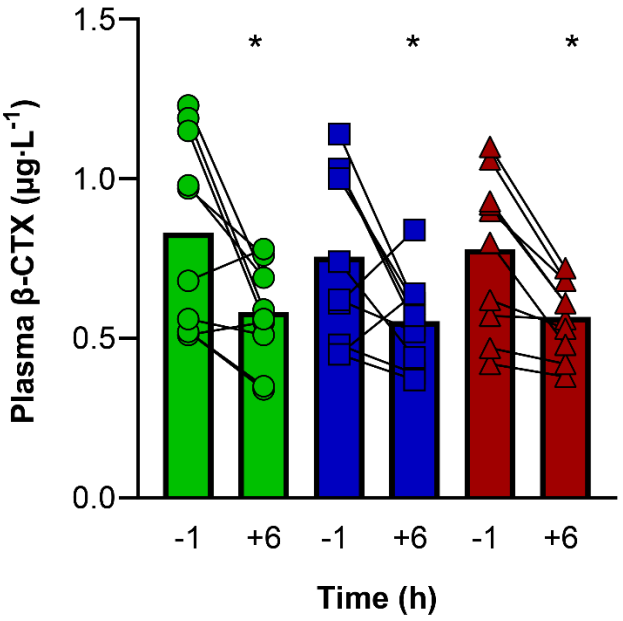

Figure 5. Collagen breakdown following hydrolysed collagen (HC) intake and performing resistance exercise. Plasma CTX-1 concentrations following 0 g HC (green circles), 15 g HC (blue squares), and 30 g HC (red triangles) ingestion. *Lower than -1 h ($P < 0.05$).

Serum amino acid concentrations

Serum concentrations over the 7-h period of each intervention of the 18 amino acids that constitute type I collagen are shown in **Figure 6**. The main effects of time, dose, and dose × time interaction effects for each amino acid are denoted in **Figure 6**. There were main effects of dose for 14 amino acids (glycine, proline, hydroxyproline, glutamic acid, alanine, arginine, aspartic acid, lysine, serine, leucine, valine, isoleucine, methionine, and glutamine), with 30 g HC demonstrating higher serum concentrations than 0 g. Of those amino acids, glycine, proline, hydroxyproline, arginine, lysine, serine, leucine, valine, isoleucine, and methionine in 30 g HC showed higher serum concentrations than 15 g HC.

All amino acids except for histidine ($P > 0.05$) showed a dose × time interaction effect, and there was no main effect of dose for histidine ($P = 0.451$).

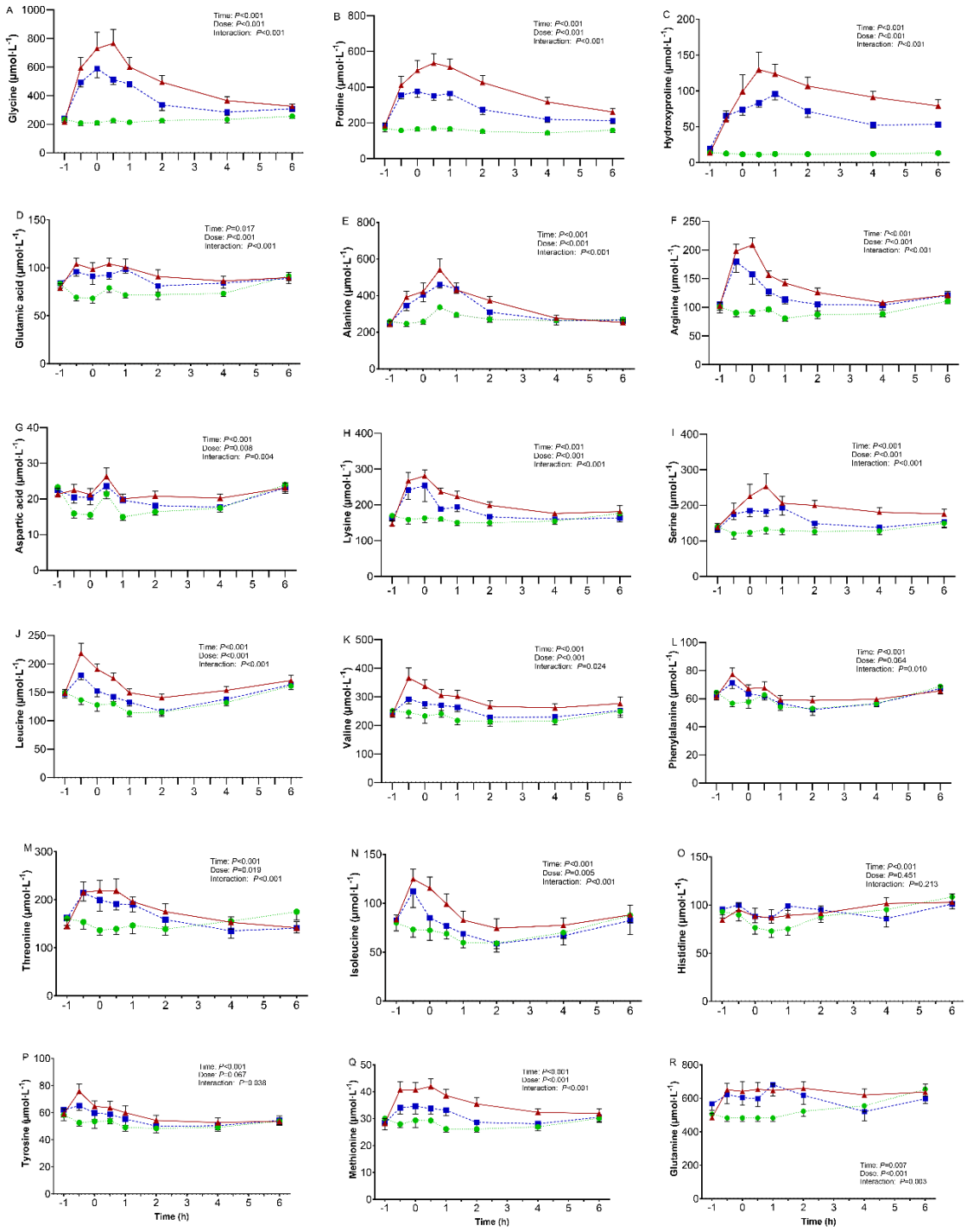

Figure 6. Concentrations of eighteen serum amino acid at before ingesting hydrolysed collagen (-1 h), 1 h after ingesting 0 g (green circles), 15 g (blue squares), or 30 g (red triangles) (HC) (+1 h) and then performing barbell back squat exercise at time point 0 h. Values represent means ± SEM.

3.4 Discussion

This study is the first to investigate the effect of *high-intensity* resistance exercise (RE) and 0, 15 and 30 g hydrolysed collagen (HC) supplementation on whole body collagen turnover in a homogenous group of resistance-trained, healthy, young men. We found that the serum PINP concentration × time area-under-the-curve (AUC) for the 30 g HC intervention was greater than for the 15 g and 0 g HC interventions. Further, these results were consistent with greater increases in the appearance of key amino acid constituents of collagen (e.g. glycine and proline) within the blood following ingestion of 30 g HC versus 15 g and 0 g. Therefore, at least 30 g HC is required to provide greater exogenous collagen amino acid availability, which appears to be a key factor for optimising collagen synthesis following high-intensity RE in resistance-trained, young men.

To address the aims of our study, we measured serum PINP following RE with different doses of HC. We chose high-intensity back squat RE to target the quadriceps muscle-tendon unit (MTU), because the human patellar tendon appears to hypertrophy only following prolonged periods of high-intensity (Kongsgaard et al., 2007, Seynnes et al., 2009) and not moderate-intensity (Lee et al., 2023) resistance training. In the current study, the 7-h experimental design was based on a significant increase in muscle and tendon collagen fractional synthetic rate (FSR) at 6 h post-exercise following 1-h RE in healthy young men (Miller et al., 2005). Although the significantly elevated muscle collagen FSR was similar at 6 h post- and 24 h post-RE (Miller et al., 2005), tendon collagen FSR appeared to be further augmented at 24 h post- compared to 6 h post-RE, although it is not stipulated in the article whether tendon collagen FSR measured at these two time points differed significantly. In a separate study, serum PICP concentration was significantly higher 48 h post-exercise following 50 maximal concentric knee extensions in healthy young men

(Virtanen et al., 1993). Thus, it is possible that, had each of our three interventions lasted 24 – 48 h post-RE, we may have observed further increases in serum PINP concentration, and possibly a larger effect of HC ingestion on these increases.

The 30 min high-intensity RE model we employed in our study was associated with peak serum PINP concentrations of ~60 $\mu g \cdot L^{-1}$ (regardless of dose), which occurred 30-60 min after the onset of RE (and 90-120 min after supplement ingestion). We chose to measure serum PINP concentration because it is a reliable biomarker of collagen synthesis, being a procollagen peptide that is cleaved off during maturation from procollagen to collagen (Heinemeier et al., 2016). The similar PINP concentration at +0.5 and +1 h post-RE for all three doses (including 0 g HC) suggests the increase in PINP concentration observed within the first hour after starting the RE occurred as a consequence of the RE, rather than HC ingestion. However, it is possible that our data at +0.5 h post-RE may have been influenced by an increase in blood flow. A distinction should be made between flux, i.e. the total amount of PINP passing through the blood registered at any given time, and concentration, i.e. the ratio of PINP to the volume of serum. Blood flow rises 20-fold and 7-fold in the calf muscle and peritendinous area of the Achilles tendon, respectively, during repeated plantar flexion contractions in healthy individuals (Boushel et al., 2000). However, an increase in blood flow to the peritendinous tendon has been shown to return to resting levels within a few minutes of finishing the same type of exercise (Langberg et al., 2001). Therefore, the fact that serum PINP concentration was still at its peak at +1 h in the current study, i.e. 30 min after RE ended, when cardiac output would be expected to have returned to resting rates following lower-limb RE (Poton and Polito, 2016), it is likely that this increase in PINP concentration was due to an increase in RE-induced collagen synthesis, rather than blood flow. Serum PINP concentration decreased to baseline

values in the 0 g and 15 g interventions for the remainder of those interventions, while it decreased but remained more elevated in the 30 g intervention, which resulted in the higher AUC in the 30 g intervention compared to the 0 g and 15 g interventions.

In contrast to our results, Aussieker et al. (2023) recently found that 30 g HC ingestion with six sets of 8–15 repetitions at 60% estimated 1-RM barbell back squat did not augment *vastus lateralis* muscle connective tissue protein FSR or circulating PINP concentration more than RE with 30 g whey protein ingestion or RE alone in different groups of young men and women. A number of differences in study design may help explain this discrepancy between studies. Firstly, the between-group design used by Aussieker et al. (2023) may have introduced more within and between intervention variability (thus potentially confounding an effect of HC) compared to a within-group cross-over design, as used in the current study. Secondly, oestrogen is known to affect skeletal muscle and tendon collagen synthesis in women (Hansen et al., 2009a, Hansen et al., 2009c), and the use of a mixed-sex cohort by Aussieker et al. (2023), rather than a 100% male cohort as used in the current study, may have increased within- and between-group variability in connective tissue protein FSR. Thirdly, vitamin C was not consumed during the interventions by Aussieker et al. (2023), which began after an overnight fast. As vitamin C is required for the biosynthesis of collagen (Murad et al., 1981) and humans are unable to store it in the body or synthesize it endogenously (Li and Schellhorn, 2007), this may have limited muscle connective tissue protein FSR. Finally, it should be noted that Aussieker et al. (2023) measured connective tissue protein FSR in skeletal muscle and not tendon or ligament. The latter tissues have a 70-85% type I collagen content (Kjaer, 2004) compared to just ~5% in skeletal muscle (Babraj et al., 2005).

Contrary to Aussieker et al. (2023) and in accordance with our findings, Shaw et al. (2017) found that serum PINP concentration was greater following jump-rope exercise with gelatine supplementation in a dose-response manner. Due to different time points used to measure serum PINP concentration, a direct comparison of peak concentration between studies is not possible. Nevertheless, the serum PINP AUC was greater in our 30 g HC intervention compared to our 15 g and 0 g interventions (with no difference between our 0 g and 15 g interventions, **Figure 4**), while Shaw et al. (2017) found a greater effect of 15 g versus 5 g and 0 g gelatine. This suggests that the different exercise models used may require different doses of exogenous collagen to optimise the collagen synthetic response for that particular exercise.

Regarding the amino acids that constitute collagen (e.g. glycine, proline, hydroxyproline, etc.), these peaked in circulation around 1 – 1.5 h after ingestion of 30 g HC in our study (**Figure 6**). This was in line with previous studies, which involved the ingestion of 15 g gelatine or 20 g or 30 g collagen peptides in healthy young populations (Shaw et al., 2017, Alcock et al., 2019, Aussieker et al., 2023). This similarity between studies indicates the maximal rate of amino acid absorption occurs approximately an hour after ingestion of collagen in healthy young men, regardless of dose and type of collagen supplementation. We also observed that the average concentrations of glycine, proline, hydroxyproline, arginine, lysine, serine, leucine, valine, isoleucine, and methionine were greater after ingestion of 30 g HC compared to 15 g and 0 g HC. The high availability of collagen amino acids in the 30 g HC intervention might have promoted a greater collagen synthetic response in two ways. Firstly, the greater abundance of key amino acids may have simply provided more of the essential components to increase collagenous tissue content follow-

ing an overload-induced stimulation of collagen synthesis in the lower-limb MTUs. Secondly, they may have stimulated mammalian target of rapamycin complex 1 (mTORC1) phosphorylation independently of muscle contraction/stretch-activated mechanisms, in a similar manner to amino acid stimulation of skeletal muscle myofibrillar protein synthesis via mTORC1 activation (Drummond et al., 2009). For example, after treating chondrocytes from bovine cartilage with 1.5 mM glycine, proline, or lysine for 15 days, type II collagen synthesis was 1.6 times greater following glycine treatment compared to proline treatment and 2 times greater compared to lysine treatment (de Paz-Lugo et al., 2018). Further, human skin fibroblasts treated with 5 mM proline for 48 h demonstrated an increase in collagen type I α1 expression (Szoka et al., 2017), while proline and hydroxyproline also increased TGF-β expression in human fibroblasts (Surazynski et al., 2010), which would be expected to cause phosphorylation of protein kinase B (Akt) and mTORC1 (thus explaining the increase in collagen synthesis and gene expression). Considering the above mechanisms and that collagen synthesis is stimulated via phosphorylation of Akt and mTORC1 in response to mechanical loading in cultured human tendon-derived stromal cells (Mousavizadeh et al., 2020), and that mechanical loading increases in TGF-β expression and type I collagen expression in rat Achilles tendon (Heinemeier et al., 2007), independent RE- and amino acid-associated signalling pathways likely explain our findings that high-intensity RE with 30 g HC supplementation stimulates collagen synthesis more than RE with 15 g HC ingestion or RE alone.

Concerning collagen breakdown, β-CTX is released into circulation from mature type I collagen during degradation, and therefore serves as a reliable biomarker of collagen breakdown (Gineyts et al., 2000). In the current study, regardless of HC dose, plasma β-CTX concentration decreased by ~30% from -1 to 6 h post RE (**Figure 5**). This may have

been caused by the high-intensity RE stimulating collagen synthesis (**Figure 4**), subsequently inhibiting collagen breakdown. This hypothesis is supported by currently unpublished data from our laboratory in middle-aged men, suggesting the decrease in circulating β-CTX concentration occurs immediately after RE and remains lower for the subsequent six hours of rest, regardless of HC dose. Alternatively, the reduction may have been associated with circadian rhythm, as Qvist et al. (2002) reported that resting serum β-CTX concentrations in men and pre- and postmenopausal women (aged 24 – 73 years) peaked at 08:00, then sharply decreased between 11:00 and 14:00. Nevertheless, as tissue turnover is determined by the rates of both synthesis and breakdown, the role of collagen degradation on the regulation of collagen turnover is crucial. For example, avian skeletal muscle exposed to mechanical loading led to an increase in collagen synthesis, which was accompanied by a decrease in the degradation of newly synthesized collagen, as well as an increase in the degradation of mature collagen (Laurent et al., 1985). Thus, further research is necessary to elucidate the effects of RE and HC supplementation on degradation of newly synthesized and mature collagen for the regulation of collagen turnover.

A limitation of this study is that our assessment of collagen synthesis was indirect, i.e. we measured serum PINP concentration rather than harvesting tendon biopsies, with which we could have assessed tendon PINP concentration or collagen FSR directly. However, human tendon (Langberg et al., 1999) and serum PINP concentration (Huang et al., 2022) increases after an acute bout of exercise, and serum PINP can remain elevated for up to four days following resistance-type exercise in healthy young men (Virtanen et al., 1993). Nevertheless, future studies should investigate the effect of HC ingestion with RE on connective tissue collagen synthesis by measuring circulating and tendon PINP concentration, and tendon collagen FSR simultaneously. Although percentage difference in PINP

AUC comparison (30 g *vs*. 0 g HC 27.9% ± 21.9% (95% CI: 12.2% – 43.6%); 30 g *vs*. 15 g HC 15.8% ± 19.7% (95% CI: 1.7% – 29.9%); 15 g *vs*. 0 g 11.6% ± 18.2% (95% CI: -1.4% – 24.6%) was higher than PINP intra-assay CV (< 10%), 95% CI of lower limit for the comparison between 30 g *vs*. 15 g HC was within the CV of PINP assay. Therefore, caution should be taken for interpreting this comparison. Future studies could consider the co-ingestion of HC with other nutrients that may augment connective tissue protein synthesis, such as whey protein/branch-chain amino acids, etc.

In conclusion, we have demonstrated for the first time that a single bout of high-intensity, lower-limb RE with 30 g HC ingestion increased whole body collagen synthesis more than RE with 15 g or 0 g HC in resistance-trained young men. This higher response was likely related to the greater availability of key amino acids following the ingestion of 30 g HC compared to 15 g and 0 g. This may have implications for augmenting tendon adaptation to high-intensity resistance training when 30 g HC is ingested in combination with resistance exercise over a prolonged period of time. Future studies should also investigate if a dose-response exists regarding high-intensity RE with HC supplementation in resistance-trained young women.

Chapter Four

Case study: effect of resistance exercise, collagen ingestion and circulating oestrogen concentration on collagen synthesis in a eumenorrheic, resistance-trained woman

Prelude

Chapter Three focused on the dose-response effect of HC and RE on collagen turnover in resistance-trained, young men. The main outcome was that 30 g HC led to the highest collagen synthesis response. Although, it is known that circulating endogenous oestrogen affects collagen metabolism in women, it is not known whether HC ingestion with resistance exercise can augment that reduced collagen synthesis response. Therefore, this chapter will investigate the effect of 30 g HC ingestion and resistance exercise on markers of collagen turnover when circulating oestrogen concentration is high and when it is low in a young, healthy, eumenorrheic, resistance-trained woman. Data collection for this study began in March 2019 and was completed in May 2019.

Abstract

We investigated the combined effect of high-intensity resistance exercise (RE), hydrolysed collagen (HC) ingestion and circulating oestrogen concentration on collagen synthesis in a eumenorrheic, resistance-trained woman. In a double-blind, randomized cross-over design, the participant (36 years; height 1.61m; mass 82.6kg) consumed 0g or 30g HC prior to performing 4×10 repetitions' back-squat RE when circulating oestrogen concentration was low (onset of menses, OM); and when it was high (late follicular phase, LF) during two consecutive menstrual cycles. Ten 5-mL blood samples were collected over the 7-h duration of each of the four interventions to analyse serum concentrations of 17β-oestradiol, procollagen type I amino-terminal propeptide (PINP, a biomarker of type I collagen synthesis), β-isomerized C-terminal telopeptide of type I collagen (β-CTX, a biomarker of type I collagen breakdown) and 18 amino acids related to collagen composition. Serum 17β-oestradiol concentration was 5-fold greater at LF (891±116 pmol·L^{-1}) than at OM (180±13 pmol·L^{-1}). The PINP concentration×time area-under-the-curve (AUC) was higher in the 30g OM intervention (201 μg·L^{-1}·h) than the 30g LF (144 μg·L^{-1}·h), 0g OM (151 μg·L^{-1}·h), and 0g LF (122 μg·L^{-1}·h) interventions. Plasma β-CTX concentration decreased 1.4-fold from pre-RE to 6h post-RE, regardless of HC dose or menstrual cycle phase. The AUCs of glycine, proline and hydroxyproline were 2.2-, 2.3-, and 6.4-fold higher in the 30g vs. 0g HC interventions. Thus, high endogenous serum oestrogen concentration was associated with lower collagen synthesis following RE in a eumenorrheic, resistance-trained woman. Ingesting 30g HC, however, augmented the collagen synthetic response at both LF and OM.

4.1 Introduction

The female sex hormone, oestrogen, has been proposed as a risk factor for sustaining soft tissue injuries (particularly to the anterior cruciate ligament) in female athletes (Hewett et al., 2006). This is due to the evidence that knee joint laxity increases around the time of ovulation during the menstrual cycle (Shultz et al., 2015), which is when circulating oestrogen concentration peaks (Park et al., 2009). An inverse relationship has also been shown between circulating oestrogen concentration and tendon stiffness in healthy young women (Hansen et al., 2013), which suggests that this hormone can affect the mechanical properties of collagenous tissues, such as tendon and ligament. Furthermore, oestrogen regulates various intracellular signalling pathways (including PI3K/Akt) by binding to its α and β receptors (Marino et al., 2006), which are found in human collagenous tissues such as tendon (Bridgeman et al., 2010). Thus, oestrogen has the potential to influence collagen protein turnover in human tendon, thereby potentially affecting the mechanical properties of the muscle-tendon unit (Hansen and Kjaer, 2014).

Chronic resistance exercise (RE) is known to alter the morphological and mechanical properties of human tendon by increasing its size, stiffness and elastic modulus (Kongsgaard et al., 2007, Seynnes et al., 2009), which may improve the efficiency of force transference from the muscle to the bone, thus increasing rate of force development and having the potential to improve physical performance (Bojsen-Møller et al., 2005). In addition, consumption of 15 g gelatine, which contains amino acids such as glycine, proline and hydroxyproline that are crucial in forming the type I collagen fibrils abundant in human muscle, tendon and ligament (Kadler et al., 2007), has been shown to increase collagen synthesis following jump-rope exercise in healthy young men (Shaw et al., 2017). However, a recent study found that 30 g hydro-

lysed collagen (HC) with moderate-intensity barbell back squat exercise did not further increase muscle connective tissue protein synthesis compared to RE alone in young healthy men and women (Aussieker et al., 2023). The between-group design and use of mixed-sex cohorts in this study may have introduced more within- and between-group variability, which together with the moderate intensity of RE, may have confounded an effect of HC on connective tissue protein synthesis. Thus, although RE is known to increase muscle and tendon collagen synthesis (Miller et al., 2005), it is not known if HC ingestion can augment the collagen synthesis response to RE in women, and whether this response is influenced by circulating oestrogen concentration.

We aimed to compare collagen turnover following an acute bout of high-intensity RE with 30 g HC in a healthy young eumenorrheic, resistance-trained woman, at different stages of her menstrual cycle (i.e. when circulating oestrogen concentration was low, and when it was high) as ingesting exogenous HC may augment the collagen synthesis response to RE and override any negative effect of endogenous oestrogen on collagen synthesis. We hypothesized that *in vivo* marker of collagen synthesis would be greater when RE was performed with 30 g HC ingestion at low circulating oestrogen concentration compared to when no HC was ingested, or when serum oestrogen concentration was high (with or without HC ingestion). We also hypothesized that 30 g HC would lead to a greater serum concentration of the amino acids necessary for collagen synthesis to occur, e.g. glycine, proline and hydroxyproline.

4.2 Methods

Presentation of subject

A healthy young eumenorrheic, resistance-trained woman (age, 36 years; height, 1.61 m; mass, 82.6 kg) provided written informed consent before completing this study. The study was registered at https://clinicaltrials.gov/ (identifier: NCT05932771), was approved by Liverpool John Moores University Research Ethics Committee (approval number: 18/SPS/059) and adhered to the Declaration of Helsinki. This participant had two years' experience of weightlifting prior to the study, and she performed RE 3 – 4 times per week, which included barbell back squat on a weekly basis. She had not used any hormonal contraceptive method for two years prior to the study and had a 'normal' menstrual cycle, as determined by responses to the 'low energy availability in females questionnaire' (LEAF-Q, score 1) (Melin et al., 2014). Furthermore, the participant was healthy (as determined via responses to a health questionnaire), she did not smoke, and she had not sustained a musculoskeletal injury in the 12 months prior to the study. Data collection for this study began in March 2019 and was completed in May 2019.

Overview of intervention

This was a double-blind, randomised cross-over designed case study. The participant was asked to attend the laboratory on five occasions, including the familiarisation and baseline test (visit 1), and two dose-response interventions (i.e. 0 g and 30 g HC consumption) at the onset of menses (OM) and two dose-response interventions at the end of the late follicular phase (LF, estimated to be the date of ovulation) of her menstrual cycle, to ensure the greatest difference in circulating oestrogen concentration. During the first visit, the participant's barbell back squat 10 repetition maximum (RM) load was determined (65 kg for this participant), which was used as the training load during the four dose-response interventions. In addition, the duration of the participant's forthcoming menstrual cycle was estimated based on self-reporting onset of menses and previous cycle length (28 days), which was repeated for the next menstrual cycle (also

28 days). Two interventions (including either 30 g or 0 g HC), interspersed by at least 12 days, were performed in a random order during each cycle of two consecutive menstrual cycles. The participant therefore completed four interventions on the following days: day 2 and day 14 of cycle 1; day 1 and day 13 of cycle 2. Each of the four interventions lasted for seven hours and started and finished at the same times during the day (i.e. 08:00 – 15:00). The participant was instructed to refrain from performing strenuous physical activity and consuming caffeine and alcohol for 48 h before each intervention. Upon arrival at the laboratory following an overnight fast (≥10 h), the participant consumed either 30 g or 0 g HC prior to performing four sets' 10-RM back squat. After completion of the exercise, the participant rested for 6 h and 10 × 5-mL blood samples were collected at regular times points over the course of the 7-h intervention (**Figure 1**). Following ingestion of the supplement, only water was allowed to be consumed *ad libitum* during each intervention. The participant was instructed to record her dietary intake in the day before their first intervention and to replicate that dietary behaviour in the day preceding each of the subsequent interventions.

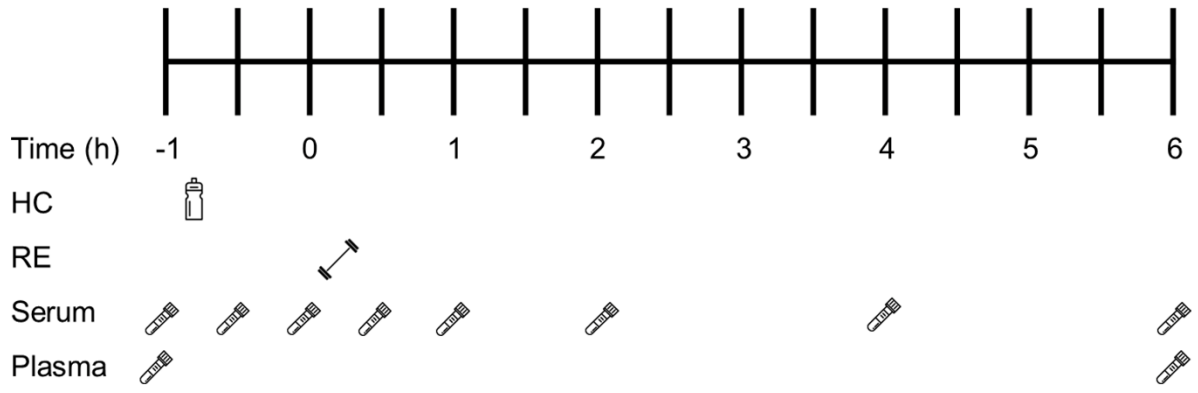

Figure 1. Schematic of experimental design. HC, hydrolysed collage; RE, resistance exercise.

Barbell back squat 10-repetition maximum (10-RM) assessment and 10-RM bout during each intervention

The squat depth during the 10-RM was standardized to ensure the muscle-tendon units bore the same mechanical loading during the four interventions. The participant placed a 20 kg Olympic barbell on her shoulders (the high bar position), placed her feet shoulder-width apart and descended until the knee joint angle reached 90° flexion, which was measured using a goniometer. While the participants held the position at 90° knee flexion, a vertical line from the ground to the ischial tuberosity was measured with a tape measure, and a resistance band was placed on both sides of the squat rack at this location to ensure the participant knew when she had reached 90° knee flexion during each repetition. A warm-up included two dynamic exercises (low lunge and squat to stand) prior to the actual 10-RM assessment, which comprised the following sets of barbell back squat: 10 repetitions with the 20 kg barbell, 8 repetitions at 50% of the estimated 10-RM, 4 repetitions at 70% and 1 repetition at 90% of the estimated 10-RM). After a 5-min rest, participants performed 10-RM attempts separated by 5-min rest periods until 10-RM load was obtained. Two researchers observed each test procedure to provide a cue when the participant's proximal hamstrings/gluteus maximus touched the resistance band, and to spot the participant. The 10-RM bout during each intervention was preceded by a similar warm-up, i.e. two dynamic exercises followed by performing 1 – 10 repetitions barbell back squat at 50 – 90% 1-RM, as stated above.

Nutritional supplementation

Two supplements (30 g HC and 0 g HC, Myprotein, Cheshire, UK) with 50 mg vitamin C (Holland and Barrett Retail Limited, Warwickshire, UK) were dissolved with 300 ml water in an opaque drinks bottle. In order to match the calories of 30 g HC, 34.1 g maltodextrin (Myprotein, Cheshire, UK) was used in the 0 g HC dose. Although both supplements were "non-

flavoured", 4 g non-caloric sweetener (Truvia®, SilverSpoon, London, UK) was added in all drinks to mask any potential difference in taste.

Blood sampling and analysis

Using the BD NexibiaTM closed IV catheter system (22 G, Becton, Dickinson and Company, Franklin Lakes, USA), a total of 10 venous blood samples were collected from the right antecubital fossa (Sampling procedures, Figure 1). Eight 5 mL blood samples were collected in BD Vacutainer™ Serum Separation Tube (SST™) II Advance (Dickinson and Company, Franklin Lakes, USA) for procollagen type 1 N-terminal (PINP), 17β-oestradiol and amino acid profile analyses while, 2 × 5 mL blood samples were collected in BD Vacutainer™ Hemogard Closure Plastic K2-Ethylenediaminetetraacetic acid (EDTA) Tubes (Dickinson and Company, Franklin Lakes, USA) for β-isomerized C-terminal telopeptide of type I collagen (β-CTX) analysis (Figure 1). The concentrations of PINP (USCN Life Sciences, Wuhan, China; intra-assay coefficient of variation (CV) <10%; inter-assay CV <12%; detection range: 2.47-200 $\mu g \cdot L^{-1}$; sensitivity <0.91 $\mu g \cdot L^{-1}$) and 17β-oestradiol (IBL International GmbH, Hamburg, Germany; intra-assay CV <10%; inter-assay CV <15%; detection range: 35.6-7340 $pmol \cdot L^{-1}$; sensitivity: 38.9 $pmol \cdot L^{-1}$) were analysed at Liverpool John Moores University by enzyme-linked immunosorbent assay (ELISA). The ELISA absorbance readings were performed at 450 nm, using a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). Plasma β-CTX concentrations were measured using electrochemiluminescence immunoassay on a Cobas e601 analyser (Roche Diagnostics, Mannheim, Germany; inter-assay CV ≤3%; detection range 0.2 and 1.5 $\mu g \cdot L^{-1}$; sensitivity 0.01 $\mu g \cdot L^{-1}$). The concentrations of 18 amino acids were measured simultaneously in serum using anionic ion-pair reverse phase liquid chromatography tandem mass spectrometry (LC-MS/MS) system (a Micromass® Quattro Ultima™ Pt (Manchester, UK))

coupled to an Agilent 1100 series (Cheadle, UK) high performance liquid chromatography (HPLC) binary pump. The assay range was 0 – 2000 μmol·L^{-1} for all 18 amino acids studied. Inter-assay CV for all amino acids were between 3.3% to 10.3%. The β-CTX and amino acid profile analyses were performed by the Bioanalytical facility at the University of East Anglia. The concentration × time area under the curve (AUC) for PINP and amino acids were calculated using Prism software (version 9.4.1, GraphPad Inc., San Diego, California USA).

4.3 Results

Endogenous oestrogen, collagen synthesis and breakdown, and amino acid profile

Serum 17β-oestradiol concentration was lower at OM than at LF (**Figure 2**). Serum PINP concentration peaked at +1 h post RE and the PINP concentration × time AUC was highest in the 30 g HC OM (201 μg·L^{-1}·h) intervention than the 30 g HC LF (144 μg·L^{-1}·h), 0 g HC OM (151 μg·L^{-1}·h), and 0 g HC LF (122 μg·L^{-1}) interventions (**Figure 3**). Plasma β-CTX concentration decreased 1.3- to 1.5-fold from rest immediately prior to HC ingestion to 6-h post RE in all four interventions (**Figure 3**). The serum concentrations of 18 amino acids that constitute collagen are shown in **Figure 4**. The concentration × time AUCs of glycine, proline and hydroxyproline in the 30 g HC interventions were 2.2, 2.3 and 6.4 times greater than in the 0 g HC interventions, respectively.

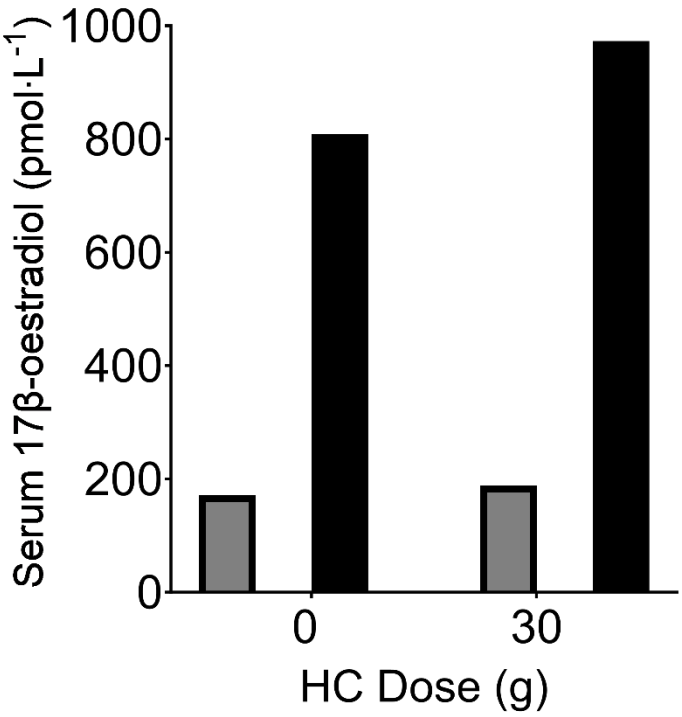

Figure 2. Oestrogen concentration at rest prior to consuming 0 g or 30 g hydrolysed collagen (HC) at the onset of menses (grey bars) and at the end of the late follicular phase (black bars).

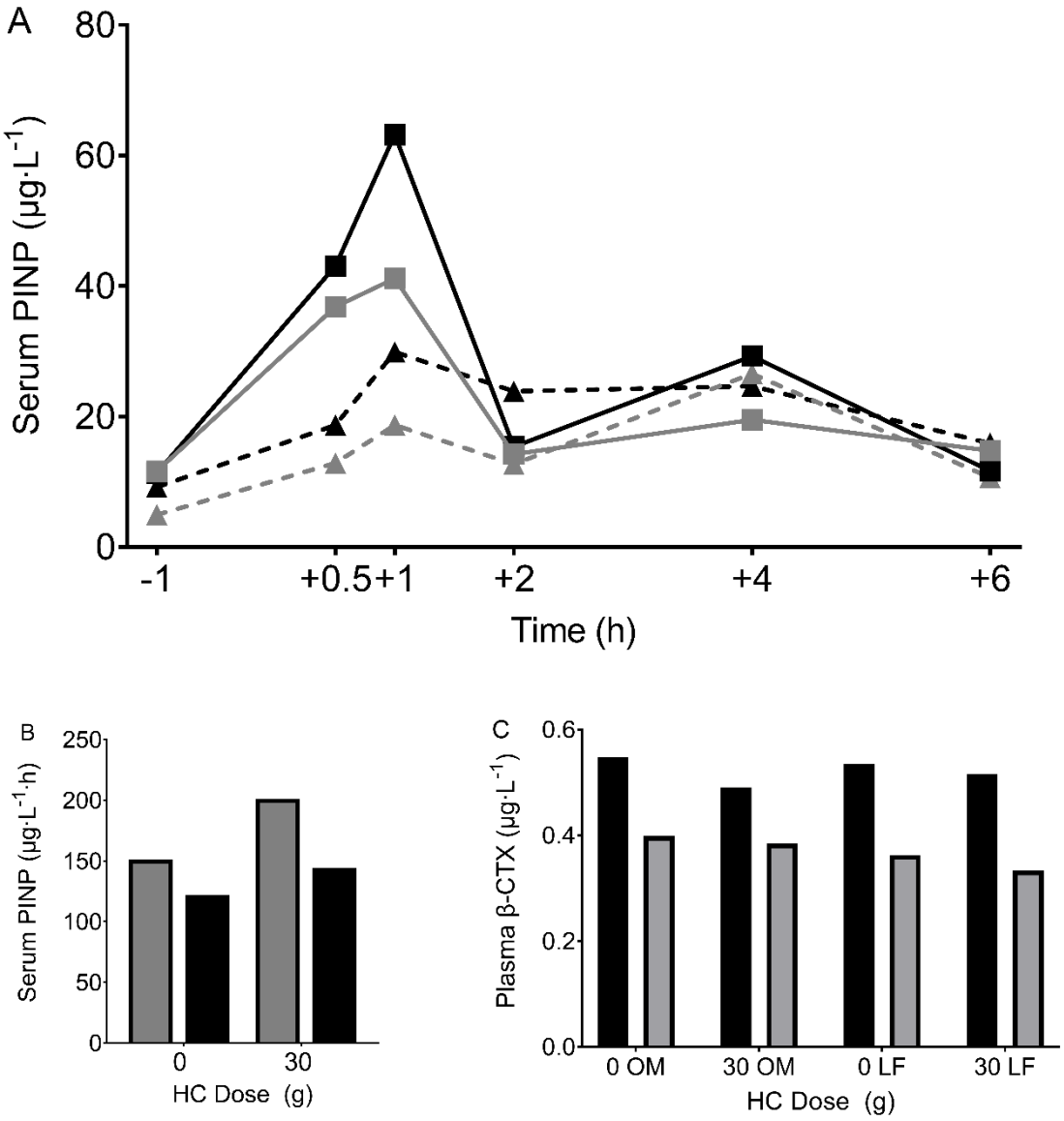

Figure 3. **A:** Time course of serum PINP concentration following 30 g hydrolysed collagen (HC) consumption and resistance exercise (RE) at the onset of menses (OM, black squares), 0 g HC at OM (grey squares), 30 g HC at the end of the late follicular phase (LF, black triangles), and 0 g HC at LF (grey triangles); **B:** serum PINP concentration × time area-under-the-curve at OM (grey bars) and LF (black bars) following either 0 g or 30 g HC and RE; **C:** plasma β-CTX concentrations at rest prior to either 0 g or 30 g HC consumption (black bars) and 6 h-post RE (grey bars) at OM and LF.

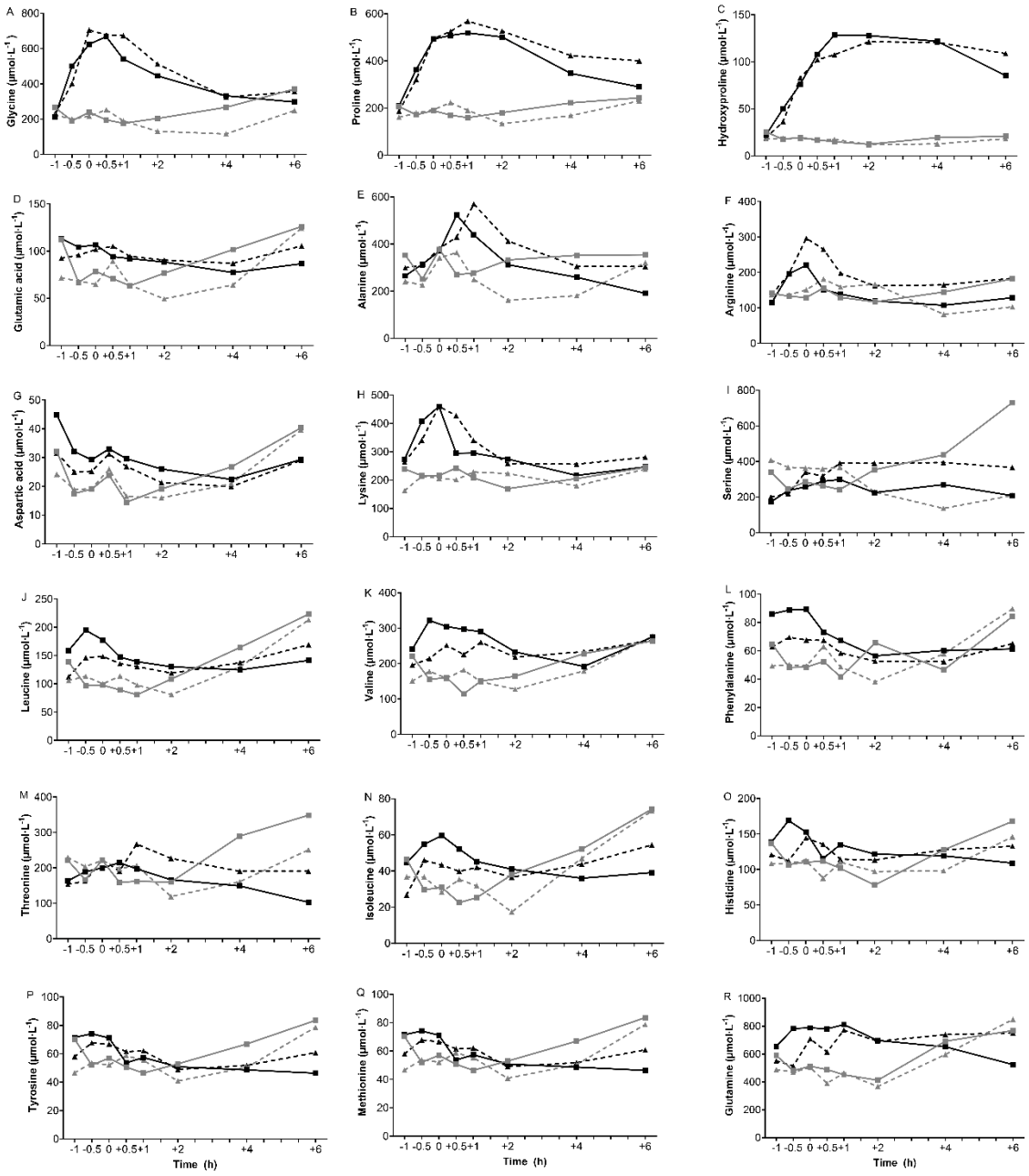

Figure 4. Serum concentrations of 18 amino acids following ingestion of 30 g hydrolysed collagen (HC, black squares) or 0 g HC (grey squares) at the onset of menses; and 30 g HC (black triangle) or 0 g HC (grey triangle) at the end of the late follicular phase.

4.4 Discussion

The aim of this case study was to investigate whether high-intensity lower-limb RE with 30 g hydrolysed collagen (HC) could augment collagen synthesis more than RE alone at high and low circulating oestrogen concentrations in a healthy, young, eumenorrheic, resistance-trained woman. The main findings were that collagen synthesis was highest when RE was performed after ingestion of 30 g HC at the onset of menses (i.e. when circulating endogenous oestrogen concentration was low) than during the late follicular phase (i.e. when circulating endogenous oestrogen concentration was high), or when 0 g HC was ingested regardless of circulating oestrogen concentration.

In the current case study, peak serum PINP concentrations were 5.6 and 3.3 times greater than baseline values following high-intensity RE at the onset of menses (OM) and during the late follicular phase (LF), respectively. This is in line with previous reports of an acute bout of resistance-type exercise increasing circulating PINP concentration in young, healthy women who did not use the oral contraceptive pill (OCP) (Miller et al., 2007, Hansen et al., 2008). Moreover, we found that the PINP concentration × time AUC in 30 g HC at OM and LF was 1.3-fold greater than when 0 g HC was ingested (regardless of menstrual cycle phase). These findings are in line with Chapter Three in which 30 g HC ingestion with a bout of RE augmented PINP concentration × time AUC greater than 0 g HC ingestion in resistance-trained young men. Although how 30 g HC with RE augmented collagen synthetic response greater than RE alone is unclear, the availability of exogenous amino acids following 30 g HC ingestion may have activated signalling pathways (i.e. Akt/mTORC1) that promote collagen synthesis (this theoretical mechanism in detail is explained in Chapter Three).

In addition to RE and 30 g HC ingestion that influenced PINP concentrations in the current case study, we observed that the serum PINP concentration × time AUC was higher at OM than at LF. Although the exact mechanism underpinning how circulating oestrogen might influence collagen synthesis is unclear, different levels of endogenous oestrogen is associated with type I collagen synthesis either directly or indirectly. When porcine anterior cruciate ligament fibroblasts are mechanically loaded and administered with different concentrations of 17β-oestradiol, an inverse relationship exists between 17β-oestradiol concentration and type I collagen mRNA expression (Lee et al., 2004). This suggests that mechanical loading of collagenous tissue, when circulating concentrations of oestrogen are high (e.g. at LF), will attenuate type I collagen gene expression, thus potentially inhibiting collagen synthesis. However, other previous studies found that 17β-oestradiol did not affect collagen synthesis in sheep ACL fibroblast (Seneviratne et al., 2004) and human ligamentum flavum cells (Chen et al., 2014). Alternatively, oestrogen may indirectly inhibit collage synthesis by interacting with insulin-like growth factor 1 (IGF-1). In postmenopausal women, systemic and tendon IGF-1 concentrations were approximately 1.4 and 2.3 times lower in hormone replacement therapy (HRT) users (who took 2 mg oral 17β-oestradiol daily), compared to age-matched non-HRT users (Hansen et al., 2009a). As mechanical loading upregulates procollagen I alpha 2 and IGF-1 gene expression in rat plantaris tendon, resulting in augmented tendon mass (Olesen et al., 2006), the lower collagen synthesis observed in the current study when circulating oestrogen was high (i.e. at LF) may be due to an inhibitory effect of oestrogen on IGF-1 secretion during muscle contraction.

Miller et al. (2007) reported that varying oestrogen levels did not affect PINP concentration following exercise in healthy young women. Despite different serum oestrogen concentrations measured at two different phases of the menstrual cycle (follicular and luteal), these two phases

were probably not the most appropriate for assessing maximal differences in circulating oestrogen, given the overlap in their oestrogen values: 0.07-0.42 nmol·L^{-1} in the follicular phase and 0.24-0.79 nmol·L^{-1} in the luteal phase (Miller et al., 2007, Hansen et al., 2008). This is likely to have influenced the lack of difference in PINP concentrations between menstrual cycle phases following exercise in the study by Miller et al. (2007), and had the investigators used similar time points as used in the present study (i.e. OM and LF), their results and conclusions may have been different.

Concerning collagen degradation, plasma β-CTX concentration was lower at 6-h post RE compared to 1-h prior to RE in the current study, regardless of collagen dose or menstrual cycle phase. Qvist et al. (2002) found that human serum CTX-I concentration was higher in the morning (08:00) and started to decrease from 11:00 to 14:00 independently of exercise. Thus, as β-CTX at 6-h post RE was measured at 15:00 in the current study, it is possible that circadian rhythm may have affected these results. However, unpublished data from our laboratory suggest the decrease in β-CTX occurs immediately after RE and remains low for the subsequent six hours of rest, regardless of HC dose. It is therefore possible that, by activating the Akt/mTORC1 pathway, the high-intensity RE model used in the current study may have inhibited collagen breakdown with immediate effect, lasting for the duration of the study.

In Chapter Four, we found an association between endogenous circulating oestrogen concentration and a marker of collagen synthesis following RE with different doses of HC ingested. However, the sample size was n = 1 and, given the variability in endogenous oestrogen concentration between and within eumenorrheic females (Shultz et al., 2011), it remains to be seen if we would have seen the same pattern of responses with a statistically powered sample size.

Therefore, future studies need to investigate the effect of different endogenous oestrogen concentrations with RE and HC ingestion on collagen synthesis with an appropriate sample size and measuring tendon collagen FSR in healthy eumenorrheic women.

To conclude, in a young, healthy, eumenorrheic, resistance-trained woman, whole body collagen synthesis following high-intensity, lower-limb RE appeared to be negatively affected when circulating endogenous oestrogen concentration was high (i.e. in the late follicular phase of the menstrual cycle). Ingesting 30 g HC prior to performing RE augmented collagen synthesis compared to RE alone, and 30 g HC ingestion with RE when circulating endogenous oestrogen concentration was low (i.e. at the onset of menses) was associated with an overall superior collagen synthesis response. These novel and important findings have implications for improving muscle-tendon health and physical performance in healthy young, eumenorrheic resistance-trained female athletes.

Chapter Five

Ten weeks' *in-season* soccer training with bodyweight strength training supplemented with 30 g hydrolysed collagen augments changes in patellar tendon properties in female *academy* soccer players

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Prelude

Chapter Three demonstrated that 30 g hydrolysed collagen (HC) ingestion with resistance exercised led to a greater collagen synthetic response compared to 15 g and 0 g HC ingestion in healthy resistance-trained young men. Further, Chapter Four found that 30 g HC ingestion with resistance exercise augmented a marker of collagen synthesis compared to exercise alone regardless of circulating endogenous oestrogen concentration. These findings suggest that chronic ingestion of 30 g HC and mechanical loading might have a greater effect on the adaptive response of connective tissue to training in female soccer players. This hypothesis is tested in Chapter Five, which builds on the previous two chapters by investigating the effect of 30 g HC ingestion with in-season soccer training (incorporating bodyweight resistance/plyometric exercise) on changes in patellar tendon properties in female academy soccer players. Therefore, adaptation of the patellar tendon and *vastus lateralis* muscle were measured using an ultrasound scan and isokinetic dynamometer in this study chapter. Data collection for this study began in February 2021 and was completed in May 2021. Thus, this study took place during a UK Government-enforced COVID-19 UK national lockdown, during which time only professional athletes were legally permitted to attend their place of work (and the University for research purposes). The general public and university students were not permitted to attend either the University laboratories or their places of work. The PhD candidate was given special dispensation from the UK Government via the University's Senior Management Team to undertake this research study at the University. The fact that no other university staff or students were available to help at the time affected the design of this study. For example, under normal conditions, this would have been a double-blind nutritional intervention study but, as the PhD candidate needed to prepare the nutritional supplements himself, he could not be blinded to participant group allocation.

Abstract

We investigated the effect of 30g hydrolysed collagen (HC) ingestion supplementation on changes in patellar tendon (PT) properties after 10 weeks' training in female soccer players from a Football Association Women's Super League Under 21s squad. We pair-matched *n*=17 players (age: 17 ± 0.9 years; height: 1.66 ± 0.06 m; mass: 58.8 ± 8.1 kg) for baseline knee extension (KE) maximum isometric voluntary contraction (MIVC) torque, age, height, and body mass, and randomly assigned them to collagen (COL) or placebo (PLA) groups (COL *n*=8, PLA *n*=9). Participants consumed 30g HC or energy-matched PLA with 500 mg vitamin C after each training session, comprising bodyweight strength-, plyometric- and/or pitch-based exercise 3 days/week for 10 weeks in-season. We assessed KE MIVC torque, vastus lateralis muscle thickness and PT morphological, mechanical, and material properties using isokinetic dynamometry and ultrasonography before and after 10 weeks' training. KE MIVC torque, muscle thickness and tendon cross-sectional area did not change after training in either group. However, COL increased PT stiffness (COL, +18.0 ± 12.2 % [*d*=1.11] vs. PLA, +5.1 ± 10.4 % [*d*=0.23], P=0.049) and Young's modulus (COL, +17.3 ± 11.9 % [*d*=1.21] vs. PLA, +4.8 ± 10.3 % [*d*=0.23], P=0.035) more than PLA. Thus, 10 weeks' in-season soccer training with 30g HC increased PT mechanical and material properties more than soccer training alone in female academy soccer players. Using 30g HC to further augment PT tensile strength with soccer training, therefore, has potential implications for reducing soft tissue injury risk in female soccer players.

5.1 Introduction

Soft tissue injury (including collagenous tissues, such as skeletal muscle, tendon and ligament) is the most common injury type in women's soccer (López-Valenciano et al., 2021, Mayhew et al., 2021), and is two-to-five times more common in female than in male soccer players (Waldén et al., 2011, Larruskain et al., 2018). Indeed, female players suffer a higher proportion of quadriceps muscle strains, knee ligament injuries and anterior cruciate ligament (ACL) ruptures than male players (Larruskain et al., 2018). Although this sex difference in injury risk is likely multifactorial, ACL injury specifically can be partially ascribed to greater knee joint laxity in women than men (Rozzi et al., 1999). This sex-difference is reflected in women having a more compliant patellar tendon than men (Onambele et al., 2007, Hicks et al., 2013), which may be linked to higher circulating levels of oestrogen in women (Hansen et al., 2013). As tendon and ligament comprise ~80% type I collagen (Frank, 2004, Kjaer, 2004), and circulating levels of oestrogen fluctuate during the menstrual cycle in eumenorrheic women, this may explain the higher incidence of muscle and tendon injuries in the late follicular phase of the menstrual cycle, i.e. when circulating oestrogen concentration peaks (Martin et al., 2021). Another injury risk factor in women's soccer is muscle weakness (Crossley et al., 2020), so it follows that incorporating chronic resistance exercise into a soccer training program should increase strength, thereby reducing injury risk. However, despite the aforementioned sex differences in tendon properties and soft tissue injury risk, tendon properties have not been investigated in female soccer players, so it is not known to what extent their tendons can adapt to strength training.

Traditional strength (Reeves et al., 2003b, Kongsgaard et al., 2007, Seynnes et al., 2009) or

plyometric (Foure et al., 2010) training is known to increase both stiffness and Young's modulus of human tendon *in vivo*. As there is a linear relationship between Young's modulus and ultimate stress (LaCroix et al., 2013), a stiffer tendon has a greater loading capacity, thus potentially reducing tendon injury risk during periods of high mechanical load, e.g. during soccer training/match play. However, a direct link between tendon loading/stretching and pathology or injury currently lacks support, so this hypothesis remains speculative. Furthermore, a stiffer tendon is associated with a faster rate of torque development (Bojsen-Møller et al., 2005), which is also linked to greater lower-limb strength and power outputs (Bojsen-Møller et al., 2005, Wdowski et al., 2022). Thus, strength training could have multiple benefits for soccer players, with changes in tendon properties likely due to the cumulative remodelling that occurs after each bout of exercise. Indeed, resistance exercise increases human patellar tendon collagen fractional synthetic rate (Miller et al., 2005), while endurance exercise increases circulating concentration of procollagen type I N-terminal propeptide (PINP, a biomarker of collagen synthesis) in humans (Langberg et al., 1999, Langberg et al., 2001). Newly synthesized procollagen molecules then undergo post-translational modifications, transport and assembly into tendon (Canty and Kadler, 2005).

Concomitant increases in serum PICP and indirect markers of human Achilles tendon collagen content after two months' strength training, followed by an increase in tendon stiffness with a further month's training, suggest that augmented tendon collagen synthesis and content are necessary to increase the tendon's mechanical properties in response to overloading the muscle-tendon unit (Kubo et al., 2012). This is further supported by the findings of Quinlan et al. (2021) and Crossland et al. (2023), which showed that patellar tendon collagen fractional synthetic rate, tendon stiffness and Young's modulus all increased after four weeks' resistance training

in healthy young and older men. However, it should be noted that tendon stiffness can be augmented by increased collagen fibril cross-linking, and the direct mechanisms of tendon stiffening are still unknown.

Moreover, collagen synthesis after a single bout of exercise in humans appears to be further augmented with the addition of exogenous vitamin C-enriched collagen in a dose-response manner, i.e. 15 g gelatine increases serum PINP concentration by more than two-fold compared to 5 g and 0 g (Shaw et al., 2017). Thus, further augmentation of collagen synthesis via collagen supplementation may cause even greater changes in tendon mechanical and material properties following a period of chronic exercise training. In support of this hypothesis, strength training with 15 g collagen supplementation has been shown to cause greater accretion of lean mass in young, healthy men (Kirmse et al., 2019). Furthermore, short-term power training with 20 g collagen supplementation appears to have a beneficial effect on the rate of force development in young male athletes (Lis et al., 2021), which suggests collagen supplementation may enhance the stiffening of the muscle-tendon unit that occurs with strength training. Interestingly, a recent study found that just 5 g collagen supplementation with strength training in young men induced greater hypertrophy of the Achilles tendon and gastrocnemius muscle than strength training alone, although tendon stiffness was not affected (Jerger et al., 2022). It is not known, however, if collagen supplementation can affect the patellar tendon, which is stiffer than the Achilles tendon (Seynnes et al., 2009, Arampatzis et al., 2010) in a similar manner, or whether the interaction of a larger dose of collagen with soccer-specific training can elicit greater changes in patellar tendon properties in high-level female soccer players, which would have implications for mitigating soft tissue injury risk in this population.

Therefore, the aim of the current study was to compare the effect of 10 weeks' soccer training (incorporating strength-, plyometric- and pitch-based training) with or without collagen supplementation on changes in patellar tendon properties in female soccer players. We hypothesized that 30 g collagen and 500 mg vitamin C ingested immediately after each training session (three times per week) would confer greater changes in patellar tendon mechanical and material properties compared to soccer training alone. As strength training with collagen supplementation has recently been shown to enhance fat-free mass in healthy young men (Kirmse et al., 2019), a secondary aim was to investigate the effect of soccer training and collagen supplementation on *vastus lateralis* muscle thickness and subcutaneous adipose tissue thickness.

5.2 Methods

Experimental Overview

The study recruited high-level female soccer players from the 'Under 21s' squad of a Football Association Women's Super League Academy Club and the study design was a single-blind, randomized controlled trial. Due to the study taking place during the UK Government-enforced national lockdown associated with the COVID-19 pandemic, the lead researcher was required to be unblinded to participant group allocation (collagen, COL, or placebo, PLA) from the outset of the study. This was to ensure the correct supplements were consumed by the participants (due to lockdown, no other researchers or technicians were available to prepare the supplements). Crucially, however, all participants were blinded to group allocation, plus the strength and conditioning coaches responsible for undertaking all training sessions were blinded to participant group allocation.

All participants attended the laboratory for muscle-tendon assessments before and after 10 weeks' soccer training. The training took place during the 2020-21 in-season and comprised a combination of lower-limb plyometric, bodyweight strength and pitch-based soccer training. All muscle-tendon measurements were performed on the right leg in the following order: maximal isometric and isokinetic strength of the knee extensors and flexors (antagonist muscle activation was measured using surface electromyography [EMG]), thickness of the *vastus lateralis* (VL) muscle and its subcutaneous adipose tissue (SAT), and morphological, mechanical, and material properties of the patellar tendon were measured via a combination of ultrasonography and isokinetic dynamometry (IKD). All tests took place between 09:00 and 17:00 and the pre/post-training tests were performed at the same time of day for each participant to avoid potential diurnal effects on intra-individual pre/post-training changes (Onambele-Pearson and Pearson, 2007). Further, participants were instructed not to participate in strenuous physical activity, not to consume alcohol or caffeine in the 24 h prior to testing. Due to the busy training and match schedule during in-season, staggered testing was performed within two weeks. Participants were then pair-matched according to age, height, body mass, maximum knee extensor strength and use (or not) of hormonal contraception, and then randomly assigned to one of two groups (COL or PLA) using block randomization. Participants were instructed to ingest their respective supplement three times a week with training for the 10-week period. Each participant completed all 30 training sessions and ingested all of their 30 respective supplements (supervised by a member of the research team). Post-training assessments were performed within three-to-five days after the final supplemented training session.

Participants

A priori sample size estimation: A minimal sample size was estimated prior to conducting the study with G*Power software (v3.1.9.6, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). Briefly, the estimation was performed using the effect size ($\eta_p^2 = 0.163$) from the time (pre and post 12 weeks' strength training) × group (COL *vs.* PLA) interaction ($p = 0.002$) regarding the change in fat-free mass in the study by (Kirmse et al., 2019). The results from our *a priori* power calculation deemed a minimum of 14 participants was necessary to detect an effect of COL *vs.* PLA (two-way analysis of variance (ANOVA); α: 0.05; power: 0.80).

Twenty-one female soccer players from the 'Under 21s' squad volunteered to take part in this study after providing written informed consent. However, three players were unable to be scheduled for baseline testing and, in the week before post-training testing, one participant suffered an injury unrelated to the study and was unable to complete the testing. Following random allocation into COL and PLA (after pair-matching, as described above), characteristics of the 17 participants, who completed the study are displayed in **Table 1**. COL comprised one goalkeeper, three defenders, two midfielders and two forwards, while PLA comprised one goalkeeper, three defenders, four midfielders and one forward.

Exclusion criteria for all participants included history of lower limb muscle/tendon injuries in the six months prior to the start of the study; consumption of nutritional supplementation that purportedly affects muscle-tendon adaptation or recovery (e.g., protein powder, vitamin C, collagen); being vegan or vegetarian (due to the mammalian source of collagen); previous anterior cruciate ligament injury where the patellar tendon was used as a graft; age < 16 years or > 39 years. The study was approved by the Liverpool John Moores University Research Ethics Committee and complied with the Declaration of Helsinki.

Table 1. Participant baseline characteristics.

Variable	COL (*n* = 8)	PLA (*n* = 9)
Age (years)	17.0 ± 0.8	16.8 ± 1.0
Height (m)	1.65 ± 0.08	1.67 ± 0.04
Body mass (kg)	62.0 ± 10.7	57.4 ± 6.2
ISO KE MVC (N·m)	185 ± 41	183 ± 48
ISO KF MVC (N·m)	84.2 ± 22.0	80.9 ± 20.2
CON KE MVC (N·m)	138 ± 31	131 ± 29

Data are mean ± SD. *COL*, collagen group; *PLA*, placebo group; *ISO*, isometric; *KE*, knee extension; *MVC*, maximal voluntary contraction torque; *KF*, knee flexion; *CON*, concentric. There were no differences between COL and PLA (all $P > 0.05$).

Identification of menstrual cycle phase and hormonal contraceptive use

During the pre-testing, participants were asked to complete a questionnaire in order to report which menstrual cycle (MC) phase they were tested in. Each participant's MC was estimated using calendar-based counting, which requires the self-reported date of the onset of menses, number of days' menstruation, and length of MC. The MC phases specified here were defined as 'early follicular', 'late follicular', 'early luteal' and 'late luteal'. Days of follicular and luteal phases with different MC length were calculated based on the regression equation from (McIntosh et al., 1980). Also, hormonal contraceptive use was identified via the questionnaire to provide more detailed participant characteristics.

Training period

The in-season training program detailed here did not deviate from the athletes' habitual soccer training program. Participants performed four training sessions (Monday, Tuesday, Friday and Saturday) and one competitive match (Wednesday) per week, and nutritional supplementation was consumed on three of those sessions every week for 10 weeks. A typical microcycle with nutritional supplementation was Monday (pitch-based session followed by lower-limb body-weight strength exercises), Tuesday (pitch-based session followed by lower-limb plyometric exercises), and Friday (pitch-based session). An additional pitch-based session was conducted on Saturday, which was only used as a supplementation day if participants missed one of their regular supplementation days or the match day. The volume of plyometric and bodyweight strength training was progressively increased on a weekly basis. Detailed training programs for lower-limb bodyweight strength and plyometric exercises are presented in **Table 2**. Individual training load during the pitch-based sessions for 10 weeks was measured using a global positioning system device (Apex, STATSports, Newry, Northern Ireland). Total running (12.2 – 19.1 $km \cdot h^{-1}$) and sprinting (> 19.4 $km \cdot h^{-1}$) (Dwyer and Gabbett, 2012) distance are presented in **Table 3**.

Table 2. Bodyweight strength and plyometric exercises.

Strength Exercise	**Volume**	**Week 1 – 2**	**Week 3 – 4**	**Week 5 – 6**	**Week 7 – 8**	**Week 9 – 10**
Isometric split squat	Set	3	3	4	4	5
(10-s hold)	Repetitions	3	3	3	3	3
Reverse Plank alternative	Set	3	3	3	3	3
heel raise	Repetitions	8	8	10	10	12

(3-s hold)						
Banded sissy squat	Set	3	3	3	3	3
	Repetitions	6	6	8	8	10
Countermovement jump	Set	3	3	3	3	3
	Repetitions	3	3	4	4	5
Reverse Nordic	Set	3	3	3	3	3
	Repetitions	3	3	4	4	5
Nordic hamstring exercise	Set	3	3	3	3	3
	Repetitions	2	2	3	3	3
Plyometric exercise						
Broad jump	Set	3	3	3	3	3
	Repetitions	4	5	6	6	6
Pogo hurdle hop	Set	3	3	3	3	3
	Repetitions	2	3	4	4	4
Lateral countermovement jump	Set	3	3	3	3	3
	Repetitions	3	3	4	4	4
Single leg multi direction hop	Set	3	3	3	3	3
	Repetitions	2	3	4	4	4
Banded acceleration 5 steps	Set	3	3	3	3	3
	Repetitions	2	3	4	4	4
Fall start acceleration 10 m	Set	2	2	2	2	2
	Repetitions	2	3	4	4	4

Table 3. Training load during pitch-based sessions for 10 weeks in collagen (COL) and placebo (PLA) groups.

Variable	COL	PLA	*P*
Distance covered			
Total (km)	143 ± 11	149 ± 15	0.449
Running (km)	28 ± 4	30 ± 5	0.383
Sprinting (km)	5 ± 2	5 ± 2	0.622

Nutritional intervention

Due to the high standard of athletes participating in this study, all supplements were required to be 'Informed Sport' certified as having been tested by LGC Group's anti-doping laboratory for contamination with banned substances. Participants in COL received 90 mL 'Collagen Liquid' (GBR Nutrition, London, UK), which contained 30 g collagen hydrolysate, dextrose monohydrate, fructose, flavouring (mango and passion fruit), stabilisers (potassium sorbate and sodium benzoate), sweetener (sucralose), citric acid and water, and comprised 180 kcal. Participants in PLA received 49.3 g 'Tropical' flavour 'GO Electrolyte' (Science in Sport, London, UK), which contained 36.5 g maltodextrin, 8.4 g fructose, citric acid, electrolytes (sodium chloride, calcium lactate, potassium chloride, sodium citrate, magnesium citrate), natural flavouring sweetener (aspartame) and phenylalanine (source) and comprised 180 kcal. Thus, PLA was calorie- and taste-matched with the COL supplement. Each supplement was mixed with water to create a total volume of 250 mL, and all participants in both groups were given a 500 mg vitamin C tablet (Elite Vitamin C, Healthspan, Guernsey, UK) to consume immediately after consuming the drink. Participants consumed their supplements in entirety immediately after each training session (i.e. three times a week for 10 weeks) under the supervision of the strength and conditioning coach, who was blinded to group allocation. All drinks were provided

in opaque bottles and, together with the taste-matching and equal volume of drink, this ensured participants were blinded to their allocated group for the entirety of the study. The number of nutritional supplements that participants consumed during the different types of training session is shown in **Table 4**.

Table 4. The number of nutritional supplements participants had with training sessions or match.

Session type	**COL (*n* = 8)**	**PLA (*n* = 9)**
PBS	11 ± 1	12 ± 1
PBS and BWSE	8 ± 0	8 ± 1
PBS and PLY	7 ± 1	8 ± 1
Match	4 ± 2	2 ± 1

Data are mean ± SD. *PBS*, pitch-based session; *BWSE*, bodyweight strength exercise; *PLY*, plyometric exercise.

Habitual dietary intake and anthropometry

Participants' height (SECA, model-217, Hamburg, Germany) and body mass (SECA, model-875, Hamburg, Germany) were measured to the nearest 0.1 cm and 0.1 kg, respectively. Participants were asked to record their habitual dietary behavior using a food and drink diary for three days (Thursday to Saturday) during the baseline testing period. This aspect of the study was completed by *n* = 14 (**Table 5**). Records were analysed with Nutritics professional dietary analysis software (version 5.09, Nutritics Ltd., Co. Dublin, Ireland) to obtain total energy, macro- and micronutrient composition. All daily nutritional composition data were presented

as absolute and relative (normalized to body mass) values. 'Total intake' was calculated as the sum of habitual intake and nutritional supplementation used in this study. Thus, the total COL supplementation on training days was 30 $g \cdot d^{-1}$, and when averaged across training and non-training days, the COL supplement increased protein intake by 12.9 $g \cdot d^{-1}$, vitamin C intake by 214 $mg \cdot d^{-1}$ and energy intake by 77.1 $kcal \cdot d^{-1}$. Each PLA supplement contained 44.9 g maltodextrin/fructose and, when averaged across training and non-training days, increased carbohydrate intake by 19.2 $g \cdot d^{-1}$, vitamin C intake by 214 $mg \cdot d^{-1}$ and energy intake by 77.1 $kcal \cdot d^{-1}$.

Table 5. Energy, macronutrient, and micronutrient intake during the pre-training assessment period.

Nutritional composition	**COL (*n* = 6)**	**PLA (*n* = 8)**	***t*-test, *P***
Energy intake			
Habitual intake ($kcal \cdot d^{-1}$)	1634 ± 162	1553 ± 200	0.433
Total intake ($kcal \cdot d^{-1}$)	1711 ± 162	1630 ± 200	0.433
Carbohydrate intake			
Habitual intake ($g \cdot d^{-1}$)	204 ± 37	187 ± 33	0.378
Habitual intake ($g \cdot kg \cdot d^{-1}$)	3.2 ± 0.7	3.3 ± 0.9	0.772
Total intake ($g \cdot d^{-1}$)	204 ± 55	206 ± 33	0.916
Total intake ($g \cdot kg \cdot d^{-1}$)	3.2 ± 0.7	3.7 ± 1.0	0.344
Protein intake			
Habitual intake ($g \cdot d^{-1}$)	79.7 ± 9.0	77.3 ± 14.7	0.733
Habitual intake ($g \cdot kg \cdot d^{-1}$)	1.2 ± 0.1	1.4 ± 0.3	0.373
Total intake ($g \cdot d^{-1}$)	92.5 ± 9.0*****	77.3 ± 14.7	**0.046**
Total intake ($g \cdot kg \cdot d^{-1}$)	1.4 ± 0.1	1.4 ± 0.3	0.615

Fat intake			
Habitual intake ($g \cdot d^{-1}$)	55.6 ± 6.6	56.0 ± 11.3	0.930
Habitual intake ($g \cdot kg \cdot d^{-1}$)	0.9 ± 0.1	1.0 ± 0.2	0.214
Vitamin C intake			
Habitual intake ($mg \cdot d^{-1}$)	76.0 ± 26.8	96.6 ± 42.9	0.323
Habitual intake ($mg \cdot kg \cdot d^{-1}$)	1.2 ± 0.5	1.7 ± 0.9	0.183
Total intake ($mg \cdot d^{-1}$)	290 ± 27	311 ± 43	0.320
Total intake ($mg \cdot kg \cdot d^{-1}$)	4.5 ± 0.7	5.5 ± 1.3	0.103

Data are mean ± SD; * Different from PLA ($P < 0.05$).

Knee extensor and flexor maximal voluntary contraction

Participants performed isometric and isokinetic knee extension (KE) and isometric knee flexion (KF) maximal voluntary contractions (MVCs) on an IKD (Humac Norm, Computer Sports Medicine Inc., Stoughton, USA) to determine KE and KF MVC torque. Knee and hip joint angles were set at 90° (0° = full knee extension) and 85° (180° = supine), respectively, and movement was restricted with the use of inextensible waist, chest, and thigh straps. Participants performed a standardized warm up comprising 10 repetitions of KE and KF ($60 \cdot s^{-1}$; full range of motion) by gradually increasing intensity from sub-maximal to maximal for preconditioning the tendon (Maganaris, 2003). Participants then performed three concentric KE MVCs at $60 \cdot s^{-1}$ (full range of motion) interspersed with 5-s rest, and the highest of the three attempts was used for subsequent analysis. After five minutes' rest, participants performed three isometric KE and KF MVCs (each lasting three seconds), alternating between KE and KF every 30 seconds. If the highest MVC differed from the next highest by >5%, a further attempt was made to ensure

the highest MVC was achieved (which was generally attained within three attempts). However, in the event that the highest KE MVC performed during the ramped KE isometric MVC (RMVC, for more details see below), then this value was used to represent KE isometric MVC instead. The torque signal was interfaced with an analogue-to-digital converter (MP150 Biopac Systems Inc., Santa Barbara, USA), sampled at 2 kHz with a PC using data acquisition software (AcqKnowledge v.5.1, Biopac Systems Inc.) and low-pass filtered (10 Hz edge frequency) off-fline.

Morphological, mechanical, and material tendon properties

Antagonist muscle co-activation

To estimate the extent of antagonist (hamstring) muscle co-activation during a KE RMVC, the EMG activity of the biceps femoris long head (BFlh) was recorded, which represents the knee flexor muscle group (Kellis and Baltzopoulos, 1999). The BFlh was identified via palpation during a submaximal knee flexion in the anatomical position. After preparation of the skin surface (shaving, skin abrasion with a sandpaper and cleaning the skin with an alcohol wipe), two bipolar Ag-AgCl surface electrodes (Neuroline, Medoicotest, Rugmarken, Denmark) with 20 mm inter-electrode distance were placed on the sagittal axis of the BFlh. The location of surface electrodes was on the distal third of the BFlh length according to SENIAM guidelines (Hermens et al., 2000) and one reference electrode was placed on the lateral tibial condyle. The EMG signal was band-pass filtered (10-500 Hz), and the root mean square (RMS) was calculated every 300 ms. Assuming a linear relationship between BFlh RMS EMG and KF MVC torque output (Kellis and Baltzopoulos, 1997, Reeves et al., 2004), KF co-activation torque during increment of RMVC and relaxation was calculated as:

$$\frac{\text{BFlh RMS EMG during KE RMVC}}{\text{BFlh RMS EMG during KF MVC}} \times \text{peak KF MVC torque}$$

The antagonist co-activation torque was subsequently added to the KE torque at the relevant RMVC to provide the net KE MVC torque. To estimate tendon force, the net KE torque was divided by the patellar tendon moment arm at 90° knee flexion, which was estimated from each participant's femur length (Visser et al., 1990).

Patellar tendon cross-sectional area

Participants were seated on the IKD in the resting state, with the knee secured at 90°. A 4-cm wide 5-18 MHz linear probe (Philips EPIQ 7 Ultrasound System, Bothel, USA) was placed sagittally on the skin overlying the patellar tendon to measure resting tendon length, defined as the distance between the patella apex and the tibial tuberosity. Using a permanent marker pen, locations corresponding to 25%, 50%, and 75% tendon length were marked on the skin and the ultrasound probe was placed on each location in the axial plane to image the tendon cross-sectional area (CSA).

Measurement of tendon elongation

Participants remained seated on the IKD and a 2-mm wide strip of surgical tape (3M, Neuss, Germany) was placed on the skin, transversely over the patellar tendon at ~50% tendon length, to act as an echo-absorptive marker that would be visible in the subsequent tendon elongation video scans. This was to ensure the transducer did not move with respect to the tendon throughout the RMVC but, if it did, the RMVC was repeated. Prior to performing the RMVC, participants competed 2 – 3 submaximal ramped isometric KE contractions to further pre-condition the tendon and ensure the participant could generate KE torque at the required loading rate. A

10-cm wide (10 − 15 MHz) linear probe (Mylab70, Esaote Biomedica, Genoa, Italy) was positioned sagitally over the tendon (imaging depth set to 7 cm), which enabled the whole tendon length to be captured in a single sagittal image/movie. Elongation of the tendon during a KE RMVC was measured in each video frame as the displacement of both the patella apex and the tibial tuberosity from the stationary marker in the action line of the tendon. The RMVC lasted for 6 s, followed immediately by a 6 s ramped relaxation to rest. At least two RMVC attempts were made with 2-min rest in between attempts and, generally, a successful attempt was achieved within two attempts. The loading rate during the 6 s RMVC was COL: 33.6 ± 5.7 $Nm \cdot s^{-1}$ *vs.* PLA: 29.0 ± 9.4 $Nm \cdot s^{-1}$ (pre-training) and COL: 32.8 ± 4.9 $Nm \cdot s^{-1}$ *vs.* PLA: 31.7 ± 6.9 $Nm \cdot s^{-1}$ (post-training). As the loading rate depended on the participant's ability to produce maximal voluntary force, real-time torque-time data were projected in front of the participant, so they could gradually and consistently increase torque output to MVC. After filtering the EMG signal (see above), the torque and EMG data in the AcqKnowledge file were resampled at 23 Hz offline to be synchronized with the ultrasound video, which was sampled at 23 Hz. The administration of a square wave pulse, which was visible simultaneously on the AcqKnowledge software and the ultrasound video via the ECG signal was used to synchronize torque and EMG data with the ultrasound video.

Vastus lateralis and its subcutaneous adipose tissue (SAT) thickness

With the participant in the anatomical position, femur length (from the mid-point of the greater trochanter to the lateral femoral epicondyle) was assessed with a measuring tape and, at 50% of this distance, the medio-lateral borders of the VL muscle were determined with ultrasound and the distance between them was assessed with a measuring tape. The point corresponding to 50% femur length and 50% VL width was marked on the skin with a permanent marker pen.

With the participant seated on the IKD in the resting state (knee and hip joint angles set at 90° and 85°, respectively), the 10-cm wide (10 − 15 MHz) linear probe (Mylab70, Esaote Biomedica, Genoa, Italy) was placed on this point in the same orientation of the VL muscle fascicles to obtain a sagittal image of the VL. Minimal pressure was exerted on the transducer to prevent compression of the muscle tissue and SAT. VL and SAT thicknesses were subsequently analysed using freely available image analysis software (ImageJ V.1.8.0, National Institute of Health, MD, USA). VL thickness (the perpendicular distance between the superficial and deep aponeuroses) and SAT thickness (the perpendicular distance between the lower border of dermis and the upper border of the superficial VL aponeurosis) were measured as an average of three sites (i.e. 25%, 50%, and 75% of the 10-cm wide image).

Analysis of tendon data

Using semi-automated tracking software (Tracker, version 6.0.10, https://physlets.org/tracker/), an average of 335 ± 44 frames during RMVC and relaxation were tracked to measure the displacement of both the patellar apex and tibial tuberosity. Individual tendon force–elongation data were subsequently fitted with a second-order polynomial ($R^2 > 0.90$ in all cases). An example of force-elongation data is represented **Figure 1**. Patellar tendon mechanical and material properties pre- and post-training were calculated using the weakest maximum tendon force for each participant, usually determined at pre-training. Patellar tendon strain was defined as tendon elongation expressed as a percentage of the tendon's original length, i.e. 100 × change in tendon length (ΔL)/ resting tendon length (L_0). Tendon stress was defined as the peak tendon force (F_t) at KE RMVC relative to the mean tendon CSA (i.e. F_t/CSA). Tendon stiffness (ΔF_t / ΔL) was calculated from the participant's highest 20% F_t interval. Young's modulus (E) was calculated by multiplying stiffness (k) with the ratio of the resting tendon length to mean tendon

CSA (i.e. $E = k \times (L_0/CSA)$). Tendon hysteresis was calculated as the difference between the area under the curves regarding the RMVC and relaxation phases and presented as a percentage.

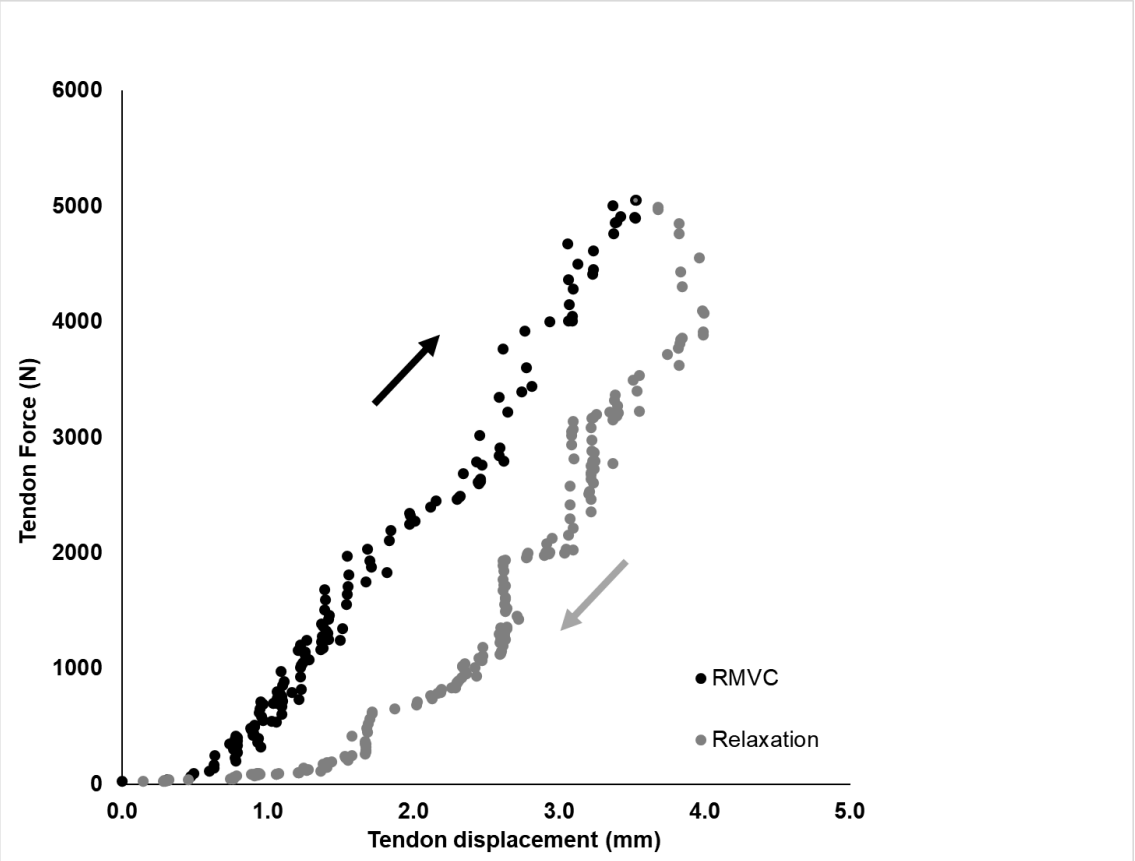

Figure 1. An example of force-elongation curve during the 6 s ramped isometric maximal voluntary contraction (RMVC).

Test-retest reproducibility of morphological, mechanical, and material tendon properties

Test–retest reproducibility of key measurements was determined on eight healthy young men (age: 24.1 ± 4.7 years; height: 1.77 ± 0.05 m; body mass: 73.7 ± 6.1 kg). Participants for this reproducibility test were asked to visit the laboratory twice with a 1-week interval. All measurements were performed by the same researcher (J.L.) and using the same methods, as described above. Coefficient of variation (CV), typical error, and intraclass correlation coefficient

(ICC) with 95% confidence intervals (CIs) were used to express inter-day reproducibility **(Table 6)**. For all variables, CVs were low except for tendon hysteresis (14%) and ICCs were high (> 0.90) with narrow 95% CIs (0.62 – 0.99) except for tendon hysteresis (0.54 – 0.98).

Table 6. Test-retest reproducibility of morphological, mechanical, and material tendon properties.

Variable	CV (%)	Typical error (95% CI)	ICC (95% CI)
PT length (mm)	1.4	0.654 (0.432 – 1.330)	0.986 (0.924 – 0.998)
Mean CSA (mm^2)	1.6	1.566 (1.035 – 3.187)	0.984 (0.912 – 0.997)
Tendon force (N)	3.9	154 (102 – 314)	0.991 (0.950 – 0.998)
Loading rate ($Nm \cdot s^{-1}$)	8.0	3.516 (2.325 – 7.157)	0.951 (0.745 – 0.991)
Stiffness ($N \cdot mm^{-1}$)	5.6	131 (86 – 266)	0.964 (0.806 – 0.994)
Young's modulus (GPa)	5.0	0.057 (0.038 – 0.117)	0.921 (0.618 – 0.986)
Stress (MPa)	4.1	1.771 (1.171 – 3.605)	0.980 (0.889 – 0.996)
Elongation (mm)	2.5	0.103 (0.068 – 0.210)	0.965 (0.815 – 0.994)
Strain (%)	4.2	0.354 (0.234 – 0.721)	0.966 (0.819 – 0.994)
Hysteresis (%)	14.0	2.03 (1.343 – 4.134)	0.902 (0.543 – 0.982)

Statistical analyses

All data are presented as means ± standard deviations (SD). Pre-training between group (COL *vs.* PLA) comparisons of physical characteristics, dietary behavior and total training load during pitch-based sessions were performed with independent *t*-tests. Two-way mixed ANOVA models (group: COL *vs.* PLA; time: pre- *vs.* post-training) were performed to detect changes in KE and KF MVC torque, resting tendon length, loading rate, mean tendon CSA, all other tendon mechanical and material properties, and thickness of the VL and SAT. When significant group × time interaction effects were found, post-hoc paired *t*-tests (pre- *vs.* post-training for

COL and PLA) were performed to reveal between-group differences. A three-way mixed ANOVA was performed to assess differences among group (COL *vs.* PLA), time (pre- *vs.* post-training), and location (25% *vs.* 50% *vs.* 75% tendon length) for tendon CSA. Two effect sizes, Cohen's *d* (for *t*-tests) and the partial eta squared, η_p^2, (for ANOVA interaction) were reported for each statistical model. The thresholds of Cohen's *d* and η_p^2 are defined as small (*d* = 0.20 and $\eta_p^2 = 0.01$), medium (*d* = 0.50 and $\eta_p^2 = 0.06$) and large (*d* = 0.80 and $\eta_p^2 = 0.14$) (Cohen, 1988). Data were analysed by using the statistical software package SPSS (version 26, SPSS Inc., Chicago, IL) and level of significance was set at $P < 0.05$.

5.3 Results

Group characteristics

Age, body mass, height and baseline isometric and concentric KE and isometric KF MVC did not differ between COL and PLA groups (all $P > 0.05$, **Table 1**). All participants were 'normally' menstruating women except for two participants (one in COL and one in PLA), who had menstrual irregularity (thus, their menstrual cycle phases could not be estimated). One participant in COL was using the combined oral-contraceptive pill (OCP, Yasmin®, 21 days) and had been doing so for six months. Due to the challenges involved with testing during in-season, it was not possible to schedule the pre- and post-training tests in the same menstrual cycle phase, but we recorded when participants were tested according to their phase (**Table 7**).

Table 7. Pre- and post-training tests performed during different phases of the menstrual cycle.

	Menstrual cycle phase			
	Early follicular	Late follicular	Early luteal	Late luteal
PRE				

COL (*n* = 6)	2	3	1	0
PLA (*n* = 8)	2	3	2	1
POST				
COL (*n* = 6)	1	1	4	0
PLA (*n* = 8)	3	3	0	2

Macro- and micronutrient intake

Habitual and total macronutrient and vitamin C intake did not differ between COL and PLA (all $P > 0.05$, **Table 5**) except for total protein intake (including the COL supplementation).

Training load during pitch-based session

Total distance and running and sprinting distance did not differ between COL and PLA ($P > 0.05$, **Table 3**).

Maximum strength

Isometric and concentric KE MVC, isometric KF MVC, antagonist co-activation, VL muscle and SAT thickness before and after training are presented in **Table 8**. There were no main effects for training, group, or interaction effects for any of the variables ($P > 0.05$).

Table 8. Knee extension (KE) and knee flexion (KF) isometric and concentric maximal voluntary contraction (MVC) torque, antagonist muscle co-activation and vastus lateralis (VL) muscle and subcutaneous adipose tissue (SAT) thickness in COL and PLA groups before (PRE) and after (POST) training.

Variable	**COL (*n* = 8)**		**PLA (*n* = 9)**		
	PRE	**POST**	**PRE**	**POST**	**g × t, *P***

Isometric					
KE (N·m)	175 ± 45	192 ± 31	182 ± 50	184 ± 29.6	0.325
KF (N·m)	84.2 ± 22.0	79.8 ± 20.4	81.9 ± 20.2	77.6 ± 19.3	0.863
Concentric					
KE (N·m)	138 ± 31	139 ± 30	132 ± 29	141 ± 22	0.849
Antagonist co-activation (%)	19.6 ± 9.0	18.2 ± 7.8	18.9 ± 9.7	18.3 ± 5.8	0.858
VL thickness (mm)	25.2 ± 2.7	25.7 ± 3.0	24.7 ± 2.9	24.9 ± 2.4	0.781
SAT thickness (mm)	7.7 ± 2.9	7.4 ± 3.0	6.0 ± 1.6	6.0 ± 2.0	0.887

Data are mean ± SD.

Morphological, mechanical, and material tendon properties

Patellar tendon length, mean CSA, force, stress, elongation, strain, and hysteresis data are presented in **Table 9**. There were no main effects for training or group, or interaction effects for resting patellar tendon length, tendon force and loading rate ($P > 0.05$; **Table 9**). Regarding mean PT CSA, there was no main effect of training ($F_{1,15} = 4.290$, $P = 0.056$, $\eta_p^2 = 0.222$), group ($F_{1,15} = 0.037$, $P = 0.851$, $\eta_p^2 = 0.002$), or training × group interaction ($F_{1,15} = 3.418$, $P = 0.084$, $\eta_p^2 = 0.186$; **Table 9**). A three-way ANOVA revealed no training × group × location interaction ($F_{2,30} = 0.067$, $P = 0.933$, $\eta_p^2 = 0.004$).

Table 9. Patellar tendon morphological, mechanical, and material properties in PLA and COL groups before (PRE) and after (POST) training. Data are mean ± SD.

PT property	COL (*n* = 8)		PLA (*n* = 9)		
	PRE	POST	PRE	POST	g × t, *P*
Resting tendon length (mm)	46.5 ± 5.0	46.4 ± 5.4	43.5 ± 5.7	43.5 ± 5.6	0.610
Mean CSA (mm^2)	76.9 ± 7.1	77.1 ± 7.2	77.7 ± 8.1	77.7 ± 8.2	0.084
Tendon force (N)	5017 ± 871	5029 ± 776	4680 ±1038	4369 ± 629	0.356
Stress (MPa)	63.3 ± 9.3	63.2 ± 9.1	55.4 ± 8.1	55.3 ± 8.1	0.134
Elongation (mm)	4.0 ± 1.1	3.4 ± 0.5	3.5 ± 0.8	3.4 ± 0.9	0.275
Strain (%)	8.9 ± 3.0	7.3 ± 1.1	8.0 ± 1.5	8.1 ± 2.5	0.133
Hysteresis (%)	35.1 ± 25.3	37.6 ± 12.6	36.7 ± 14.9	28.2 ± 12.0	0.897

Tendon stiffness, Young's modulus, and hysteresis

Regarding stiffness at 80–100% RMVC, there was a main effect of training ($F_{1,15}$ = 18.040, P < 0.001, η_p^2 = 0.546) and a training × group interaction ($F_{1,15}$ = 4.589, P = 0.049, η_p^2 = 0.234) but no main effect of group. Post-hoc paired *t*-tests revealed that post-training stiffness was greater than pre-training in COL (P = 0.002) but not in PLA (P = 0.186; **Figure 2**). Regarding Young's modulus, there was a main effect of training ($F_{1,15}$ = 19.916, P < 0.001, η_p^2 = 0.570) and a training × group interaction ($F_{1,15}$ = 5.379, P = 0.035, η_p^2 = 0.264; **Figure 2**). Post-hoc paired *t*-tests revealed that post-training Young's modulus was greater than pre-training in COL (P = 0.003) but not in PLA (P = 0.142). Regarding hysteresis, there was no main effect of training ($F_{1,15}$ = 4.004, P = 0.064, η_p^2 = 0.211), main effect of group ($F_{1,15}$ = 0.025, P = 0.877, η_p^2 = 0.002) and training × group interaction ($F_{1,15}$ = 0.017, P = 0.897, η_p^2 = 0.001).

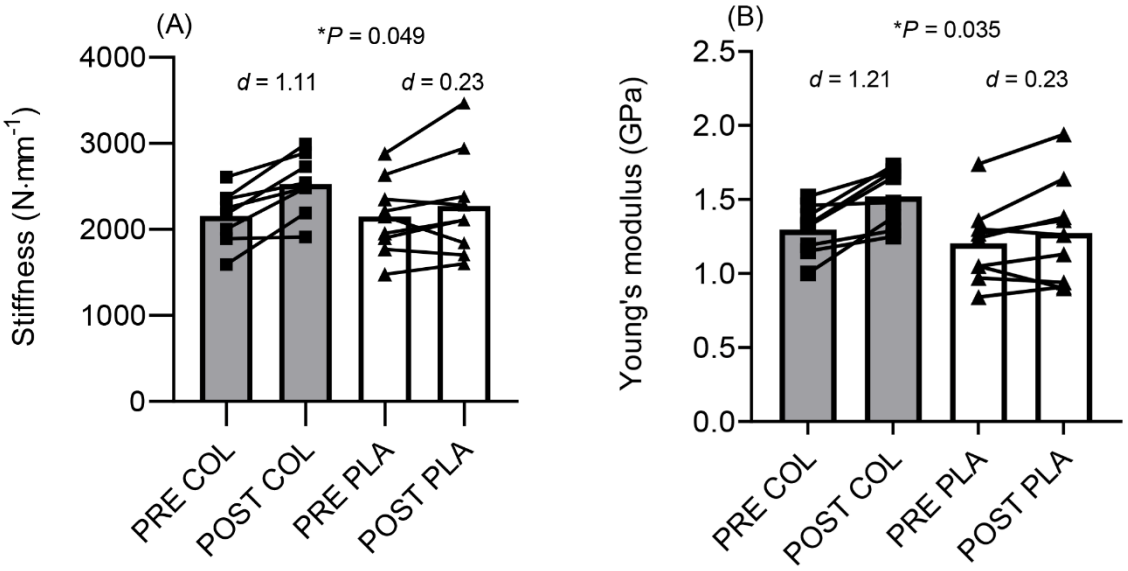

Figure 2. Changes in tendon stiffness (A) and Young's modulus (B) in collagen (COL) and placebo (PLA) groups before (PRE) and after (POST) training. *d* = Cohen's *d*; *Significant interaction effect.

5.4 Discussion

The aim of this chapter was to investigate the effect of collagen supplementation on the changes in patellar tendon morphological, mechanical, and material properties after 10 weeks' in-season soccer training (which incorporated lower-limb bodyweight strength/plyometric training) in the U21 squad of a FA Women's Super League soccer club. The main findings were greater increases in tendon stiffness and Young's modulus in the players who consumed collagen hydrolysate supplementation with their soccer training compared to those players who received placebo.

To our knowledge, this is the first study to investigate the changes in patellar tendon properties in high-level female soccer players following a period of training with or without collagen supplementation. We observed no changes in tendon CSA, which suggests the 18% increase in

stiffness was explained predominantly by changes in the tendon's material properties, which is supported by the 17% increase in Young's modulus. These greater changes in tendon stiffness and Young's modulus in COL *vs.* PLA, may be due to augmented mechanical loading-induced tendon collagen synthesis in the presence of high serum concentrations of the necessary exogenous amino acids (i.e., glycine, proline, and hydroxyproline) and vitamin C for synthesizing collagen (Shaw et al., 2017, Lis and Baar, 2019). These key amino acids may have increased the concentration of the collagen-specific cross-linking compound, hydroxylysyl pyridinoline, and/or may have increased collagen fibril density, both of which have the potential to increase tendon stiffness (Couppé et al., 2014, Couppé et al., 2021) in the absence of tendon hypertrophy. Further, orally administered collagen is absorbed within the connective tissue of rodents (Oesser et al., 1999, Watanabe-Kamiyama et al., 2010), while arginine and glycine intake for seven days increases the amount of hydroxyproline deposition, which indirectly indicates increased collagen synthesis in wounded rat muscle (Chyun and Griminger, 1984). Also, 5 g collagen intake for 12 months in postmenopausal women increased plasma PINP compared to baseline (König et al., 2018), which suggests that long-term chronic collagen supplementation augments tissue collagen synthesis. When repeated mechanical loading and 20 g collagen supplementation are combined, Lis et al. (2021) found that lower limb rate of force development (RFD) in healthy male athletes improved. Although tendon stiffness was not directly measured in the study by Lis et al. (2021), muscle-tendon stiffness is related to RFD (Bojsen-Møller et al., 2005), and a training-induced change in tendon stiffness likely affects the change in RFD (Maffiuletti et al., 2016). Thus, augmenting (loading-induced) tendon collagen synthesis with collagen supplementation may have increased connective tissue stiffness in the study by Lis et al. (2021), leading to improved RFD, which would support our main findings.

Although previous studies using high-intensity resistance training for 9 – 12 weeks found an increase in patellar tendon CSA at the proximal and distal ends (Kongsgaard et al., 2007; Seynnes et al., 2009), the intensity of training in the current study was probably insufficient to induce tendon hypertrophy. In contrast to our findings, a recent study by Jerger et al. (2022) showed that moderate-to-high intensity strength training (70 – 85% single repetition maximum) for 14 weeks with 5 g daily collagen supplementation further increased Achilles tendon CSA compared to training alone in previously untrained, healthy, young men. However, the increase in tendon stiffness after training did not differ between PLA and COL in this study (Jerger et al., 2022). The discrepancies between our findings and those of Jerger et al. (2022) are likely linked to the numerous methodological differences between studies. For example, the intensity and frequency of strength training in the study by Jerger et al. (2022) probably facilitated a greater hypertrophic response (+5% in PLA and +11% in COL). However, given the similar PINP response to 0 g *vs.* 5 g vitamin C-enriched gelatine and exercise in the study by Shaw et al. (2017), it is noteworthy that just 5 g collagen (with no co-ingestion of vitamin C) led to more than a two-fold increase in tendon size in the COL group in the study by Jerger et al. (2022), although this may be due to the *daily* consumption of 5 g collagen. Further, as tendon stiffness is influenced by both the material properties (e.g. collagen fibril density and cross-linking) and CSA of the tendon (Heinemeier and Kjaer, 2011), it is also notable that this greater hypertrophic adaptation in COL did not confer greater changes in tendon stiffness. Unfortunately, modulus was not reported in the study by Jerger et al. (2022), so it is not known whether between group differences in material properties may explain these results. The other methodological differences between studies, e.g. collagen dose, timing of ingestion, participants' training history, participants' sex [e.g. compared to men's tendons, women's tendons show a lower collagen synthesis rate following acute exercise and attenuated hypertrophy following training

(Magnusson et al., 2007)], the investigated tendon (Achilles *vs.* patellar), etc., may explain our contrasting findings. Nevertheless, our study is the first to show that a relatively large dose of hydrolysed collagen (plus vitamin C) in combination with soccer training (incorporating bodyweight strength/plyometric exercises) increases patellar tendon stiffness and modulus in female soccer players more than soccer training alone.

Due to the viscoelastic properties of human tendon, tendon hysteresis *in vivo* has been included in this chapter, as it is associated with tendon's elastic recoil to store elastic energy. In this chapter, PT hysteresis was not changed after 10 weeks' in-season soccer training regardless of nutritional supplements used. However, Reeves et al. (2003b) found that 14 weeks' high-intensity RT reduced PT hysteresis by 28% in older individuals. As RT frequency and intensity were different between this chapter and the study by Reeves et al. (2003b), further research is required to investigate whether high-intensity RT with HC ingestion would reduce PT hysteresis greater than RT alone. Although PT hysteresis in this chapter is comparable with the study by Reeves et al. (2003b), PT hysteresis in male elite athletes was 12 – 15% (Wiesinger et al., 2017). It should be however, noted that this discrepancy between this chapter and the study by Wiesinger *et al.,* (2017) is likely due to different methodology (e.g. 100% *v*s. 80% RMVC and length of probe (recording two site or one) and sampling frequency (not specifically stated in Wiesinger et al. (2017).

Despite the novelty of our study and the important findings, certain limitations should be considered. Firstly, the current study did not attempt to assess muscle-tendon properties during the same menstrual cycle phase pre- and post-training due to the club's and players' restricted timeframe during the research period. It has been suggested that fluctuating serum oestrogen concentration does not affect maximum strength (Dasa et al., 2021), tendon properties (Burgess et

al., 2010), or patellar tendon collagen synthesis (Miller et al., 2007) in young, active, eumenorrheic women. However, the limitations in these studies (Hansen, 2018) suggest it is still unclear whether menstrual cycle phase does affect these variables. While future studies should attempt to conduct pre- and post-testing in the same menstrual cycle phase (ideally determined using an ovulation test), this is not practically feasible in elite athletes, given their extremely limited availability. It is also not known whether the inclusion of the single participant using the oral contraceptive pill (OCP) had any impact on our results. (Hansen et al., 2009b) reported that patellar tendon collagen synthesis and PINP is lower in OCP users than in non-OCP users. However, the lower serum oestrogen concentration in OCP-users does not appear to affect tendon properties (Hansen et al., 2013, Hicks et al., 2017) and, given the fact that only one participant used OCP in our study, this is unlikely to have affected our results. Secondly, our study incorporated bodyweight strength and plyometric exercises, while high-intensity strength training may have induced greater tendon adaptation, particularly with regards to tendon hypertrophy. Future studies may therefore wish to implement this mode of chronic exercise to test the hypothesis that COL in conjunction with *high intensity* strength training also improves tendon properties more than strength training alone. Thirdly, regarding research design, although the lead researcher was not blinded to participant group allocation, the participants themselves and the strength and conditioning coaches responsible for the training programs were blinded. Furthermore, in contrast to the manual method of calculating tendon force-elongation curves (used to measure tendon stiffness, Young's modulus, strain and hysteresis), the semi-automated tracking software used in this study removed significant subjectivity from the data analysis and risk of bias from the lead researcher. The lack of bias is reflected in the lack of group × time interaction regarding MVC force, tendon loading rate and tendon CSA. It should also be noted that tendon stiffness and modulus (the only variables to demonstrate a significant group × time

interaction) were calculated using the last 20% of each participant's *lowest absolute* MVC force (usually recorded at baseline), and MVC force did not differ between COL and PLA at baseline. Thus, it is highly unlikely that our single-blind study design had any impact on the comparative outcome of our data. Fourthly, although our test-retest data demonstrate high reproducibility for the main measurements used in this study, these assessments were performed in healthy young men, who have greater knee extensor strength and patellar tendon stiffness than age-matched women (Hicks et al., 2013). Finally, it is acknowledged that energy intake in Chapter Five does not represent the energy intake during the 10 week's intervention. However, as the dietary records were collected immediately before the start of the intervention, it is not expected that habitual behaviour would have changed significantly.

In conclusion, 10 weeks' in-season soccer training (incorporating bodyweight lower-limb strength and plyometric training), supplemented with 30 g collagen (and 500 mg vitamin C) three times a week, conferred greater gains in patellar tendon stiffness and Young's modulus compared to soccer training alone in a FA Women's Super League Under 21s squad. These novel findings have significant implications for sport science support in female soccer players. Future studies should investigate if collagen supplementation can improve specific aspects of female soccer performance requiring rapid transference of force, and if it can help mitigate injury risk in this under-researched population.

Chapter Six

Ten weeks' *pre-season* soccer training with high-intensity resistance training supplemented with 30 g hydrolysed collagen confers greater changes in patellar tendon properties than training alone in *professional* female soccer players

Prelude

In Chapter Five, 30 g hydrosylsed collagen (HC) with 10 weeks' *in-season* soccer training (incorporating *bodyweight* resistance/plyometric exercises) increased patellar tendon stiffness and Young's modulus more than soccer training alone in female *academy* soccer players, with no tendon hypertrophy. Based on existing literature showing changes in morphological, mechanical and material properties of patellar tendon following chronic *high-intensity* resistance exercise, the exercise intensity used in Chapter Five was probably insufficient to induce tendon hypertrophy. Furthermore, it is unknown whether 30 g HC ingestion with resistance exercise would induce greater tendon adaptation in *professional* rather than *academy* female soccer players, and whether the timing of the intervention (i.e. within the competitive season or during pre-season, when there is generally more time to be devoted to training) will influence any effect of HC supplementation. Therefore, this chapter will investigate the effect of 30 g HC with 10 weeks' *pre-season* soccer training incorporating high-intensity resistance/plyometric exercise on changes in patellar tendon properties in *professional* female soccer players (using the same methods as used in Chapter Five). Data collection for this study was performed between July and September 2021 (also a time of heavy restrictions associated with the COVID-19 pandemic). For the reasons stated in the prelude for Chapter Five, this study was also a single-blind design. Furthermore, this study was affected by numerous participants developing injuries (unrelated to the study), and some contracting COVID-19 and being unavailable for testing, which limited the sample size. Nevertheless, the relatively long duration of the exercise-nutritional intervention (10 weeks) likely led to a relatively large effect size for changes in PT properties in *professional* female soccer players.

Abstract

This study investigated whether 10 weeks' *pre-season* soccer training (including high-intensity resistance exercise) with hydrolysed collagen (COL) supplementation leads to greater changes in patellar tendon (PT) morphological, mechanical, and material properties compared to placebo (PLA) in *professional* female soccer players. We pair-matched *n*=11 players (age: 25.7±4.2 years; height: 1.68±0.04 m; mass: 64.0±4.6 kg) from the first team squad of a Football Association Women's Championship football club for baseline knee extensor (KE) maximum isometric voluntary contraction (MIVC) torque, age, height, and mass, and randomly assigned them to COL (*n*=6) or PLA groups (*n*=5). Participants were provided with 30 g COL, or energy-matched (36.5 g maltodextrin, 8.4 g fructose) PLA, plus 500 mg vitamin C before each training session, which comprised high-intensity (75–90% 1-repetition maximum) lower-limb strength, plyometric- and/or pitch-based exercise 3 days/week for 10 weeks during pre-season. We assessed KE MIVC torque and PT properties using isokinetic dynamometry and ultrasonography before and after the training period. PT stiffness (COL, +15.4±3.1% [*d*=0.81] *vs.* PLA, +4.6±3.0% [*d*=0.32], *P*=0.002) and Young's modulus (COL, +14.2±4.0% [*d*=0.65] *vs.* PLA, +3.4±2.8% [*d*=0.15], *P*=0.004) increased more in COL than PLA. Although there was a main effect of training on PT cross-sectional area (P=0.027), there was no group×time interaction (P=0.934). Thus, 10 weeks' *pre-season* soccer training with 30g COL increased PT mechanical and material properties more than training alone in professional female soccer players. Given the limited time for interventions during the competitive season, future studies should investigate if COL can affect tendon properties in athletic populations over shorter training durations in-season.

6.1 Introduction

The adaptation of human skeletal muscle and tendon to mechanical loading is well documented (Kjaer, 2004). A single bout of resistance exercise (RE) is able to increase tendon collagen synthesis (Miller et al., 2005), while chronic resistance exercise, i.e. resistance training (RT) (Reeves et al., 2003a, Kongsgaard et al., 2007, Seynnes et al., 2009) and chronic plyometric exercise (Foure et al., 2010) are known to improve morphological and mechanical properties of human tendon. Also, when tendon is exposed to habitual loading, its stiffness and thickness are increased (Couppé et al., 2008, Couppé et al., 2021).

In addition to the effect of resistance exercise alone, recent studies have investigated the acute effect of combining resistance exercise with hydrolysed collagen (COL) ingestion on markers of collagen synthesis (e.g. Chapters Three and Four), while others have investigated the effect of RT and COL supplementation on tendon adaptation (Chapter Five, Jerger et al., 2022; 2023). Shaw et al. (2017) showed that a marker of collagen synthesis (procollagen type I amino-terminal propeptide, or PINP) increased in the blood of healthy young men in a dose-dependent manner following exercise supplemented with 0, 5 or 15 g gelatine, i.e. 15 g gelatine ingestion led to a greater serum PINP response compared to 5 and 0 g (Shaw et al., 2017). In Chapter Three, we showed that 30 g COL ingested prior to high-intensity RE in resistance-trained men led to an even greater serum PINP response compared to 15 and 0 g COL. In Chapter Four, we found that 30 g COL increased the serum PINP response after RE in a resistance-trained woman regardless of her menstrual cycle phase.

Regarding a long-term effect of RT with COL supplementation on tendon properties, in Chapter Five, we investigated for the first time how COL supplementation combined with 10 weeks' in-season soccer training affected muscle tendon properties in female academy soccer players.

This intervention incorporated bodyweight strength and plyometric exercises, as well as pitch-based exercise, and we found that COL led to greater changes in PT stiffness and Young's modulus compared to PLA, with no change in PT cross-sectional area (CSA) in either group. Two recent studies in young healthy men have found that daily ingestion of 5 g COL, together with high-intensity RT performed three times a week for 14 weeks led to greater increases in the CSA of the Achilles and patellar tendons, compared to RT alone (Jerger et al., 2022, Jerger et al., 2023). Therefore, it is likely that the relatively low-intensity of the bodyweight exercises incorporated in Chapter Five limited the tendon's ability to hypertrophy, and it remains to be seen whether *high-intensity* RT with COL supplementation in female soccer players would confer greater changes in tendon CSA compared to PLA.

Therefore, the primary aim of this study was to investigate the effect of 10 weeks' pre-season soccer training (incorporating high-intensity resistance training plus plyometric exercises and pitch-based training) with 30 g COL supplementation on changes in tendon properties. As previous studies have found that RT with COL supplementation increased fat-free mass (Kirmse et al., 2019) and muscle size (Balshaw et al., 2023a) more than RT alone, a second aim was to investigate the effect of soccer training and COL on changes in muscle thickness. We hypothesized that 30 g COL and 500 mg vitamin C ingested immediately before each training session (three times per week) would improve morphological and mechanical properties of the patellar tendon more than soccer training alone (with no effect on muscle thickness).

6.2 Methods

Experimental Overview

The study recruited professional female soccer players from the first team squad of a Football Association Women's Championship Club. The study design was a single-blind (participants were unaware of their group allocation), randomized controlled trial. All participants attended the laboratory for muscle-tendon assessments before and after 10 weeks' soccer training, which took place during the 2021-22 pre-season period (July to September 2021) and comprised a combination of strength, plyometric and pitch-based soccer training. All muscle-tendon measurements were performed on the right leg in the following order: morphology or the patellar tendon (PT), a standardized warm-up on isokinetic dynamometer, maximal isometric and isokinetic strength of the knee extensors and flexors (antagonist muscle activation was measured using surface electromyography [EMG]), and mechanical and material properties of PT were measured via a combination of ultrasonography and isokinetic dynamometry. All tests took place between 09:00 and 17:00 and the pre/post-training tests were performed at the same time of day for each participant to avoid potential diurnal effects on intra-individual pre/post-training changes (Onambele-Pearson and Pearson, 2007). Further, participants were instructed not to participate in strenuous physical activity, not to consume alcohol or caffeine in the 24 h prior to testing. Following the baseline assessments, participants were pair-matched for age, height, body mass, maximum knee extensor torque. Participants were then randomly assigned to one of two groups: collagen (COL) or placebo (PLA) group and instructed to ingest their respective supplement three times a week with training for the 10-week period. Each participant completed all 30 training sessions (implemented the soccer club's strength and conditioning coach, who was blinded to participant group allocation) and ingested all of their 30 respective supplements (supervised by the soccer club's strength and conditioning coach). Post-training assessments were performed within three-to-five days after the final supplemented training session.

Participants

Eighteen female soccer players from the first team squad of a FA Women's Championship soccer club provided written informed consent to take part in this study, which was approved by Liverpool John Moores University Research Ethics Committee and complied with the Declaration of Helsinki. However, three participants contracted COVID-19 and were unavailable for testing and therefore had to withdraw; three participants developed injuries during the course of the study (unrelated to the study), which forced them to withdraw, and one player withdrew due to personal reasons. Therefore, following random allocation into COL and PLA (after pair-matching, as described above), the characteristics of the 11 participants who completed this study are presented in **Table 1**. COL comprised two defenders, three midfielders and one forward, while PLA comprised one goalkeeper, two defenders, one midfielder and one forward. Two participants in COL were using an intrauterine device and had been doing so for 3.8 ± 4.5 years. One participant in COL was using an oral contractive pill (OCP) (Gedarel® 30/150) and had been doing so for six years, while three participants in PLA were using OCP (Rigevidon®, Lucette® and Microgynon® 30), and had been doing so for 7.3 ± 4.2 years. The remaining five participants were 'normally' menstruating women, as determined by responses to the 'low energy availability in females questionnaire' (LEAF-Q, score COL: 3 ± 3; PLA: 5 ± 2) (Melin et al., 2014). Exclusion criteria for all participants included history of lower limb muscle/tendon injuries in the six months prior to the start of the study; consumption of nutritional supplementation that purportedly affects muscle-tendon adaptation or recovery (e.g., protein powder, vitamin C, collagen); being vegan or vegetarian (due to the mammalian source of collagen); previous anterior cruciate ligament injury where the patellar tendon was used as a graft; age < 16 years or > 39 years.

Table 1. Baseline participant characteristics.

Variable	COL (*n* = 6)	PLA (*n* = 5)
Age (years)	24.3 ± 3.0	27.4 ± 5.1
Height (m)	1.68 ± 0.03	1.69 ± 0.06
Body mass (kg)	64.1 ± 4.8	64.1 ± 5.6
ISO KE MVC (N·m)	190 ± 32	204 ± 37
ISO KF MVC (N·m)	82.1 ± 15.6	85.6 ± 21.3
CON KE MVC (N·m)	147 ± 26	158 ± 22

Data are mean ± SD. *COL*, collagen group; *PLA*, placebo group; *ISO*, isometric; *KE*, knee extension; *MVC*, maximal voluntary contraction torque; *KF*, knee flexion; *CON*, concentric. There were no differences between COL and PLA (all $P > 0.05$).

Training and nutritional intervention period

Participants performed four training sessions (Tuesday, Wednesday, Friday and Saturday) and one friendly match (Sunday) per week, which was part of the athletes' regular training and competition during the pre-season period. The nutritional supplementation was consumed on three of those sessions every week for 10 weeks. A typical microcycle with nutritional supplementation was Tuesday (pitch-based session followed by externally-loaded upper-body strength exercises), Wednesday (pitch-based session followed by externally-loaded lower-limb strength training exercises), and Friday (externally-loaded lower-limb plyometric exercises followed by pitch-based sessions). An additional pitch-based session was conducted on Saturday,

which was only used as a supplementation day if participants missed one of their regular supplementation days or the match day. At the beginning of pre-season, three-repetition maximum (3-RM) testing was performed for the split squat exercise and rear-foot elevated split squat, to predict 1-RM for each player's training load for these exercises. In addition to the split squat and rear-elevated split squat exercises, the externally loaded lower-limb strength exercises comprised bilateral ballistic exercises, hip dominant posterior chain exercises, and unilateral plantar flexor exercises. Further, Copenhagen adduction and Nordic hamstring exercises were also included in the programme. During the 10-week training period, exercise selection, and the number of sets and repetitions were individualized due to different training history within the squad of players. However, the general approach was to progressively increase volume on a weekly basis by first increasing the number of sets, followed by increasing intensity, which generally coincided with a reduction in repetition number. The volume of plyometric exercise varied on a weekly basis due to the incorporation of a match (or not) and travelling for an away game. Detailed training programs for the externally loaded lower-limb strength and plyometric exercises are presented in **Table 2.**

Table 2. The externally loaded lower-limb strength and plyometric exercises.

Lower-limb strength exercise				
Weeks 1 – 8				
Exercise	Volume and intensity	Week 1	Week 2	Week 3 – 8
Bilateral ballistic exercise				

Clean pull from rack or loaded jump or trap bar pulls or medicine ball countermovement jump	Set	2	3	4
	Repetitions	4	4	4
Unilateral anterior exercise				
Rear-foot elevated split squat or split squat	Set		3 – 4	
	Repetitions		2 – 6	
	Intensity		75 – 90 % 1RM	
Hip dominant posterior chain exercise				
Staggered Romanian deadlift or hip thrust	Set	2	3	4
	Repetitions	5	5	5
Unilateral plantar flexor exercise				
Single-leg calf raise or calf-raise isometric hold (5- or 10-s hold)	Set	2	3	3
	Repetitions	8	8	6
Copenhagen adduction exercise	Set	2	2	2 – 3
	Repetitions	6	8	8 – 15
Nordic hamstring exercise	Set	1	2	3

	Repetitions	3	3	3

Week 9 – 10		
Exercise	Volume and intensity	Week 9 – 10
Bilateral ballistic exercise		
Hang high pull or loaded jump or trap bar pulls or medicine ball countermovement jump	Set	2 – 3
	Repetitions	4 – 6
Unilateral anterior exercise		
Rear-foot elevated split squat	Set	3
	Repetitions	4
	Intensity	75 – 90% 1RM
Hip dominant posterior chain exercise		
Romanian deadlift or single-leg glute-hamstring raise	Set	2 – 4
	Repetitions	4 – 6
Unilateral plantar flexor exercise		
Single-leg calf raise or calf-raise isometric hold (5 s hold)	Set	2 – 3
	Repetitions	3

Drop jump to box	Set	2 – 3
	Repetitions	3
Nordic hamstring exercise	Set	2 – 3
	Repetitions	3
Plyometric exercise		
Week 1 – 10		
Broad jump	Set	2 – 4
	Repetitions	5
Seated box jump	Set	2 – 4
	Repetitions	5
Pogo jumps	Set	2 – 4
	Repetitions	10

1RM, one-repetition maximum.

Nutritional supplementation

Due to the high standard of athletes participating in this study, all supplements needed to be 'Informed Sport' certified as having been tested by LGC Group's anti-doping laboratory for contamination with banned substances. Participants in COL received 90 mL 'Collagen Liquid' (GBR Nutrition, London, UK), which contained 30 g collagen hydrolysate, dextrose monohydrate, fructose, flavouring (mango and passion fruit), stabilisers (potassium sorbate and sodium benzoate), sweetener (sucralose), citric acid and water, and comprised 180 kcal. Participants in PLA received 49.3 g 'Tropical' flavour 'GO Electrolyte' (Science in Sport, London,

UK), which contained 36.5 g maltodextrin, 8.4 g fructose, citric acid, electrolytes (sodium chloride, calcium lactate, potassium chloride, sodium citrate, magnesium citrate), natural flavouring sweetener (aspartame) and phenylalanine and comprised 180 kcal. Thus, PLA was calorie- and taste-matched with the COL supplement. Each supplement was mixed with water to create a total volume of 250 mL, and all participants were given a 500 mg vitamin C tablet (Elite Vitamin C, Healthspan, Guernsey, UK) to consume immediately after consuming the drink. Participants consumed their supplements in entirety immediately before each training session under the supervision of one of the investigators. All drinks were provided in opaque bottles and, together with the taste-matching and equal volume of drink, this ensured participants remained blinded to their allocated group for the entirety of the study. The number of nutritional supplements participants consumed during the different types of training session is shown in **Table 3**.

Table 3. The number of nutritional supplements participants had with training sessions or match.

Session type	**COL (*n* = 6)**	**PLA (*n* = 5)**
PBS	13 ± 1	13 ± 1
PBS and ST	7 ± 1	8 ± 1
PBS and PLY	10 ± 0	9 ± 1
Match	0 ± 0	0 ± 0

Data are mean ± SD. *PBS*, pitch-based session; *PLY*, plyometric exercise; *ST*, strength exercise.

Habitual dietary intake and anthropometry

Participants' height (SECA, model-217, Hamburg, Germany) and body mass (SECA, model-875, Hamburg, Germany) were measured to the nearest 0.1 cm and 0.1 kg, respectively. Participants were asked to record their habitual dietary behaviour using a food and drink diary for three days (Thursday to Saturday) during the baseline testing period. This aspect of the study was completed by $n = 10$ (**Table 4**). Records were analysed with Nutritics professional dietary analysis software (version 5.09, Nutritics Ltd., Co. Dublin, Ireland) to obtain total energy, macro- and micronutrient composition. All daily nutritional composition data were presented as absolute and relative (normalised to body mass) values. 'Total intake' was calculated as the sum of habitual intake and nutritional supplementation used in this study. Thus, the total COL supplementation on training days was 30 $g \cdot d^{-1}$, and when averaged across training and non-training days, the COL supplement increased protein intake by 12.9 $g \cdot d^{-1}$, vitamin C intake by 214 $mg \cdot d^{-1}$ and energy intake by 77.1 $kcal \cdot d^{-1}$. Each PLA supplement contained 44.9 g maltodextrin/fructose and, when averaged across training and non-training days, increased CHO intake by 19.2 $g \cdot d^{-1}$, vitamin C intake by 214 $mg \cdot d^{-1}$ and energy intake by 77.1 $kcal \cdot d^{-1}$.

Knee extensor and flexor maximal voluntary contraction

Isometric and concentric knee extension (KE) and isometric knee flexion (KF) maximal voluntary contractions (MVCs) on an isokinetic dynamometer (IKD, Humac Norm, Computer Sports Medicine Inc., Stoughton, USA) were measured. Knee and hip joints angles were set at 90° (0° = full knee extension) and 85° (180° = supine), respectively, and movement was restricted with the use of inextensible waist, chest, and thigh straps. Isometric MVCs were then measured while participants were performing isometric KE and KF for 3 s. The true isometric MVC was measured until the difference between two MVCs from two attempts was < 5%. For concentric KE MVCs, the highest concentric KE at $60 \cdot s^{-1}$ (full range of motion) was chosen

from three consecutive attempts. The torque signal was interfaced with an analogue-to-digital converter (MP150 Biopac Systems Inc., Santa Barbara, USA), sampled at 2 kHz with a PC using data acquisition software (AcqKnowledge v.5.1, Biopac Systems Inc.) and low-pass filtered (10 Hz edge frequency) offline.

Morphological, mechanical, and material tendon properties

Details and reliability of the measurements have been documented in Chapter Five. Due to preconditioning of tendon, the order of muscle-tendon assessment was as follows: scanning PT cross-sectional area (CSA), a standardized warm up (10 repetitions of KE and KF at 60 s^{-1} with full range of motion by gradually increasing intensity from submaximal to maximal efforts) for preconditioning tendon, 3-s isometric and concentric KE and isometric KF, and 6-s isometric KE ramped MVC (RMVC). Therefore, this assessment order provided enough preconditioning tendon to prevent the residual tendon elongation (Maganaris, 2003). Briefly, PT CSA was measured at 25%, 50%, and 75% tendon length using a 4-cm wide 5-18 MHz linear probe (Philips EPIQ 7 Ultrasound System, Bothel, USA) while participants were seated on the IKD in the resting state, with the knee secured at 90°. After measuring tendon CSA, participants performed an isometric KE RMVC, which lasted 6 s and was followed immediately by a 6 s ramped relaxation to rest. During the RMVC, a 10-cm wide (10−15 MHz) linear probe (Mylab70, Esaote Biomedica, Genoa, Italy) was positioned sagitally over the tendon (imaging depth set to 7 cm) to record tendon elongation (sampling frequency: 23 Hz). At least two RMVC attempts were made with 2-min rest in between attempts and generally, a successful attempt was achieved within two attempts. As the loading rate ($Nm \cdot s^{-1}$) depended on the participant's ability to produce maximal voluntary force, real-time torque-time data were projected in front of the participants, so they could gradually and consistently increase torque output to

MVC. The loading rate during the 6 s RMVC was COL: 29.6 ± 5.3 Nm·s^{-1} vs. PLA: 29.4 ± 5.5 Nm·s^{-1} (pre-training) and COL: 32.6 ± 7.2 Nm·s^{-1} vs. PLA: 33.7 ± 7.9 Nm·s^{-1} (post-training).

Patellar tendon force was estimated by dividing KE torque during the RMVC by the tendon moment arm, which was estimated using following equation from individual's femur length where moment arm (d) is expressed as a percentage of segment length, θi is expressed in degrees and A_1 and A_2 are the constants of *Vastus lateralis* (VL) muscle (Visser et al., 1990).

$$d = \{femur\ length \times (A_1 + 2A_2\,\theta_i) \times 180/\pi\}/100$$

The patellar tendon force was subsequently corrected for antagonist (hamstring) co-activation via electromyography (EMG). Using semi-automated tracking software (Tracker, version 6.1.2, https://physlets.org/tracker/), the displacement of both the patellar apex and tibial tuberosity was measured during RMVC. Individual tendon force–elongation data were subsequently fitted with a second-order polynomial ($R^2 > 0.93$ in all cases). Patellar tendon mechanical and material properties pre- and post-training were calculated using the weakest maximum tendon force for each participant, usually determined at pre-training. Patellar tendon strain was defined as tendon elongation expressed as a percentage of the tendon's original length, i.e. 100 × change in tendon length (ΔL)/ resting tendon length (L_0). Tendon stress was defined as the peak tendon force (F_t) at KE RMVC relative to the mean tendon CSA (i.e. F_t/CSA). Tendon stiffness (ΔF_t / ΔL) was calculated from the participant's highest 20% F_t interval. Young's modulus (E) was calculated by multiplying stiffness (k) with the ratio of the resting tendon length to mean tendon CSA (i.e. $E = k \times (L_0/CSA)$).

Thickness of subcutaneous adipose tissue and skeletal muscle

VL muscle and its subcutaneous adipose tissue (SAT) thickness were measured using the 10-cm wide (10−15 MHz) linear probe (Mylab70, Esaote Biomedica, Genoa, Italy) while the participant sat on the IKD in the resting state (with knee and hip angles as stated above). The thickness of both the VL and its SAT were an average of three measurements for each tissue, i.e. 25%, 50%, and 75% of the 10 cm wide field of view in the ultrasound image (**Figure 1**).

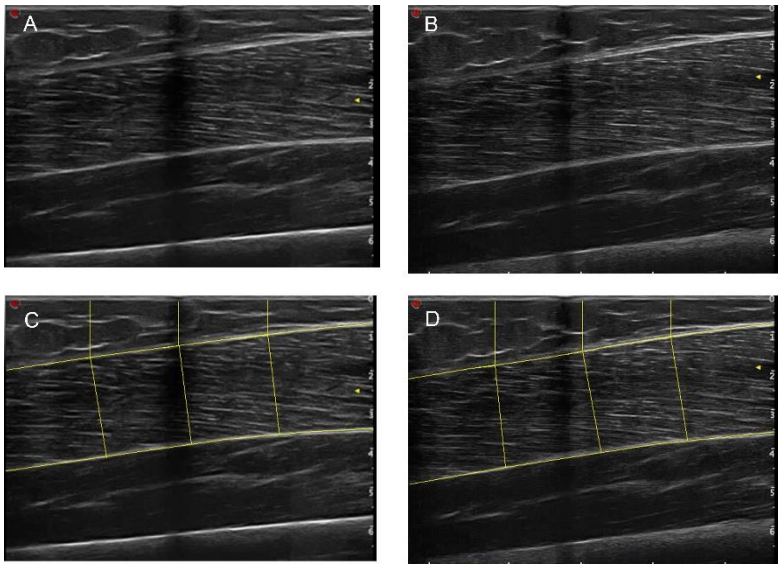

Figure 1. Example ultrasound images of *vastus lateralis* muscle thickness and its subcutaneous adipose tissue. (A) Pre-training original image, (B) Post-training original image, (C) Pre-training analysed image, (D) Post-training analysed image.

Statistical analyses

All data are presented as means ± standard deviations (SD). Pre-training between group (COL vs. PLA) comparisons of physical characteristics and dietary behaviour were performed with independent t-tests. Two-way mixed ANOVA models (group: COL vs. PLA; time: pre- vs. post-training) were performed to detect changes in KE and KF MVC torque, resting tendon length, loading rate, mean tendon CSA, VL and its SAT thickness, and all other tendon mechanical and

material properties. When significant group × time interaction effects were found, post-hoc paired t-tests (pre- vs. post-training for COL and PLA) and independent t-tests (COL vs. PLA for percentage changes in tendon properties) were performed to reveal between-group differences over time. A three-way mixed ANOVA was performed to assess differences among group (COL vs. PLA), time (pre- vs. post-training), and location (25% vs. 50% vs. 75% tendon length) for tendon CSA. Two effect sizes, Cohen's d (for t-tests) and the partial eta squared, η_p^2, (for ANOVA interaction) were reported for each statistical model. The thresholds of Cohen's d and ηp2 are defined as small (d = 0.20 and $\eta_p^2 = 0.01$), medium (d = 0.50 and $\eta_p^2 = 0.06$) and large (d = 0.80 and $\eta_p^2 = 0.14$) (Cohen, 1988). Data were analysed by using the statistical software package SPSS (version 26, SPSS Inc., Chicago, IL) and level of significance was set at $P < 0.05$.

6.3 Results

Group characteristics

Age, body mass, height and baseline isometric and concentric KE and isometric KF MVC did not differ between COL and PLA groups (all $P > 0.05$, **Table 1**).

Macro- and micronutrient intake

The habitual and total macronutrient and vitamin C intake did not differ between COL and PLA (all $P > 0.05$, **Table 4**).

Table 4. Energy, macronutrient, and micronutrient intake during the pre-training assessment period. Data are mean ± SD.

Nutritional composition	**COL (*n* = 5)**	**PLA (*n* = 5)**	***t*-test, *P***

Energy intake			
Habitual intake (kcal·d^{-1})	1743 ± 242	1584 ± 328	0.408
Total intake (kcal·d^{-1})	1820 ± 242	1661 ± 328	0.408
Carbohydrate intake			
Habitual intake (g·d^{-1})	213 ± 31	192 ± 32	0.336
Habitual intake (g·kg·d^{-1})	3.4 ± 0.4	3.0 ± 0.4	0.112
Total intake (g·d^{-1})	213 ± 31	212 ± 32	0.956
Total intake (g·kg·d^{-1})	3.4 ± 0.4	3.3 ± 0.4	0.592
Protein intake			
Habitual intake (g·d^{-1})	103 ± 39	83.1 ± 25.9	0.367
Habitual intake (g·kg·d^{-1})	1.6 ± 0.5	1.3 ± 0.3	0.239
Total intake (g·d^{-1})	116 ± 39	83 ± 26	0.155
Total intake (g·kg·d^{-1})	1.8 ± 0.5	1.3 ± 0.3	0.083
Fat intake			
Habitual intake (g·d^{-1})	51.9 ± 13.5	49.8 ± 14.7	0.818
Habitual intake (g·kg·d^{-1})	0.8 ± 0.2	0.8 ± 0.2	0.547
Vitamin C intake			
Habitual intake (mg·d^{-1})	82.5 ± 64.0	125 ± 31	0.218
Habitual intake (mg·kg·d^{-1})	1.4 ± 1.1	2.0 ± 0.5	0.279
Total intake (mg·d^{-1})	297 ± 64	339 ± 31	0.218
Total intake (mg·kg·d^{-1})	4.8 ± 1.2	5.3 ± 0.5	0.384

Maximum strength and muscle thickness

Isometric and concentric KE MVC, isometric KF MVC, and antagonist co-activation before and after training are presented in **Table 5**. There were no main effects for training, group, or interaction effects for any of the variables ($P > 0.05$). Regarding VL and its SAT thickness, there were no main effects for training, group, or interaction effects ($P > 0.05$).

Table 5. Knee extension (KE) and knee flexion (KF) isometric and concentric maximal voluntary contraction (MVC) torque, antagonist muscle co-activation and vastus lateralis (VL) muscle and subcutaneous adipose tissue (SAT) thickness in COL and PLA groups before (PRE) and after (POST) training. Data are mean ± SD.

Variable	COL (*n* = 6)		PLA (*n* = 5)		
	PRE	POST	PRE	POST	g × t, *P*
Isometric					
KE (N·m)	190 ± 32	196 ± 47	204 ± 37	213 ± 27	0.850
KF (N·m)	82.0 ± 15.7	85.1±19.2	87.0 ± 20.5	89.6 ± 21.0	0.932
Concentric					
KE (N·m)	147 ± 26	140 ± 24	159 ± 22	162 ± 28	0.191
Antagonist co-activation (%)	17.3 ± 5.9	16.7 ± 6.6	21.8 ± 7.4	16.8 ± 8.0	0.136
VL thickness (mm)	8.0 ± 3.1	8.2 ± 3.1	9.2 ± 4.1	8.2 ± 3.0	0.611
SAT thickness (mm)	27.0 ± 2.6	27.1 ± 2.7	26.9 ± 2.9	26.8 ± 2.8	0.163

Morphological, mechanical, and material tendon properties

Resting tendon length and tendon CSA

There were no main effects for training or group, or interaction effects for resting tendon length. $P > 0.05$; **Table 6**). Regarding the mean tendon CSA, there was a main effect for training ($F_{1,9} = 6.914$, $P = 0.027$, $\eta_p^2 = 0.434$) but no main effect for group ($F_{1,9} = 0.249$, $P = 0.630$, $\eta_p^2 = 0.07$) and no training × group interaction ($F_{1,9} = 0.007$, $P = 0.934$, $\eta_p^2 = 0.001$; **Table 6**). A three-way ANOVA for tendon location revealed only a main effect of training ($F_{1,9} = 6.910$, $P = 0.027$, $\eta_p^2 = 0.434$), i.e. no regional hypertrophy in either group.

Table 6. Patellar tendon morphological, mechanical, and material properties in PLA and COL groups before (PRE) and after (POST) training. Data are mean ± SD.

	COL (*n* = 6)		PLA (*n* = 5)		
Variable	**PRE**	**POST**	**PRE**	**POST**	**g × t, *P***
Resting tendon length (mm)	47.2 ± 5.6	47.3 ± 5.0	49.3 ± 4.1	49.3 ± 4.3	0.844
Mean CSA (mm^2)	73.9 ± 5.7	74.7 ± 5.4	72.1 ± 6.1	72.9 ± 5.9	0.934
Tendon force (N)	4709 ± 754	4959 ± 1012	4323 ± 752	4721 ± 717	0.895
Stress (MPa)	59.7 ± 9.9	59.0 ± 10.0	55.3 ± 8.2	56.0 ± 7.8	0.145
Elongation (mm)	4.0 ± 0.4	3.9 ± 0.8	3.8 ± 0.9	3.5 ± 0.7	0.414
Strain (%)	8.5 ± 1.0	8.2 ± 1.5	7.8 ± 2.0	7.1 ± 1.6	0.508

Mechanical and material properties of tendon

Regarding loading rate, there was a main effect of training ($F_{1,9} = 11.993$, $P = 0.007$, $\eta_p^2 = 0.571$) but no main effect of group ($F_{1,9} = 0.011$, $P = 0.919$, $\eta_p^2 = 0.001$) and no interaction effect ($F_{1,9} = 0.387$, $P = 0.549$, $\eta_p^2 = 0.041$). Therefore, any group differences in changes in mechanical and material tendon properties were not affected by loading rate in the current study. Regarding tendon stiffness, there was a main effect of training ($F_{1,9} = 65.348$, $P < 0.001$, $\eta_p^2 = 0.879$) and an interaction effect ($F_{1,9} = 18.968$), $P = 0.002$, $\eta_p^2 = 0.666$) but no main effect of group ($F_{1,9} = 0.066$, $P = 0.804$, $\eta_p^2 = 0.007$, **Figure 2A**). Regarding Young's modulus, there was a main effect of training ($F_{1,9} = 47.424$, $P < 0.001$, $\eta_p^2 = 0.840$) and an interaction effect ($F_{1,9} = 15.005$), $P = 0.004$, $\eta_p^2 = 0.625$) but no main effect of group ($F_{1,9} = 0.578$, $P = 0.467$, $\eta_p^2 = 0.060$, **Figure 2B**). Regarding the other tendon properties (tendon stress, strain, and elongation), there were no main effects of training or group, and no interaction effects (all $P > 0.05$, **Table 6**). Post-hoc paired t-test revealed that tendon stiffness and was significantly increased after 10-week soccer training in both COL [$t(5) = -7.865$, $P < 0.001$] and PLA [$t(4) = -3.471$, $P < 0.026$] groups.

Also, Young's modulus was significantly increased in both COL [t(5) = -6.664, *P* = 0.001] and PLA (t(4) = 0.020, *P* = 0.039) groups. Consequently, post-hoc independent t-test revealed that the percentage changes in tendon stiffness [t(9) = 5.891, P < 0.001, +15.4 ± 3.1% vs. +4.6 ± 3.0] and Young's modulus [t(9) = 4.910, *P* < 0.001, +14.2 ± 4.0 vs. +3.4 ± 2.8%] were significantly higher in COL vs. PLA.

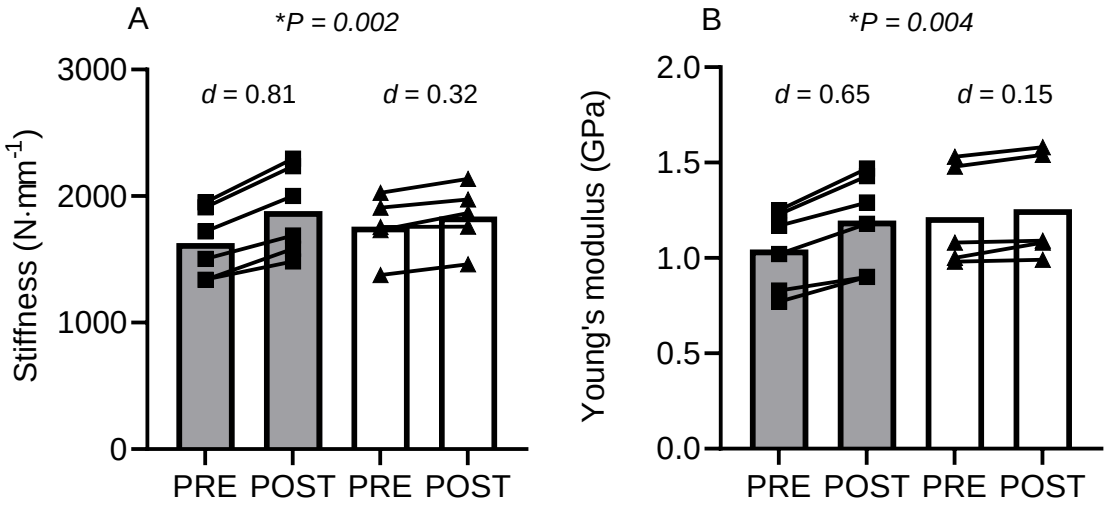

Figure 2. Tendon stiffness (A) and Young's modulus (B) before (PRE) and after (POST) the 10-week soccer training in collagen (grey bar and black squares) and placebo (white bar and black triangles). * Group × time interaction effects; *d*, Cohen's *d* effect size.

6.4 Discussion

The main aim of this study was to investigate the effect of collagen supplementation on the changes in patellar tendon morphological, mechanical, and material properties after 10 weeks' soccer training during pre-season (incorporating high-intensity lower-limb strength/plyometric training) in professional female soccer players. The main findings were greater increases tendon stiffness and Young's modulus in COL compared to PLA. However, although there was a

main effect of training on the change in patellar tendon CSA, the degree of tendon hypertrophy did not differ between COL and PLA. Regarding parameters of skeletal muscle adaptation, KE MVC and VL thickness were not changed after 10-week's in-season soccer training in both groups.

We observed large effect sizes for changes in tendon stiffness (d = 0.81 *vs.* d = 0.32) and moderate effect sizes for changes in Young's modulus (d = 0.65 *vs.* d = 0.15) in COL compared to small effects sizes in PLA. In Chapter Five, 10 weeks' *in-season* soccer training incorporating lower-limb *bodyweight* strength/plyometric exercise supplemented with 30 g COL increased tendon stiffness and Young's modulus more than PLA in a women's soccer academy squad, although no patellar tendon hypertrophy was observed in either group.

The exercise intensity used in Chapter Five, was probably insufficient to induce patellar tendon hypertrophy (probably a consequential repair and/or a remodelling process) at the proximal and distal ends of the patellar tendon (Kongsgaard et al., 2007, Seynnes et al., 2009, Dalgaard et al., 2019). Therefore, the current study implemented high intensity (75 – 90% 1RM) lower-limb RT once a week (in addition to loaded plyometric training once a week and regular pitch-based training), with the intention of inducing tendon hypertrophy. Indeed, there was a main effect of training, but COL did not show an augmented hypertrophic adaptation, and the effect sizes for the change in tendon CSA for both COL and PLA were small (d = 0.1). Dalgaard et al. (2019) reported that 10-weeks' progressive resistance training (three times per week at 10 – 15 RM loads) increased patellar tendon CSA in young healthy women. Furthermore, Jerger et al. (2022) and Jerger et al. (2023) found that 14-week's resistance training (three times per week at 70 – 85% 1RM) with 5 g HC intake increased Achilles and patellar tendon CSA more than PLA in young healthy men. Therefore, trivial changes in patellar tendon CSA in the current

study may be due to the relatively low frequency of high-intensity strength training, or the fact that the athletes' tendons had already adapted to this intensity and frequency of training prior to the study. Long-term habitual loading (e.g. running) is known to induce tendon hypertrophy (Couppé et al., 2014) and considering the pitch-based session is frequently implemented in soccer training, future studies should explore whether a longer period soccer training incorporating strength training once a week would increase tendon CSA or not. Regarding skeletal muscle adaptation, the fact that this was not an untrained population at the start of the study, and that the frequency of resistance exercise may not have been sufficient to elicit a change maximal leg strength and muscle thickness.

Greater changes in tendon stiffness and Young's modulus in COL *vs.* PLA are likely due to augmented mechanical loading-induced tendon collagen synthesis in the presence of high serum concentrations of the necessary exogenous amino acids (i.e. glycine and proline) for synthesising collagen (Shaw et al., 2017, Lis and Baar, 2019). Interestingly, Clifford et al. (2019) found that short-term HC intake might be beneficial for recovery following an acute bout of strenuous physical activity. Recreationally active young men consumed 20 g hydrolysed collagen for seven days and on the next day they performed 150 drop jumps from a 60 cm box and they consumed additional 20 g hydrolysed collagen from the day of performing the exercise to 48 h post exercise (Clifford et al., 2019). The effect sizes for subjective muscle soreness at 24 h and 48 h post exercise were large in the COL group and reduction of counter movement jump height at 48 h post exercise was statistically significant in COL group (Clifford et al., 2019). Despite the perceived importance of strength training in soccer, strength and conditioning coaches in soccer have been concerned about implementing strength exercise due to muscle soreness and fatigue (McQuilliam et al., 2023), the chances of which are especially higher in

pre-season (following a break from training and matches during the off-season). Therefore, future studies should investigate whether long-term COL intake could have a beneficial impact on recovery when soccer players return from the off-season.

One limitation of the current study is the sample size. However, the large effect sizes for changes in tendon stiffness and Young's modulus in COL vs. the small effect sizes in PLA reflected the statistically significant results regarding the group-dependent changes in these properties. There were no other significant interactions between group and time and effect sizes for changes in all other tendon properties were small, which suggests that even if the sample size had been greater, it was unlikely that we would have seen any more significant interaction effects between group and time. Nevertheless, one solution for future studies to optimise sample size in this population may be to collaborate with multiple women's soccer clubs, although soccer clubs have different training programmes, so standardisation between clubs may be challenging. Furthermore, we were not able to assess the participants' muscle-tendon properties during the same menstrual phase in the pre- and post-training tests due to the club's and players' limited availability during the research period, and we acknowledge that this might have affected our findings.

In conclusion, 10 weeks' pre-season soccer training (incorporating high-intensity lower-limb strength and plyometric training), supplemented with 30 g collagen (and 500 mg vitamin C) three times a week, conferred greater gains in patellar tendon stiffness and Young's modulus compared to soccer training alone in professional female soccer players from a FA Women's Championship First Team squad. Future studies should investigate a direct link between collagen consumption and recovery following strenuous physical activities especially when soccer players return from a period of no training or matches (e.g. start of pre-season).

Chapter Seven

Effect of six weeks' resistance training with 30 g hydrolysed collagen supplementation on patellar tendon properties in resistance-trained, young men

Prelude

Chapters Five and Six showed that 30 g hydrolysed collagen (HC) with 10 weeks' soccer training comprising bodyweight or externally loaded resistance-, plyometric- and/or pitch-based exercise 3 days/week in female soccer players increased patellar tendon (PT) stiffness and Young's modulus more than training alone. Although the high-intensity resistance training (RT) led to an increase in PT cross-sectional area (CSA), the low frequency of this training (once per week) may have precluded HC from augmenting PT hypertrophy more than training alone. Thus, a higher frequency of high-intensity RT may enable HC to elicit a greater effect on PT CSA. These hypotheses are tested in this final experimental chapter, which implements high-intensity lower-limb RE twice a week for six weeks in resistance-trained, young men. *The order of this chapter in the thesis does not reflect the timing of the study implementation.* For example, the study described in this final chapter was the first to be severely impacted by the COVID-19 pandemic. Data collection for this study started in January 2020 and the training duration was due to last 12 weeks. However, due to the sudden closure of the University in March 2020 (a consequence of the UK's first full national lockdown), the PhD candidate was forced to bring the study to an abrupt end after the participants had completed just six weeks' training. Although post-training tests were completed in the few days prior to university closure, this inevitably led to a smaller sample size than planned. The PhD candidate tried to repeat this training study on subsequent occasions to increase sample size, but numerous ensuing national lockdowns made this impossible. Therefore, after discussion with the supervisory team, it was decided to report the data collected in the original six-week study, being mindful and transparent about the probable lack of statistical power.

Abstract

The current study investigated the effects of six weeks' resistance training (RT) with hydrolysed collagen (HC) supplementation on patellar tendon (PT) properties in resistance-trained, young men. Twelve men (age: 22.7±8.1 years; height: 1.76±0.07m; mass: 77.0±9.7kg) were randomly allocated into two groups (collagen, COL, *n*=5; and placebo, PLA, *n*=7), based on age, mass, height, and baseline knee extension maximum voluntary contraction torque. Participants consumed 30g HC (COL) or 34.1g energy-matched maltodextrin (PLA) with 100mg vitamin C dissolved in 250mL water immediately before each RT session. Participants completed the 6-week RT programme, with each RT session (performed twice weekly) comprising 4×10 repetitions of lower-limb exercises at 10-repetition maximum load. Pre- and post-training assessments included PT cross-sectional area (CSA), stiffness and Young's modulus, *vastus lateralis* (VL) muscle morphology and architecture, using isokinetic dynamometry and ultrasonography. PT stiffness (+10.1±6.8%; P =0.019; d=1.7 vs. +4.5±5.0%; P=0.061; d=0.9) increased in COL but not in PLA, respectively. Young's modulus (+8.7±7.4%; P=0.085; d=1.0 vs. +3.3±5.2%; P =0.151; d =0.6) did not increase in either group, although the change was approximately two-fold greater in COL than PLA and the effect size was large in COL and small in PLA. Mean PT CSA increased in both groups (+1.4±0.7%, $P<0.001$) but the change did not differ between groups ($P=0.092$). Finally, indices of muscle morphology increased in both groups ($P<0.05$), with no difference between groups ($P>0.05$). In conclusion, six week's RT supplemented with 30 g HC in resistance-trained, young men did not further alter mechanical and material properties of PT.

7.1 Introduction

Tendons are collagen-containing connective tissues that attach muscle to bone and thus, their main role is transmitting force produced by skeletal muscle to the bone to generate movement. Due to the anatomical location of tendons, they must bear stress, which is approximately four to eight times greater than body weight during physical activities such as walking, running, and jumping (Finni et al., 2000, Giddings et al., 2000). However, excessive repetitive stretching of tendon causes a non-rupture injury in tendon, termed tendinopathy (Scott et al., 2015). Incidence of tendinopathy is common in professional and recreational athletes and workers (Ferretti, 1986, Frost et al., 2002, Woods et al., 2002, Zwerver et al., 2011), which might require surgical intervention and a period of rehabilitation. Thus, a combination of exercise and nutritional strategies are highly sought after by practitioners and athletes to improve tendon health and function.

It is evident that human tendons have a degree of plasticity in response to external anabolic stimuli (e.g. mechanical loading and nutrition). In addition to the effect of chronic mechanical loading (e.g. resistance training, RT) on tendon adaptation, e.g. augmented tendon size, stiffness and Young's modulus (Seynnes et al., 2009; Kongsgaard et al., 2007), Chapter Three showed that 30 g hydrolysed collagen (HC) ingested prior to high-intensity (4 × 10 repetition maximum (RM)) back squat resistance exercise (RE) in resistance-trained, healthy, young men led to a greater collagen synthesis response, i.e., increased serum procollagen I amino-terminal peptide (PINP) concentration, compared to 15 g and 0 g HC. Furthermore, Chapters Five and Six showed that 10 weeks' RT in female soccer players supplemented with 30 g HC three times a week increased patellar tendon (PT) stiffness and Young's modulus more than RT alone. In line with this finding, Jerger et al. (2022) found that 14 weeks' RT (performed three times a

week), with *daily* consumption of 5 g HC, increased Achilles tendon cross-sectional area (CSA) more than RT alone. As part of the same project, Jerger et al. (2023) found that *daily* consumption of 5 g HC and 14 weeks' RT had a similar effect on PT CSA. However, no study has yet investigated the effect of just six weeks' high-intensity RT, supplemented with 30 g HC, on changes in PT properties. Therefore, the main aim of this study was to investigate the effect of a 6-week lower-limb RT (performed twice weekly), supplemented with 30 g vitamin C-enriched HC, on changes in PT morphological and mechanical properties in resistance-trained, healthy, young men. We hypothesized that 30 g HC and 100 mg vitamin C (ingested immediately before each training session) would confer greater changes in PT morphological and mechanical properties following RT compared with RT alone.

7.2 Methods

Participants

Twenty-three resistance-trained young men provided written informed consent before participating this 6-week exercise and nutritional intervention study, which complied with the Declaration of Helsinki and was approved by the Liverpool John Moores University Research Ethics Committee (approval number: 19/SPS/054). Data collection for the study commenced on 13/01/2020 and was completed on 20/03/2020. Participants were pair-matched for age, height, mass and knee extensor (KE) maximum isometric voluntary contraction (MVC) torque, then randomly assigned to one of the two experimental groups, collagen (COL) or placebo (PLA). Five participants were excluded from the study due to not completing at least 75% (i.e. nine) of the 12 scheduled training sessions. In addition, six participants were unable to be tested post-training, due to the sudden University closure following the start of the Covid-19 pandemic

and the first UK Government-enforced national lockdown in May 2020. Consequently, 12 participants who had 6 ± 4 years' RE experience completed this 6-week randomised control trial (participant characteristics can be found in **Table 1**). Volunteers were excluded from taking part in the study if they were vegans, consumed nutritional supplements purported to have hypertrophic effects on either the muscle or tendon and/or a potential positive effect on strength gains (e.g. protein, antioxidants, etc.), were taking any medication considered to influence tendon size or function, had a history of lower limb injuries in the previous six months or were <18 or >40 years old. An a priori power analysis using G*Power 3.1.9.7 software (G*Power Software Inc., Kiel, German) and mean patellar tendon CSA increase of 4 mm^2 following RT (Seynnes et al., 2009), and a hypothetical further 1 mm^2 increase due to collagen supplementation indicated that a minimal sample size of $n = 12$ would be required with $\alpha = 0.05$, $\beta = 0.80$).

Table 1. Group characteristics of placebo (PLA) and collagen (COL) groups before the 6-week supplemented resistance training period. Data are represented as mean ± SD.

Characteristic	**PLA (*n* = 5)**	**COL (*n* = 7)**	***t*-test, *P***
Age (years)	22.4 ± 8.0	23.0 ± 9.1	0.910
Body mass (kg)	75.8 ± 9.9	76.4 ± 10.5	0.922
Height (m)	1.77 ± 0.06	1.75 ± 0.09	0.757
Isometric KE MVC (N·m)	268 ± 52	238 ± 63	0.382

Study overview

The study was a single-centre, single-blind design (i.e. participants but not researchers were unaware of participant group allocation). All participants attended the laboratory on two occasions before and after 6-week resistance training (RT). Both groups were of similar age, an-

thropometric characteristics, and baseline isometric knee extensor strength (**Table 2**). All muscle-tendon measurements were performed on the dominant (kicking) leg before and after the 6-week RT period in the following order: isometric and isokinetic KE and knee flexor (KF) MVC (KF muscle activation was measured using surface electromyography), and patellar tendon (PT) morphological and mechanical properties were measured via ultrasonography and isokinetic dynamometry. All testing took place between 9:00 a.m. and 5:00 p.m. and each participant's pre/post-training tests were performed at the same time of day to avoid diurnal effects on intra-individual pre/post training changes. During the 6-week RT, participants received maltodextrin (PLA) or hydrolysed collagen (COL) supplementation before each training session, which was performed twice a week. Participants were instructed not to participate in strenuous physical activity and not to consume alcohol or caffeine in the 24 h before testing sessions.

Resistance training

During the 6-week RT period, participants performed two RT sessions per week (Monday and Friday). Each session included 4 × 10 at 10 repetition maximum (10-RM) load for each of the two exercises performed, which were changed on a weekly basis. Training load was progressively increased on a weekly basis by re-evaluating the 10-RM at the start of the first session of the week. Exercises included leg press, barbell back squat, box jump, dumbbell lunges, and Bulgarian split squat (**Table 2**). All training sessions were supervised by a member of the research team. RT compliance was 98% (i.e. one participant in PLA completed nine RT sessions, while the other 11 participants completed all 12 RT sessions).

Table 2. Outline of the resistance training programme.

Week	Exercise

1	One-legged press Box jump
2	Barbell back squat Dumbbell lunges
3	Barbell back squat Dumbbell step-up
4	Barbell back squat Bulgarian split squat
5	Barbell back squat Dumbbell lunges
6	Barbell back squat Bulgarian split squat

Nutritional supplementation and dietary intake

After participants were pair-matched for their age, height, mass, and KE isometric MVC, they were randomly assigned to PLA or COL groups using randomisation software (Excel 2016, Microsoft, Washington, USA). Participants received their supplementation in a single-blind manner in the form of an opaque drinks bottle, which was fully consumed immediately before each RT session. The COL supplement included 30 g hydrolysed collagen (HC) (Myprotein, Cheshire, UK), while the PLA supplement included 34.1 g calorie-matched maltodextrin (Myprotein, Cheshire, UK). Both supplements were mixed with 250 mL water, 100 mg vitamin C powder (Holland and Barrett Retail Limited, Warwickshire, UK) and 4 g non-caloric sweetener (Truvia®, SilverSpoon, London, UK). During the 6-week RT period, participants were instructed to maintain their habitual diet and physical activity, and to refrain from consuming nutritional supplements that may assist training capacity and/or recovery. Using a food and drink diary for 3 days (Thursday to Saturday) during the baseline testing period, participants were asked to record their habitual dietary behaviour. This aspect of the study was completed by $n = 6$ (**Table 3**). Records were analysed with Nutritics professional dietary analysis software

(version 5.09, Nutritics Ltd. Co. Dublin, Ireland) to obtain total energy, macro- and vitamin C composition. All daily nutritional composition data were presented as absolute and relative (normalized to body mass) values. “Total intake” was calculated as the sum of habitual intake and nutritional supplementation used in this study. Therefore, the total COL supplementation on training days was 30 g HC·d^{-1}, and when averaged across a week, the HC increased protein intake by 8.6 g·d^{-1}, vitamin C intake by 28.6 mg·d^{-1} and energy intake by 34.3 kcal·d^{-1}. Each PLA supplement contained 30.5 g maltodextrin when averaged across a week, increased carbohydrate intake by 8.7 g·d^{-1}, vitamin C intake by 28.6 mg·d^{-1} and energy intake by 34.3 kcal·d^{-1}.

Table 3. Energy, macronutrient, and vitamin C intake during the pre-training assessment period. Data are mean ± SD.

Nutritional composition	COL (*n* = 2)	PLA (*n* = 4)
Energy intake		
Habitual intake (kcal·d^{-1})	1793 ± 411	1431 ± 458
Total intake (kcal·d^{-1})	1913 ± 411	1551 ± 458
Carbohydrate intake		
Habitual intake (g·d^{-1})	191 ± 120	169 ± 92
Habitual intake (g·kg·d^{-1})	2.3 ± 1.5	2.3 ± 1.1
Total intake (g·d^{-1})	191 ± 120	178 ± 92
Total intake (g·kg·d^{-1})	2.3 ± 1.5	2.4 ± 1.1
Protein intake		
Habitual intake (g·d^{-1})	96.9 ± 27.1	80.5 ± 29.6
Habitual intake (g·kg·d^{-1})	1.2 ± 0.3	1.1 ± 0.4
Total intake (g·d^{-1})	106 ± 27	80.5 ± 29.6
Total intake (g·kg·d^{-1})	1.3 ± 0.3	1.1 ± 0.4

Fat intake		
Habitual intake ($g \cdot d^{-1}$)	59.3 ± 14.2	48.2 ± 22.9
Habitual intake ($g \cdot kg \cdot d^{-1}$)	0.7 ± 0.2	0.7 ± 0.2
Vitamin C intake		
Habitual intake ($mg \cdot d^{-1}$)	53.0 ± 36.7	116 ± 180
Habitual intake ($mg \cdot kg \cdot d^{-1}$)	0.7 ± 0.5	1.6 ± 2.5
Total intake ($mg \cdot d^{-1}$)	81.6 ± 36.7	145 ± 180
Total intake ($mg \cdot kg \cdot d^{-1}$)	1.0 ± 0.5	2.0 ± 2.5

Vastus lateralis (VL) muscle morphology and architecture

The VL muscle was scanned using B-mode ultrasonography (Philips EPIQ 7 Ultrasound System, Bothel, USA) to measure muscle anatomical cross-sectional area (ACSA), volume and architecture. While participants were in the supine position on a manual therapy bed, proximal and distal ends of the resting VL were identified using a 4-cm wide 5 – 18 MHz linear transducer and ultrasound gel. The distance between these two locations (i.e. the VL length) was measured using a measuring tape. At 25%, 50%, and 75% VL length, the mid-muscle locations were identified and marked on the skin with a permanent marker, after measuring the distances between the medial and lateral aspects of the muscle at these three locations. A straight line was drawn with a permanent marker pen between each of these points at 25%, 50% and 75% muscle length. Furthermore, at each of these three locations, 2-mm wide strips of surgical tape (3M, Neuss, Germany) were placed on the skin to act as echo-absorptive markers on the subsequent ultrasound images.

To measure VL muscle architecture, ultrasound gel was applied to the skin over the line drawn between all three points and the 4-cm wide 5 – 18 MHz linear transducer was placed on the skin in the sagittal plane at 25% VL length (in line with the trajectory of the muscle fascicles).

With minimal pressure applied to the skin (so as not to deform the muscle), the transducer was slowly but consistently moved along the mid-belly line from 25% to 75% VL length using the extended field-of-view (EFOV) function. VL muscle thickness was measured as the shortest distance between the upper and lower aponeuroses at 25%, 50% and 75% muscle length, measured using freely available image analysis software (ImageJ V.1.8.0, National Institute of Health, MD, USA), and the average of the three measurements was used in subsequent analyses. VL muscle fascicle pennation angle (θ_p, the angle at which the fascicle inserts into the lower aponeurosis) and fascicle length (L_f) were measured from at least three fascicular paths along the muscle length, and the mean of these three measurements for each variable was used in subsequent analyses.

To measure VL muscle ACSA, EFOV was also used at 25%, 50% and 75% VL length via transverse ultrasound scans, with the transducer scanning from the lateral to medial aspect of the VL at each location. The VL ACSAs were outlined using ImageJ. Images of VL CSA at all three points were taken until an acceptable scan was performed in which the entire VL border was clearly identifiable (**Figure 1**).VL muscle volume (V_m) was measured using the truncated cone formula (Erskine et al., 2017) as follows:

$$V_m = \frac{1}{3} \cdot h \cdot [a + \sqrt{(ab) + b}]$$

Where h is the distance between two ACSAs and a and b are two ACSAs of the VL. The sum of the four cones provided total VL V_m. VL physiological cross-sectional area (PCSA) was obtained dividing VL V_m by L_f.

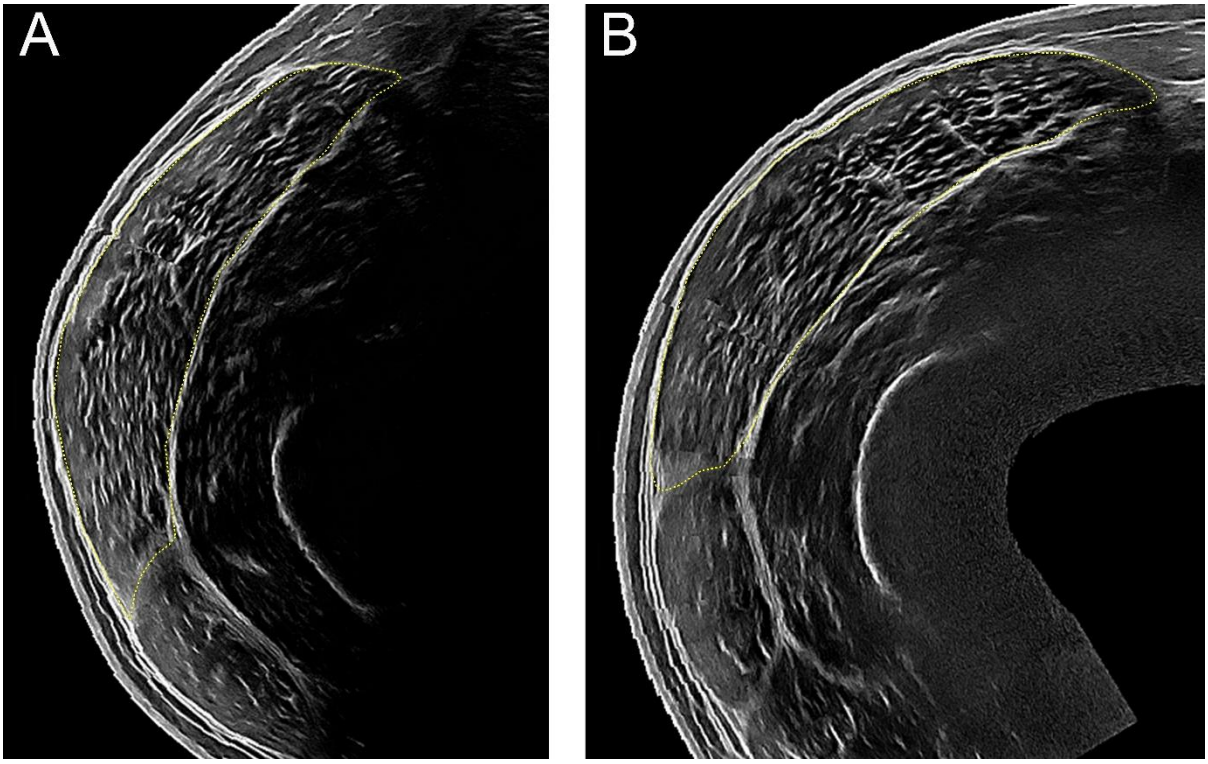

Figure 1. Example images of *vastus lateralis* (VL) muscle cross-sectional area at 25% pre- (A) and post-training (B).

Maximal strength measurement

Isometric and isokinetic knee extension (KE) and knee flexion (KF) maximal voluntary contractions (MVCs) were measured on an isokinetic dynamometer (IKD, Human Norm, Computer Sports Medicine Inc., Stoughton, USA), with the hip joint set at 85° (180° = supine). The seat was adjusted for each participant by aligning the rotational axis of the dynamometer with the lateral tibiofemoral junction and recorded for the post-training test. The lever arm of the dynamometer was attached to the lower leg and the bottom of the shin pad was fixed at 2 cm proximal to the lateral malleolus. Participants were firmly strapped at the chest, hip, and distal right thigh with inextensible straps to minimize movement. The participants performed a warm-

up session, comprising 10 isokinetic (60°/s; full range of motion) contractions, gradually increasing in intensity from sub-maximal to near-maximal (~25 – 90% MVC to ensure preconditioning of the tendon (Maganaris, 2003)). For isometric MVCs, the knee joint angle was set at 90° knee flexion (0° = full knee extensions) and participants were asked to perform a minimum of two KE and two KF MVCs (alternating between KE and KF). If the second MVC was >5% higher than the first MVC, a third MVC was performed. Each isometric MVC lasted 2 – 3 s with 30 s rest in between attempts. For isokinetic KE and KF MVCs, participants were asked to perform three continuous concentric KE and KF MVCs at 60°/s, and the highest KE and KF MVC torque from the three repetitions was recorded for subsequent analysis.

Patellar tendon (PT) morphology

Resting PT length and CSA were assessed with B-mode ultrasonography (Philips EPIQ 7 Ultrasound System, Bothel, USA) by taking sagittal and transverse images of the PT, respectively. For these measurements, participants sat on the IKD chair (knee joint angle set at 90°). The PT length was defined as the distance between the patella apex and the point at which the tendon inserted into the tibial tuberosity. These reference points were identified by positioning a 10 cm wide linear ultrasound transducer (10- to 15-MHz, Mylab70, Esaote Biomedica, Genoa, Italy) over the tendon in the sagittal plane, and marking the locations on the participant's skin with an indelible pen. A further three marks were made along the PT length, denoting 25%, 50%, and 75% PT length, which were used to take transverse images by placing the 4-cm wide ultrasound probe (Phillips Epiq 7) perpendicular to the skin surface to subsequently enable the assessment of PT CSA at these three locations. Images of PT CSA at all three points were taken until an acceptable scan was performed in which the entire PT border was clearly identifiable (**Figure 2**). PT CSA was subsequently analysed offline using ImageJ analysis software.

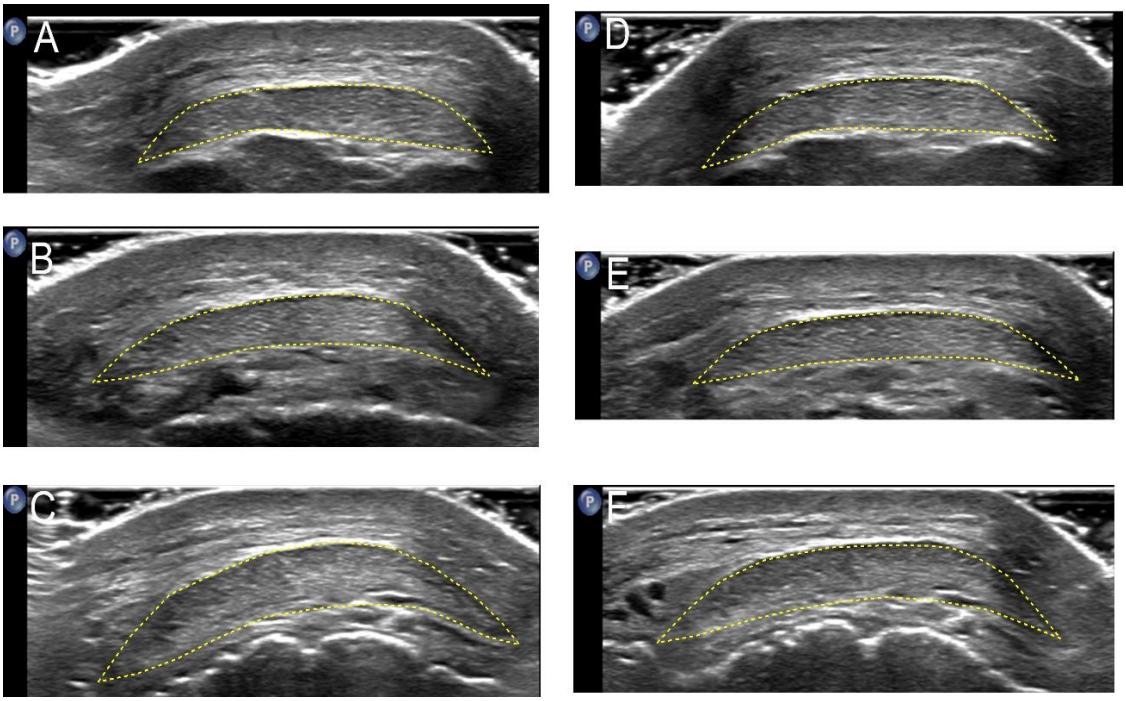

Figure 2. Example ultrasound images of patellar tendon (PT) cross-sectional area at 25% pre-(A) and post-training (D), 50% pre-(B) and post-training (E), and 75% pre-(C) and post-training (F) of resting PT length.

Tendon elongation

After scanning the PT length, participants remained sitting on the IKD chair and a 2-mm wide strip of surgical tape (3M, Neuss, Germany) was placed on the skin, transversely over the PT at ~50% PT length, to act as an echo-absorptive marker that would be visible in the subsequent tendon elongation movie files. This was to check that the transducer did not move with respect to the skin surface throughout the ramped isometric KE MVC (RMVC) but if it did, the RMVC was repeated. The 10-cm wide ultrasound transducer (Mylab70, Esaote Biomedica) was positioned sagittally over the PT and elongation of the PT during a RMVC was measured in each video frame as the displacement of both the patella apex and the tibial tuberosity from the stationary marker in the action line of the PT. The RMVC lasted for 6 s, followed immediately

by a 6 s ramped relaxation to complete rest. At least two RMVC attempts were made with 2-min rest in between attempts and, generally, a successful attempt was achieved within two attempts. As the loading rate (Nm/s) depends on the participant's ability to produce maximal voluntary force, visual feedback of force production was provided on a large screen in front of the participant. This ensured the KE MVC torque was produced gradually and consistently. Torque and EMG data during the RMVC were sampled using data acquisition software (AcqKnowledge, Biopac Systems Inc., Goleta, CA, USA). Torque (sampling frequency: 2 kHz), antagonist electromyographic (EMG, sampling frequency: 2 kHz) and ultrasound video (sampling frequency: 23 Hz) were synchronised during the RMVC by administering a square wave pulse (ECG signal), which was visualized on the AcqKnowledge software and the ultrasound movie file simultaneously.

Antagonist muscle co-activation

To estimate the extent of antagonist (hamstring) muscle co-activation during a KE RMVC, the EMG activity of the biceps femoris long head (BFlh) was recorded, which represents the knee flexor muscle group (Kellis and Baltzopoulos, 1999). The BFlh was identified via palpation during a submaximal knee flexion in the prone position. After preparation of the skin surface (shaving, skin abrasion with a sandpaper and cleaning the skin with an alcohol wipe), two bipolar Ag-AgCl surface electrodes (Neuroline, Medoicotest, Rugmarken, Denmark) with 20 mm inter-electrode distance were placed on the sagittal axis of the BFlh. The location of surface electrodes was on the distal third of the BFlh length according to SENIAM guidelines (Hermens et al., 2000) and one reference electrode was placed on the lateral tibial condyle. The EMG signal was band-pass filtered (10 – 500 Hz) and the root mean square (RMS) every 200 ms (400 data points) was calculated. Thereafter, 50 ms BFlh RMS EMG was recorded at rest

and every 10% increment of RMVC and ramped relaxation. Assuming a linear relationship between BFlh RMS EMG and KF MVC torque output (Kellis and Baltzopoulos, 1997, Reeves et al., 2004), KF co-activation torque was calculated as (BFlh RMS EMG during RMVC/BFlh RMS EMG during KF knee flexion) × peak KF MVC torque. This antagonist co-activation torque was subsequently added to the gross KE torque to provide the net KE torque. To estimate PT force, the corrected KE torque was divided by the PT moment arm at 90° knee flexion, which was estimated using each participant's femur length (Visser et al., 1990).

Analysis of tendon data

Using semi-automated tracking software (Tracker, version 6.0.10, https://physlets.org/tracker/), the patella apex and tibial tuberosity displacements were measured during a 6 s RMVC, providing an average of 352 ± 30 frames. Individual tendon force–elongation data were fitted with a second-order polynomial ($R^2 > 0.90$ in all cases). PT mechanical and material properties pre- and post-training were calculated using the weakest maximum absolute tendon force for each participant, determined during pre-training testing. For example, PT elongation pre-training was calculated as the absolute change in tendon length (ΔL) from resting tendon length (L_0) to RMVC tendon length (L_{max}), while PT elongation post-training was calculated as ΔL from L_0 to tendon length at the same absolute force value as RMVC pre-training. Tendon strain was PT elongation expressed as a percentage, i.e. $100 \times \Delta L/L_0$. Tendon stress was defined as the tendon force (F_t) at RMVC relative the mean tendon CSA (i.e. F_t/CSA). Tendon stiffness ($\Delta F_t/\ \Delta L$) was calculated from the highest individual 20% F_t interval. Young's modulus (E) was calculated by multiplying stiffness (k) with the ratio of the resting tendon length to mean tendon CSA (i.e., $E = k \times (L_0/CSA)$).

Statistics

Data were analysed by using the statistical software package SPSS (Version 26, SPSS Inc., Chicago, IL). The level of statistical significance was set at $P < 0.05$ and all data are presented as mean ± standard deviations. Comparisons between group characteristics before training was performed with independent *t*-tests. A two-way mixed analysis of variance (ANOVA) (group: PLA *vs.* COL; time: pre- *vs.* post-training) performed for changes in KE and KF MVC, muscle morphology and architecture, resting PT length, mean PT CSA and PT mechanical properties. A three-way mixed ANOVA was performed to assess differences between group (PLA *vs.* COL), time point (pre- *vs.* post-training), and tendon location (25% *vs.* 50% *vs.*75% PT length) regarding PT CSA along the tendon length. Two effect sizes, Cohen's d (for t-tests) and the partial eta squared, η_p^2, (for ANOVA interactions) were reported for each statistical model. The thresholds of Cohen's d and η_p^2 are defined as small ($d = 0.20$ and $\eta_p^2 = 0.01$), medium ($d = 0.50$ and $\eta_p^2 = 0.06$) and large ($d = 0.80$ and $\eta_p^2 = 0.14$) (Cohen, 1988).

7.3 Results

Group characteristics

Age, body mass, height and baseline isometric KE MVC did not differ between PLA and COL groups (all $P > 0.05$, **Table 1**).

Maximum strength

Isometric and isokinetic KE and KF MVC and antagonist co-activation before and after training are presented in **Table 4**. There were no main effects for training, group, or interaction effects for any of the variables ($P > 0.05$).

Table 4. Knee extension (KE) and knee flexion (KF) isometric and isokinetic maximal voluntary contraction (MVC) and antagonist co-activation in PLA and COL groups before (PRE) and after (POST) training. Data are represented as mean ± SD.

	PLA (*n* = 5)		**COL (*n* = 7)**		
Variable	**PRE**	**POST**	**PRE**	**POST**	**g × t, *P***
Isometric					
KE (N·m)	268 ± 52	277 ± 51	238 ± 63	247 ± 56	0.997
KF (N·m)	111 ± 34	110 ± 32	93 ± 39	94 ± 35	0.868
Isokinetic					
KE (N·m)	202 ± 40	207 ± 31	185 ± 33	175 ± 53	0.300
KF (N·m)	138 ± 25	134 ± 41	116 ± 27	116 ± 36	0.635
Antagonist co-activation (%)	14.8 ± 8.9	13.1 ± 5.8	13.6 ± 5.2	14.2 ± 3.2	0.482

Muscle architecture

The results regarding VL muscle morphology and architecture are presented in **Table 5**. Regarding θ_p, there was no main effect of training ($F_{1,10}$= 0.344, P = 0.570, η_p^2= 0.033), group ($F_{1,10}$= 0.005, P = 0.943, η_p^2= 0.001), or interaction effect ($F_{1,10}$= 1.145, P = 0.310, η_p^2= 0.103). Regarding L_f, there was no main effect of training ($F_{1,10}$= 1.079, P = 0.323, η_p^2= 0.097), group ($F_{1,10}$= 1.188, P = 0.301, η_p^2= 0.106), or interaction effect ($F_{1,10}$= 1.918, P = 0.196, η_p^2= 0.161).

Muscle morphology

Regarding muscle thickness, there was a main effect of training ($F_{1,10}$= 14.159, P = 0.004, η_p^2= 0.586) but there was no main effect of group ($F_{1,10}$= 0.208, P = 0.658, η_p^2= 0.020) and no interaction effect ($F_{1,10}$= 0.597, P = 0.458, η_p^2= 0.056). Regarding the mean ACSA, there was

a main effect of training ($F_{1,10}$= 18.650, P = 0.002, η_p^2= 0.651) but there was no main effect of group ($F_{1,10}$= 0.003, P = 0.955, η_p^2> 0.001) and no interaction effect ($F_{1,10}$= 4.359, P = 0.063, η_p^2= 0.304). Regarding PCSA, there was a main effect of training ($F_{1,10}$= 10.547, P = 0.009, η_p^2= 0.513) but there was no main effect of group ($F_{1,10}$= 0.446, P = 0.520, η_p^2= 0.043), and no interaction effect ($F_{1,10}$= 0.028, P = 0.870, η_p^2 = 0.003). Regarding muscle volume, there was a main effect of training ($F_{1,10}$= 27.517, P < 0.001, η_p^2= 0.773) but there was no main effect of group ($F_{1,10}$= 0.002, P = 0.963, η_p^2 < 0.001) and no interaction effect ($F_{1,10}$= 0.107, P = 0.751, η_p^2= 0.011).

Table 5. Vastus lateralis muscle morphology and architecture before (PRE) and after (POST) training in collagen (COL) and placebo (groups). Data are represented as mean ± SD.

Variable	COL (*n* = 5)		PLA (*n* = 7)		
	PRE	POST	PRE	POST	g × t, *P*
θ_p (°)	17.5 ± 1.0	17.6 ± 0.6	17.6 ± 2.4	17.3 ± 2.4	0.310
L_f (mm)	75.2 ± 8.4	77.2 ± 7.9	81.9 ± 9.1	81.6 ± 9.1	0.196
Thickness (mm)	23.2 ± 3.7	24.0 ± 3.3	24.2 ± 3.5	24.7 ± 2.9	0.458
$ACSA_{mean}$ (cm^2)	24.2 ± 2.5	25.8 ± 1.7	24.8 ± 3.5	25.4 ± 3.2	0.304
PCSA (cm^2)	61.5 ± 8.9	65.4 ± 9.9	57.7 ± 13.7	60.9 ±13.3	0.870
V_m (cm^3)	458 ± 60	500 ± 54	459 ± 94	495 ± 106	0.751

θ_p, fascicle pennation angle; L_f, fascicle length; ACSA, anatomical cross-sectional area; PCSA, physiological cross-sectional area; V_m, muscle volume.

Resting tendon length and tendon CSA

There was no main effect of training ($F_{1,10}$= 0.542, P = 0.478, η_p^2= 0.051), group ($F_{1,10}$= 0.444, P = 0.521 η_p^2= 0.042), or interaction effect ($F_{1,10}$= 1.182, P = 0.302, η_p^2= 0.106; **Table 6**).

Regarding mean tendon CSA, there was a main effect of training ($F_{1,10}$= 76.864, P < 0.001, η_p^2= 0.885) but there was no main effect of group ($F_{1,10}$ = 0.17, P = 0.689, η_p^2= 0.017) or interaction effect ($F_{1,10}$= 3.472, P= 0.092, η_p^2= 0.258). A three-way ANOVA revealed that there was no three-way interaction between group, training, and location ($F_{2, 20}$ =1.999, P = 0.162, η_p^2= 0.167; **Figure 3**).

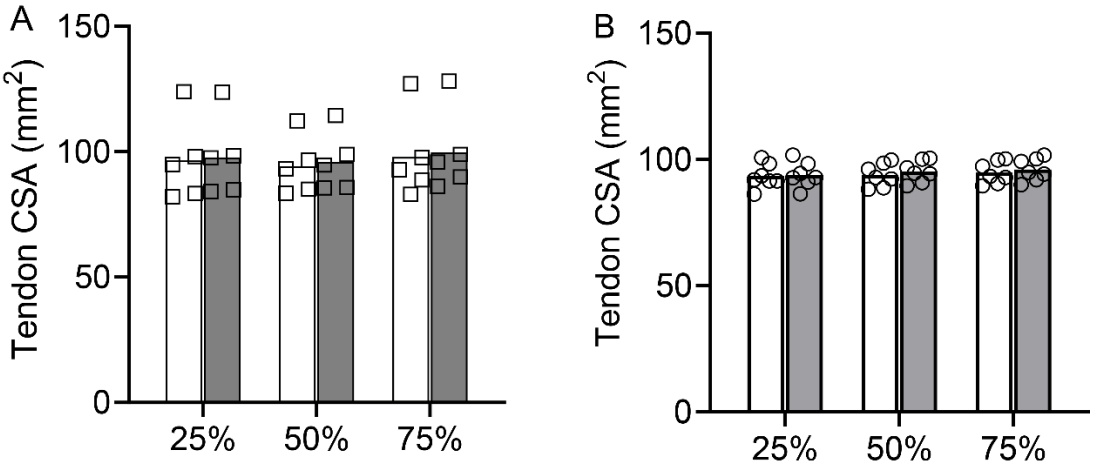

Figure 3. Patellar tendon cross-sectional area (CSA) at 25%, 50% and 75% of the resting length before (white bar) and after (grey bar) training in COL (A, n = 5) and PLA (B, n = 7).

Mechanical and material tendon properties

Tendon force, stress, elongation, and strain are represented in **Table 6**. Regarding tendon force, there was a main effect of training ($F_{1,10}$= 10.644, P = 0.009, η_p^2= 0.516) but there was no main effect of group ($F_{1,10}$= 2.292, P = 0.161, η_p^2=0.186) or interaction effect ($F_{1,10}$= 0.004, P = 0.948, η_p^2 < 0.001). Regarding tendon stiffness (**Figure 4A**), there was a main effect of training ($F_{1,10}$= 20.126, P= 0.001, η_p^2= 0.668) but there was no main effect of group ($F_{1,10}$= 0.265, P = 0.618, η_p^2= 0.026) or interaction effect ($F_{1,10}$= 2.603, P = 0.138, η_p^2=0.207). However, Cohen's d effect sizes for tendon stiffness changes were large (d = 1.7) and medium (d = 0.9) in COL and PLA,

respectively. Regarding Young's modulus (**Figure 4B)**, there was a main effect of training ($F_{1,10}$= 9.017, P = 0.013, η_p^2= 0.474) but there was no main effect of group ($F_{1,10}$= 0.473, P = 0.507, η_p^2=0.045) and no interaction effect ($F_{1,10}$= 1.647, P = 0.228, η_p^2= 0.141). However, Cohen's d effect sizes for Young's modulus changes were large and medium for COL (*d* = 1.0) and PLA (*d* = 0.6), respectively. Regarding tendon stress, there was a main effect of training ($F_{1,10}$= 2.547, P < 0.001, η_p^2= 0.765) but there was no main effect of group ($F_{1,10}$= 2.296, P = 0.161, η_p^2=0.187) and no interaction effect ($F_{1,10}$= 1.096, P = 0.320, η_p^2=0.099). Regarding tendon elongation, there was no main effect of training ($F_{1,10}$= 2.934, P = 0.118, η_p^2= 0.227), no main effect of group ($F_{1,10}$= 2.533, P = 0.143, η_p^2= 0.202) and no interaction effect ($F_{1,10}$= 2.723, P = 0.130, η_p^2= 0.214). Regarding tendon strain, there was no main effect of training ($F_{1,10}$= 3.291 P = 0.100, η_p^2= 0.248), no main effect of group ($F_{1,10}$= 0.850, P = 0.777, η_p^2= 0.008) and no interaction effect ($F_{1,10}$= 3.228, P = 0.103, η_p^2= 0.244).

Table 6. Patellar tendon (PT) mechanical and material properties in collagen (COL) and placebo (PLA) groups before (PRE) and after (POST) resistance training. Data are presented as mean ± SD.

Variable	**COL (*n* = 5)**		**PLA (*n* = 7)**		
	PRE	**POST**	**PRE**	**POST**	**g × t, *P***
Resting tendon length (mm)	48.2 ± 10.8	47.8 ± 10.6	52.0 ± 8.3	52.0 ± 8.4	0.302
Tendon force (N)	4323 ± 1378	5162 ± 1176	5383 ± 1323	6189 ± 1141	0.948
Stress (MPa)	41.7 ± 19.2	40.8 ± 18.7	55.9 ± 14.2	55.3 ± 14.1	0.320
Elongation (mm)	3.8 ± 0.4	3.3 ± 0.7	4.3 ± 1.0	4.3 ± 0.8	0.130
Strain (%)	8.5 ± 2.7	7.3 ± 2.5	8.2 ± 1.4	8.2 ± 1.1	0.103

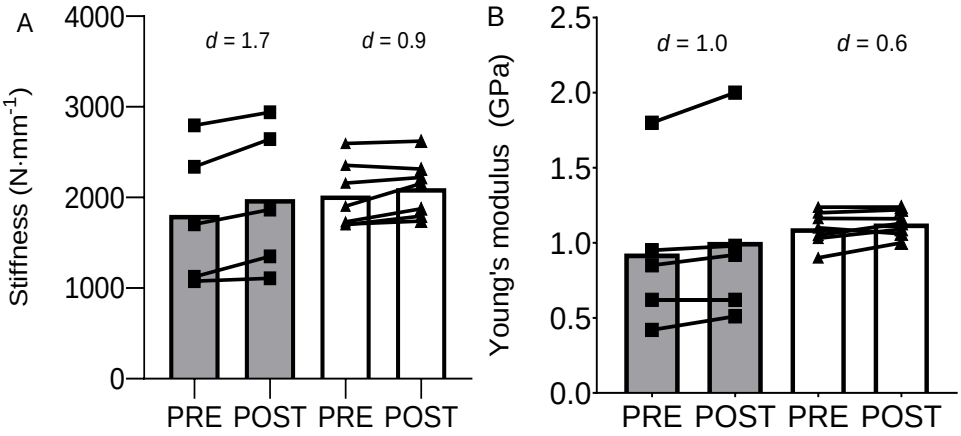

Figure 4. Patellar tendon stiffness (A) and Young's modulus (B) before (PRE) and after (POST) 6-week resistance training in COL (grey bars) and PLA (white bars) groups. Cohen's *d* effect sizes are represented.

7.4 Discussion

The aim of this study was to investigate the combined effect of HC and high-intensity RT performed twice a week for six weeks on changes in PT morphological, mechanical, and material properties in resistance-trained, young men. Contrary to our hypothesis, 30 g HC with RT did not induce significantly greater tendon adaptation compared to RT alone.

In Chapter Five, 10 weeks' in-season soccer training (incorporating bodyweight plyometric and strength exercises) with 30 g HC supplementation significantly increased both PT stiffness and Young's modulus more than soccer training alone in female academy soccer players, without any discernible changes in PT CSA. Similarly, in Chapter Six, changes in both tendon stiffness and Young's modulus (but not changes in PT CSA) were greater after 10 weeks' pre-season soccer training (incorporating high-intensity RT and overloaded plyometric exercises) when 30 g HC was ingested prior to each training session in professional female soccer players. This

discrepancy between the non-significant changes in the current study and the results from Chapters Five and Six maybe due to the shorter RT and supplementation period (i.e. six weeks vs. 10 weeks), combined with the relatively small sample size in the current study. The RT duration in the present study was due to last 12 weeks but it was forced to finish at six weeks due to the start of a national lockdown in the UK as a consequence of the COVID-19 pandemic, and six participants were unable to be tested post-training due to limited time.

Two recent studies found that 14 weeks' high-intensity RT with daily ingestion of just 5 g HC in healthy young men augmented the increase in both AT (Jerger et al., 2022) and PT (Jerger et al., 2023) CSA more than RT alone, although HC did not affect changes in ten-don stiffness in either study. The apparent discrepancies between the current study and those by Jerger et al. (2022) and Jerger et al. (2023) are not clear but are likely influenced by methodological variation. For example, the HC doses and frequencies of ingestion differed between studies (i.e. 30 g HC twice weekly in the current study vs. 5 g daily in the studies by Jerger et al.) The frequency of RT sessions and duration of RT (i.e. twice weekly for six weeks in the current study vs. three times a week for 14 weeks in the studies by Jerger et al.) led to a lower overall training volume in the current study, which may have limited the tendon's ability to adapt to the overloading stimulus, thus reducing any amplification effect of HC supplementation.

PT hypertrophy occurs after chronic RT (Kongsgaard et al., 2007, Seynnes et al., 2009, Farup et al., 2014, McMahon et al., 2018), and does so more at the distal end (Kongsgaard et al., 2007, Seynnes et al., 2009). Contrary to these previous findings, the current study did not find regional hypertrophy following 6-week RT, either with or without HC supplementation, although there was a main effect of RT on PT CSA. It has been suggested that the number of loading cycles rather than contraction mode is a critical factor for exercise-induced tendon adaptation

(Kjaer et al., 2009, Bohm et al., 2015) and thus, free-weight resistance exercises (a combination of concentric and eccentric contractions) used in the current study should have sufficiently stressed the PT. However, the current study showed just a 1.8% and 1.1% increase in the mean PT CSA in COL and PLA, respectively, which was ~3 – 5% lower than other studies that incorporated 9 to 12 weeks' RT (three times per week) at a high intensity (70 – 80% 1-RM) in healthy young men (Kongsgaard et al., 2007, Seynnes et al., 2009). Thus, the relatively short duration (6 weeks) and/or frequency (two times per week) of RT used in the current study might not have been sufficient to induce substantial tendon hypertrophy.

Regarding muscle hypertrophy, the 6-week duration in the current study was sufficient to induce an increase in vastus lateralis (VL) muscle size, as observed through 3.2 ± 3.4%, 5.0 ± 4.7%, 8.5% ± 5.5% and 7.4 ± 7.3%, increases in VL thickness, ACSA, volume, and PCSA, respectively. However, supplementation with 30 g HC did not further augment VL muscle size, which was to be expected given the relatively low abundance of essential amino acids (particularly branched chain amino acids) in HC, which are known to be po-tent stimulators of muscle protein synthesis independently of resistance exercise (Drummond et al., 2009). Surprisingly, however, a recent study did show that 15 weeks' lower-limb RT (three times a week) with daily HC supplementation of 15 g HC led to a ~50% greater increase in the volume of just one of the quadriceps femoris muscles (i.e. the vastus medialis muscle) compared to PLA (Balshaw et al., 2023a). The authors proposed that the HC may have stimulated superior muscle hypertrophy via satellite cell activation (Bentzinger et al., 2013, Urciuolo et al., 2013), although it is unclear why just one of a group of muscles would have benefitted from this mechanism. Although Jerger et al. (2022) found that daily supplementation with 5 g HC during a 12-week RT programme led to augmented increase in the thickness of the gastrocnemius muscle compared to

RT alone in healthy young men (Jerger et al., 2022), they did not report any further enhancement of quadriceps CSA with HC supplementation (Jerger et al., 2023).

The main limitation of the current study was the modest sample size and RT period, which was heavily influenced by the first UK national lockdown, suddenly enforced in March 2020 due to the COVID-19 pandemic. Despite non-statistically significant group × time interactions, it should be noted that changes in PT stiffness (+10.1 ± 6.8% vs. +4.5 ± 5.0%) and Young's modulus (+8.7 ± 7.4% vs. +3.3 ± 5.2%) were approximately 2.2-fold greater in COL than in PLA. Furthermore, Cohen's d effect sizes for changes in PT stiffness and Young's modulus were large in COL (d = 1.7 and d = 1.0, respectively), and only medium in PLA (d = 0.9 and d = 0.6, respectively). Thus, a larger sample size would have increased statistical power, potentially leading to a significant group × time interaction. The sudden start of the national lockdown also forced the study to end sooner than planned and it is possible that a longer training duration (e.g. the originally planned 12 weeks' RT) would have provided the tendon with more time to optimally adapt to the progressively increased overload. Therefore, future studies should ensure a larger sample size and longer training duration are employed to ensure the study is powered to detect a group × time interaction regarding tendon adaptation to RT and HC supplementation. Regarding dietary intake, participant compliance (n = 6) was low, and dietary intake was recorded immediately before the start of the six week's RT. This did not account for the possibility of changes in dietary intake when the nutritional supplements were given during the training period. Therefore, caution is needed when interpreting the dietary intake results in Chapter Seven.

In conclusion, high-intensity lower-limb RT (performed twice weekly) with 30 g HC ingestion in young, healthy men did not confer significantly greater changes in these muscle-tendon

properties. However, given that changes in tendon stiffness and Young's modulus in COL were approximately 2.2-fold greater compared to PLA and large effect sizes for those two variables in COL, future studies may wish to investigate the effect of 30 g HC ingestion with high-intensity RT for at least 10 weeks with a larger sample size.

Chapter Eight

Synthesis of findings

8.1 Synthesis

The purpose of this chapter is to synthesize the PhD project that comprises three parts. Firstly, a theoretical interpretation of the major findings in experimental chapters will be provided to achieve the aims and objectives of the PhD thesis. Secondly, limitations related to both an acute and a chronic effect of HC intake on muscle-tendon unit in young population will be discussed. Thirdly, recommended directions for future research in use of HC with RE in a young athletic population to investigate changes in MTU will be provided.

8.2 Achievement of aims and objectives

The overarching aims of this PhD thesis were to investigate (i) the acute effect of HC intake with a single bout of RE on markers of collagen turnover; and (ii) the chronic effect of HC supplementation with 6 – 10 weeks' RT on changes in muscle-tendon properties, all in young, resistance-trained male and female athletes. These aims were achieved by completing the five following objectives:

1. To investigate a dose-response relationship between HC ingestion with an acute bout of RE and markers of collagen turnover in young, resistance-trained men. This objective was achieved in Chapter Three.

2. To investigate how resistance exercise, collagen ingestion and circulating oestrogen concentration are associated with markers of collagen turnover in a eumenorrheic, resistance-trained woman. This objective was achieved in Chapter Four.

3. To establish the effect of chronic HC intake with 10 weeks' in-season soccer training (incorporating bodyweight plyometric and strength exercises) on changes in muscle-tendon properties in female academy soccer players. This objective was achieved in Chapter Five.

4. To determine the effect of 10 weeks' pre-season soccer training with high-intensity resistance training supplemented with HC on changes in muscle-tendon properties in professional female soccer players. This objective was achieved in Chapter Six.

5. To ascertain the effect of six weeks' high-intensity resistance training with HC supplementation on changes in muscle-tendon properties in resistance-trained young men. This objective was achieved in Chapter Seven.

8.3 General discussion

Findings from Chapter Three demonstrated that ingesting 30 g HC prior to high-intensity RE further augmented a marker of collagen synthesis (serum PINP concentration) compared to 15 g and 0 g in healthy resistance-trained, young men. Further, AUCs of the key serum amino acids that constitute collagen (e.g. glycine and proline) were greater in 30 g HC compared to 15 g and 0 g HC. Previous studies investigated the effect of different doses of HC (15 – 20 g) with an acute bout of impact exercise on collagen synthesis in healthy, young men. Shaw et al. (2017) first reported that 15 g gelatine and 48 mg vitamin C with 5 min jump-rope exercise increased serum PINP concentration approximately two-fold more than 5 g gelatine intake in healthy, young men. Chapter Three suggests that 30 g HC may have shown an even greater serum PINP response in the study by Shaw et al. (2017). However, Aussieker et al. (2023) found that serum PINP concentration and muscle connective tissue protein synthesis were not changed after an acute bout of RE with 30 g HC ingestion in healthy young men and women. Exercise intensity may be a major factor that explains the discrepancy between the results of Aussieker et al. (2023) and those in Chapters Three and Four. In these chapters, the exercise intensity for the barbell back squat was 10-RM load, whereas in the study by Aussieker et al. (2023), 60% 1-RM load was used and, therefore, moderate-intensity RE may not have been a

sufficient stimulus to elicit changes in serum PINP concentration or skeletal muscle connective tissue protein synthesis. This is supported by changes in collagen synthesis following both acute and chronic RE. For example, 50 maximal concentric knee extensions increased serum PICP concentration in healthy young men (Virtanen et al., 1993) and 12 weeks' RT at 85 – 95% 1-RM increased serum PINP concentration in healthy young women (Mosti et al., 2014).

In eumenorrheic women, oestrogen affects exercise-induced collagen synthesis (Hansen et al., 2009a, Hansen et al., 2009c). Therefore, in Chapter Four, markers of collagen turnover were measured following 0 g or 30 g HC intake with an acute bout of RE when circulating oestrogen concentration was low and when it was high in a eumenorrheic, resistance-trained woman. Interestingly, peak serum PINP concentration was affected by HC dose and endogenous oestrogen level, whereby the highest PINP concentration and its AUC were observed following 30 g HC intake with RE when oestrogen concentration was low (i.e. the onset of menses).

Regarding collagen breakdown, plasma β-CTX concentrations were measured as an indirect marker of collagen breakdown. Regardless of HC dose, plasma β-CTX concentration decreased at 6-h post RE in resistance-trained, young individuals. Also, endogenous oestrogen concentration did not affect β-CTX in the resistance-trained woman. This decrease in plasma β-CTX may be explained by circadian rhythm, which may have outweighed the effect of HC and RE. Qvist et al. (2002) reported that resting serum β-CTX concentrations in men and women aged 25 – 73 years were affected by circadian rhythm, whereby plasma β-CTX concentrations were higher in the morning (~08:00) and began to decrease at 11:00 to 14:00. In the study by Hilkens et al. (2023), serum CTX-I concentrations started to decrease an hour after HC intake (~08:00), and the lowest serum CTX-I concentrations were at 4 h post-exercise (12:00) following 20 g HC intake with 5-min high-impact (jumping) exercise in healthy, young men. Alternatively, the

high-intensity RE in Chapters Three and Four may have inhibited collagen breakdown, leading to a reduction in serum β-CTX concentration. Unpublished data from our laboratory found that, after ingestion of HC at 08:00 and completion of 10-RM leg press RE at 09:00, plasma β-CTX concentrations decreased at 0.5 h post-RE and levelled off until 6 h post-RE (leg-press exercise at 10-RM load) regardless of HC dose (0 g, 15 g and 30 g) in resistance-trained middle-aged men (49 ± 8 years).

Despite the lack of evidence showing an acute effect of HC intake with RE on collagen synthesis, findings from Chapters Three and Four suggest that 30 g HC intake with RE elevated a marker of collagen synthesis over time compared to 15 g and 0 g HC, while a marker of collagen break down were not affected by HC dose in young individuals experienced in RE. Therefore, the main purpose of Chapter Five and Six was to investigate the longer-term effect of HC intake with RT on changes in MTU properties in female soccer players. In Chapter Five, female soccer players from a Football Association Women's Super League Under 21s squad completed 10 weeks' soccer training incorporating plyometric- and bodyweight resistance exercise with 30 g HC intake and 1,000 mg vitamin C during the latter half of the competitive season. Soccer training with 30 g HC increased PT tendon stiffness (+18.0 ± 12.2% *vs.* +5.1 ± 10.4%) and Young's modulus (+17.3 ± 11.9% *vs*. + 4.8 ± 10.3%) more than soccer training alone, although maximum strength, PT CSA and VL thickness did not change. It should be noted that plyometric training increases tendon stiffness (Foure et al., 2010) and an acute bout of small-sided soccer games increases whole body collagen synthesis (Bowtell et al., 2016). The latter is supported by changes in tendon properties induced by long-term habitual loading (Couppé et al., 2008). Further, as outlined in Chapter Three, together with mechanical loading in the form of

pitch-based, plyometric, and bodyweight resistance exercise, exogenous amino acid availability may lead to an increase in collagen synthesis, resulting in a greater increase in tendon stiffness and Young's modulus compared to soccer training alone.

It should be noted, however, that 10 weeks' soccer training with 30 g HC intake implemented in Chapter Five did not incorporate high-intensity lower-limb RE as this training and nutritional intervention was implemented during COVID-19 and the female soccer players were not permitted access to a gym. PT CSA did not increase following the 10-week soccer training with or without HC in Chapter Five. Moderate-intensity RE might not be a sufficient stimulus to increase patellar tendon CSA, as previous studies have found that high-intensity RT increased PT CSA (Kongsgaard et al., 2007, Seynnes et al., 2009, Dalgaard et al., 2019). Therefore, the aim of Chapter Six was to investigate whether implementing RE in soccer training with 30 g HC would change tendon properties especially tendon hypertrophy more than training alone. In Chapter Six, professional female soccer players from the first team squad of a Football Association Women's Championship Club completed 10 week's *pre-season* soccer training incorporating *high-intensity* resistance/plyometric exercise with 30 g HC. The intensity and frequency of RE were 75 – 90% 1RM once a week. The results showed that soccer training with 30 g HC induced greater changes in PT stiffness (+15.4 ± 3.1% *vs*. +4.6 ± 3.0) and Young's modulus (+14.2 ± 4.0 *vs.* +3.4 ± 2.8%) compared to soccer training alone, with no group × training interaction regarding VL thickness and PT CSA. Further, although the main effect of training on PT CSA was significant, the 1% change was marginal. Considering 9 – 12 weeks' high-intensity RE increased PT CSA by approximately 5% (Kongsgaard et al., 2007, Seynnes et al., 2009) and 12 weeks' high-intensity RE with 5 g HC intake increased AT CSA more than training alone (Jerger et al., 2022), the relatively low frequency of high-intensity RE in Chapter

Six may not have been sufficient to elicit a group × training interaction regarding PT CSA. Together, findings from Chapters Five and Six suggest that 30 g HC with soccer training further increased PT stiffness and Young's modulus, while the relatively low intensity (Chapter Five) and the relatively low frequency (Chapter Six) may explain the lack of group × training interaction regarding PT CSA.

In contrast to Chapters Five and Six, 5 g HC intake with 14 weeks' RT further increased AT and PT CSA compared to training alone but changes in tendon stiffness did not differ between COL and PLA groups in healthy young men (Jerger et al., 2022, Jerger et al., 2023). Therefore, the aim of the final experimental study (Chapter Seven) was to investigate the effect of 6 weeks' RT on changes in MTU properties in resistance-trained, young men. Changes in PT properties did not differ in both COL and PLA groups following 6-week RT period. It should be, however, noted that low sample sizes and short RT duration in Chapter Seven (caused by the COVID-19 pandemic) might have influenced the lack of differences in these variables. However, changes in PT stiffness (+10.1 ± 6.8%; d = 1.7 vs. +4.5 ± 5.0%; d = 0.9) and Young's modulus (+8.7 ± 7.4%; d = 1.0 vs. +3.3 ± 5.2%; d = 0.6) were approximately 2.2-fold greater (and with large effect sizes) in COL compared to PLA after the 6-week RT period. Thus, further research is needed to implement long-term RT at least 10 weeks with 30 g HC ingestion to investigate the effects of RT with 30 g HC ingestion on changes in PT properties in young men. Regarding muscle and tendon morphology, changes in all variables that represent VL size (muscle thickness, mean ACSA, PCSA, and muscle volume) and PT CSA were trivial in both COL and PLA. Mean PT CSA increased by 2% and 1% in COL and PLA, respectively, and this may be a consequence of the relatively short training period (six weeks) not being long enough to induce tendon hypertrophy. Findings from Chapter Seven are in contrast to those from the studies by

Jerger et al. (2022, 2023), in which 5 g HC with 14 weeks' RT increased AT and PT CSA (but not stiffness) more in COL than PLA. However, due to different methodologies between Chapter Seven and the studies by Jerger et al. (2022, 2023), such as the training duration (six *vs*. 14 weeks) and HC dose (30 g three twice a week *vs.* 5 g daily), a direct comparison is not possible.

8.4 Project limitations and recommendations for future research

The experimental chapters presented within this thesis provide novel information regarding the combined effect of RE and HC (both acute and chronic) on MTU adaptation in healthy young men and women. However, despite achieving the aims and objectives in each of the experimental chapters, there were several limitations contained and therefore, the aim of this section is to discuss those limitations and recommend areas of future research within this field.

Suggestions arising from Chapter Three

In Chapters Three and Four, indirect measures of collagen turnover following HC intake prior to an acute bout of RE were obtained by assessing serum PINP concentration (collagen synthesis) and plasma β-CTX concentration (collagen breakdown). However, those two measurements represent whole body collagen turnover and normally indicate bone turnover, as the rate of collagen turnover in bone is faster than in soft tissue (Koivula et al., 2012). Therefore, future studies should measure collagen synthesis directly via the fractional synthetic rate of collagen within both skeletal muscle and tendon, which requires harvesting muscle and tendon biopsies and an amino acid tracer. Although the studies presented in Chapters Three and Four received ethical approval to harvest muscle biopsies to assess markers of collagen synthesis, we were unfortunately unable to take biopsies due to our medical doctor being taken seriously ill and

no replacement found in time. Without a medical doctor responsible for screening our participants for suitability to provide a biopsy, no studies could proceed with muscle biopsies. Consequently, we decided to proceed with the less invasive method of measuring PINP and β-CTX concentration in the blood. Furthermore, it is possible that our observed changes in plasma β-CTX concentration in Chapters Three and Four may be have been affected by circadian rhythm (Qvist et al., 2002), rather than solely the exercise and/or HC supplement. Previous studies have found that matrix metalloproteinase-9 (MMP-9) activity (Koskinen et al., 2004) and interleukin (IL)-6 concentration (Langberg et al., 2002) in the peritendinous tissue around the AT were increased following a bout of exercise in healthy, young men, and that cyclic stretching increased IL-1 expression in human PT fibroblasts (Yang et al., 2005). As those proteolytic enzymes and inflammatory mediators are associated with adaptation of healthy tendon (Kjaer et al., 2013), if future studies aim to measure tendon adaptation in response to exercise and nutrition, MMPs and ILs should be measured.

Suggestions arising from Chapter Four

In Chapter Four, RE-induced collagen synthesis was affected by HC dose (30 g or 0 g) and endogenous oestrogen concentration in a resistance-trained, young woman. However, it should be noted that the study in Chapter Four was a case study (i.e. $n = 1$) and therefore, inter-individual variability in circulating oestrogen concentration and the responses to HC ingestion and RE were not accounted for. Thus, future studies should measure direct collagen turnover following HC intake with an acute bout of RE on different phases of menstrual cycle in a sample of eumenorrheic women. Alternatively, a comparison between OCP users and non-OCP users is recommended, as endogenous oestrogen level is regulated by ethinyloestradiol in the former.

Suggestions arising from Chapters Five and Six

In Chapter Five, all female academy soccer players except for one participant in COL did not use any form of hormonal contraceptive method, and it is unlikely that this had any bearing on the results from this study. In Chapter Six, two participants in COL were using an intrauterine device and had been doing so for 3.8 ± 4.5 years, while one participant was using an oral contractive pill (OCP) (Gedarel® 30/150) and had been doing so for six years. Three participants in PLA were using OCP (Rigevidon®, Lucette® and Microgynon® 30), and had been doing so for 7.3 ± 4.2 years. The remaining five participants were 'normally' menstruating women, as determined by responses to the 'low energy availability in females' questionnaire (Melin et al., 2014). Due to the relatively small squad of players available for recruitment, it was decided not to exclude volunteers on the basis of hormonal contraceptive use and, instead, to ensure there were equal numbers of users in each group. However, due to a relative high number of withdrawals due to COVID-19 (n = 3), injury (n = 3), and personal reasons (n = 1), this was not possible, and it remains to be seen if hormonal contraceptive use had any effect on the results from this study. Thus, future studies should attempt to recruit solely non-OCP users, although this is particularly challenging when recruiting participants from a relatively small cohort of elite athletes. Further, the relatively large number of withdrawals was also a limitation. Although the sample size was large enough to detect an effect of HC supplementation on changes in tendon stiffness and Young's modulus, it remains to be seen if a larger sample size might have enabled an effect of HC supplementation on tendon hypertrophy to be detected. One solution is to recruit from multiple squads at the same competitive level, however, training and recovery strategies tend to differ between football clubs, which adds an additional chal-

lenge in ensuring all participants are undergoing the same loading pattern. Regarding estimation of energy intake in Chapters Five, Six and Seven, dietary intake was recorded immediately before the start of the intervention which did not account for altered habitual dietary intake when nutritional supplements were given. Therefore, future studies should estimate the energy intake during the intervention.

Suggestions arising from Chapter Seven

In the final experimental study presented in Chapter Seven, the post-training tests for half of the participants were not performed as the University suddenly closed due to the COVID-19 pandemic with limited warning, leaving insufficient time to test the full cohort. Although the sample size was similar to that of Chapter Six, this combined with a much shorter training and supplementation duration probably led to this study being underpowered to detect an effect of HC supplementation on changes in tendon properties. Thus, future studies should ensure the duration of training and supplementation is longer than six weeks (e.g. minimum 10 weeks, as per Chapters Five and Six) and/or that the sample size is sufficient to compensate for a smaller effect size due to the shorter RT period.

8.5 Conclusion

There are relatively few studies that have investigated the effect of HC with RE on tendon properties in young men or women. The findings of this thesis are the first to demonstrate that 30 g HC had a greater effect on whole body collagen synthesis compared to 15 g and 0 g HC with an acute bout of RE in resistance-trained young men (Chapter Three). Furthermore, Chapter Four showed that high circulating oestrogen concentration (during the late follicular phase of the menstrual cycle) was associated with reduced collagen synthetic response to RE in a

eumenorrheic resistance-trained woman. However, 30 g HC ingestion prior to RE appeared to counter that inhibitory effect of oestrogen as collagen synthetic response was higher after ingesting 30 g HC with RE when circulating oestrogen concentration was low (i.e. at the onset of menses) compared to RE alone. This finding led to the question of whether long-term 30 g HC ingestion with exercise could confer greater changes in tendon properties in young men and women. In Chapter Five, female academy soccer players completed 10 weeks' soccer training towards the end of the competitive season, which incorporated plyometric/bodyweight resistance training with 30 g HC. In Chapter Six, professional female soccer players completed 10 weeks' soccer training during the pre-season period, which incorporated *high-intensity* (externally-loaded) resistance and plyometric training with 30 g HC. The findings from Chapters Five and Six suggest that 30 g HC with soccer training further increased PT tendon stiffness and Young's modulus, regardless of athlete status (i.e. academy or professional level) and whether the intervention occurred during pre-season or in-season. Although the higher intensity of RE in Chapter Six probably led to tendon hypertrophy being observed in Chapter Six and not Chapter Five, the low frequency of high-intensity RE in Chapter Six may have limited the effect of HC to further augment tendon hypertrophy. Consequently, Chapter Seven included a relatively higher frequency of high-intensity RE, albeit over a shorter training duration. The latter factor probably limited the ability of this study to observe a significant group × time interaction effect regarding tendon morphological and mechanical properties, although the effect sizes for changes in tendon stiffness and modulus were greater in COL than PLA. Thus, over a longer period of time, these changes may have become statistically significant. Future studies should therefore ensure the duration of training and supplementation is longer than six weeks (e.g. minimum 10 weeks, as per Chapters Five and Six) and/or that the sample size is sufficient to compensate for a smaller effect size due to the shorter RT period.

Chapter Nine

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Chapter Ten

Appendices

Appendices

APPENDIX 1 – Participant information sheet for the experimental studies in Chapter Three and Four

Participant Information Sheet

Faculty of Science

LJMU's Research Ethics Committee Approval Reference: 18/SPS/059

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

Title of Study:

The dose-response of vitamin C-enriched collagen on markers of collagen synthesis in healthy young men and women following resistance exercise

Name and Contact Details of the Principal Investigator:

Mr. Joonsung Lee

e: J.Lee1@2017.ljmu.ac.uk

Name and Contact Details of Supervisor:

Dr. Robert Erskine

t: +44 (0)151 904 6256 e: R.M.Erskine@ljmu.ac.uk

You are being invited to take part in a research study. Before you decide it is important that you understand why the research is being done and what it involves. Please take time to read the following information. Ask us if there is anything that is not clear or if you would like more information. Take time to decide if you want to take part or not.

What is the purpose of the study?

Tendons, which attaches muscle to bone play an important role in body movement by transmitting the force produced by muscles and consist of mainly different types of collagens. Young women are at a greater risk of sustaining sports injuries in collagen-rich tissues, such as ligament and tendon, than young men and it has been reported that the incidence of anterior cruciate ligament injuries is 2 – 6 times higher in women. Therefore, maintaining tendon health is important regarding athletic performance and daily of life. Recent indirect evidence suggests that collagen supplementation with vitamin C might augment collagen synthesis following exercise, thus potentially improving tendon function and health. However, the optimal dose of collagen for maximal tendon collagen synthesis might differ between men and women as a reduced response of exercise-induced tendon collagen synthesis in young women compared to men has been found probably due to different level of sex hormone (oestrogen). Our aim is to investigate the optimal dose of collagen and vitamin C supplementation for maximal tendon collagen synthesis in healthy young and men and women.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form – if applicable. You can withdraw at any time by informing the investigators without giving a reason and without it affecting your rights/any future treatment/service you receive.

May I be excluded from the study?

Yes. You can be excluded from the study at any time point if you do not adhere to the study conditions.

Why have I been invited to participate?

You are likely to be eligible for this study if you fulfil the following criteria.

Be healthy men or women:

- Age 18-39 years
- No history of patellar tendon injuries
- No history of lower limb musculoskeletal injuries in the past 6 months
- Currently performing a structured resistance training programme (including the lower limbs) (at least 2 days per week)
- Resistance training experienced (barbell back squat) for at least 6 months
- Nullipara (a woman who has never given birth)
- Use of oral contraceptive pills and non-users (for young women) for at least 6 months
- Non-smokers (including e-cigarettes)

You MUST NOT take part in this study if:

- You are younger than 18 or older than 39 years
- You have an irregular menstrual cycle (for young women)
- Vegans and vegetarians (collagen is derived from mammals and fish)
- Smokers (including e-cigarettes)

What will happen to me if I take part?

If you are a young man or woman, who uses oral contraceptive pills (OCP) or not, you will be required to visit the laboratories located in Liverpool John Moores University over different time periods. Please see Table 1.

Table 2. Required number of visits.

Participant	Number of visits
Young men	4 (weekly basis)
Young women (OCP users)	3 (weekly basis)
Young women (non-OCP users)	5 (over 2 months)

On the first visit, there will be a familiarization session and three experimental tests. After the familiarization session, **young men** will be asked to perform 3 different trials including ingestion of different doses of collagen (0 g, 15 g, and 30 g) with vitamin C (50 mg) and performing barbell back squat (See Figure 1). **For women (OCP users)**, the experimental design is the same except for ingestion of different doses of collagen (0 g and 30 g) (See Figure 1).

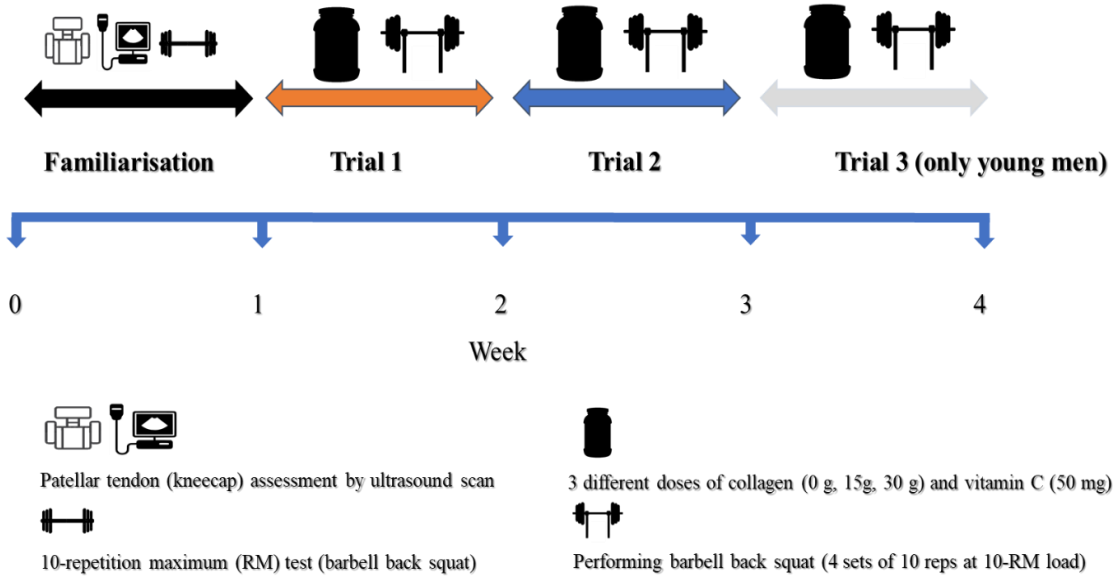
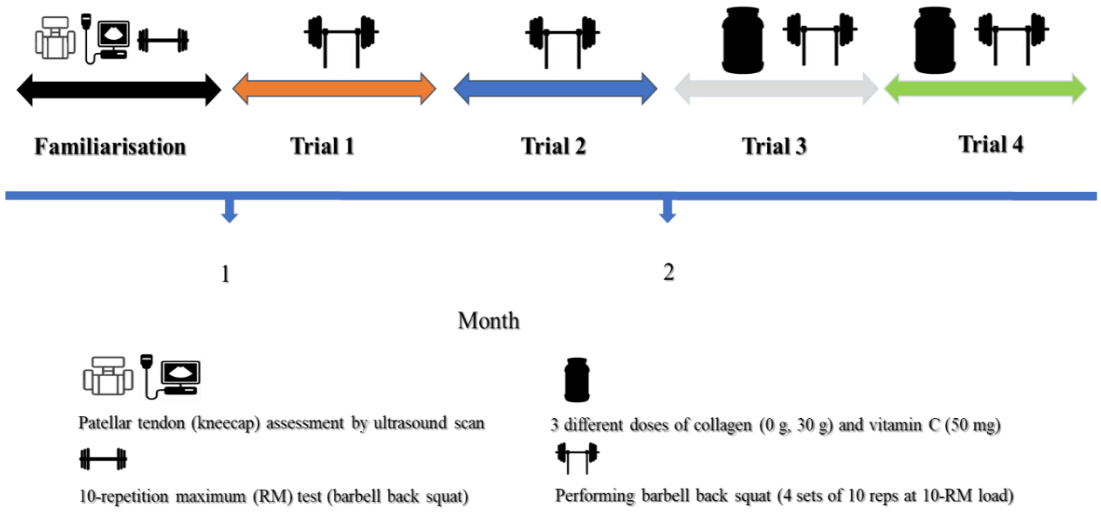


Figure 7. Study overview for young men and women (OCP users).

For young women (non-OCP users), you will be asked to visit the laboratories two different time points in a monthly basis; One is before starting the menstrual cycle and the other is 14th day of the menstrual cycle (peak oestrogen level) in order to investigate how different levels of oestrogen affect tendon collagen synthesis. Thus, you will perform the barbell



back squat and either the calorie-matched drink (30 g of maltodextrin with 50 mg of vitamin C) or collagen (30 g) drink with vitamin C (50 mg) will be provided in the main trials in a random order (See Figure 2).

Figure 8. Study overview for women (non-OCP users).

24 hours before visiting the laboratory for the required number of visits, you will be asked to fast 12-hour overnight except for water and refrain from strenuous physical exercises, consumption of alcohol and caffeine.

Familiarization session (80 minutes)

1) Tendon properties assessment (40 – 50 minutes)

: You will be seated on an isokinetic dynamometer (specialized equipment recording muscle force around a joint) and the knee joint will be set at 90 ° knee flexion, in order to measure the length of the patellar tendon (just below the knee cap) by using an ultrasound probe. The probe will then be placed on the skin over the points associated with 25, 50 and 75% tendon length, and the scans will be recorded to measure tendon cross sectional area at these points. During a ramped isometric maximum voluntary contraction (MVC, which is a contraction with a gradual increase in force to a full MVC) the ultrasound probe will be placed longitudinally over the tendon and the tendon elongation will be recorded. These measurements will subsequently be used to assess differences in tendon size and stiffness. All measurements will be performed on the right leg of the participants.

2) Familiarization and 10 RM test (30 minutes)

: A strength and conditioning professional will instruct a proper technique of barbell back squat. In order to prescribe individualized training load for the main testing session, you will perform a 10-repetition maximum (10 RM) test for and barbell back squat. 10 RM load will be determined within four trials with rest periods of 3 - 5 minutes. You will start with a warm-up session, consisting of lower limb stretching and performing and barbell back squat (5 to 10 repetitions with a light-to-moderate load). An initial weight that is within the participant's perceived capacity (50%– 70% of maximal capacity) will be selected and resistance will be progressively increased by 2.5-20 kg until the participant cannot complete more than 10 repetitions. The final weight lifted successfully with the proper technique will be as the 10-RM load.

3) Filling in a food and drink diary (8 minutes per day for three days)

: In order to maintain similar eating patterns over experimental periods, you will be asked to fill in a drink and food diary at your home for 3 days. You will need to fill in the dairy on Thursday, Friday, and Saturday, which will represent your weekly eating patterns. The diary will be provided during the first visit and you will be shown how to complete it correctly.

Main testing sessions consisting of different trials (7 hours)

1) Rest condition

: You will be asked to visit the laboratory 1 h before performing the resistance exercise. Upon arrival to the laboratory, 10 ml of resting blood sample will be taken from a superficial fore-arm vein. Then, you will ingest one of different doses of collagen (0 g, 15 g, and 30 g) with vitamin C (50 mg) in 400 ml of water. Two more 5 ml blood samples will be drawn at 0.5 h and 1 h after consumption of the supplement.

2) Performing resistance exercise

: After warming-up targeting the lower limb, you will perform barbell back squat (4 sets of 10 RM interspersed with 2-minute rest periods) and one blood sample will be taken immediately after the exercises.

3) Post-exercise condition

: After completion of the resistance exercises, four blood samples will be taken at 1 h-, 2 h-, 4 h-, and 6 h-post exercise. At the time point of '6-h post exercise', 10 ml of blood will be

drawn. **As you will rest in the laboratory for 6 hours, it is suggested that bring a laptop or books to spend the time in the laboratory.**

In each visit, eight blood samples will be taken except for the familiarization session and please see Figure 3, which illustrates the time course of samplings.

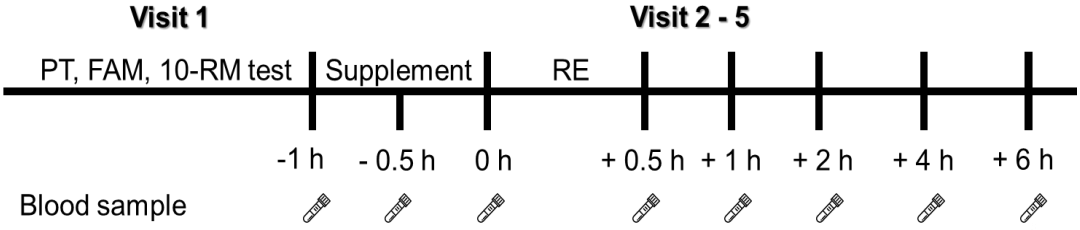


Figure 3. Schematic timeline of study. PT, patella tendon assessment; FAM, familiarization; 10-RM test, 10-repetition maximum test; RE, resistance exercise.

Note. As two different blood analyses will be used, at the time point '-1 h' and '+ 6 h' 10 ml of blood will be drawn and stored in two different tubes.

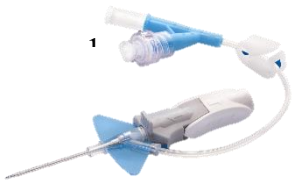
Vitamin C-enriched collagen supplement

: Hydrolyzed collagen is produced from collagen found in the bones, skin, and connective tissue of animals. On different occasions, you will be asked to ingest a different dose of hydrolyzed collagen (0 g, 15 g, or 30 g) each with 50 mg of vitamin C in 400 ml of water. Each solution will be mixed with maltodextrin (a product made from starch, usually used as a food addictive) to ensure each solution contains the same number of calories. Lastly, a small

amount of non-calorie sweetener will be added. The total kilogram calorie of vitamin C-enriched collagen supplementation is 122 Kcal (3.6 Kcal for 1 g of hydrolyzed collagen and 4 Kcal for 1 g of maltodextrin).

Blood sample

: 5 ml of blood will be drawn from a superficial forearm vein at the following time points: at rest, 0.5 h and 1 hour after consumption of the supplement, immediately after the exercise, 1h, 2h, 4h and 6 h-post exercise. A total of eight blood samples will be taken in each visit except for the familiarization session. Cannulation, a small plastic tube (1.) inserted into the



vein to allow for multiple blood sampling and thereby reducing the need for repeated needle insertions, shall be performed at the start of the day and removed at the end. This is a routine procedure and should result in no more than a small amount of discomfort on insertion of the needle.

Are there any risks/benefits involved?

Benefits

: In participating in this study no direct benefit to you will occur but it is hoped that if the optimal dose of collagen will be found, this result will be linked to a future study, which will investigate whether long-term resistance training with collagen supplementation affects muscle

and tendon properties compared to resistance training alone. Whilst participating you may experience and learn about ideas around how tendons play an important role during resistance exercise. Also, taking part in this study will help us understand how we can improve athletic performance and health, and reduce injury risk in young people, as tendons also respond to exercise and nutrition.

Possible risks

: The most obvious risks to you involve blood sampling. If at any point during the protocol you feel uncomfortable or unable to continue, testing will be ceased immediately.

Risk of muscle pain caused by resistance exercise

: Proper techniques of the resistance exercise will be provided in a familiarization session. A standard warm up, sufficient rest periods and controlled range of motion will be provided. The exercises will be immediately ceased if participants feel uncomfortable. As you are already resistance trained and experienced with barbell back squat exercises, we expect you to experience minimal muscle soreness in the days following the resistance exercise.

Blood sampling

: Blood samples will be taken on several occasions. You will feel a sharp pain when the needle is inserted but this will be short-lived. The researchers are also experienced in this technique so the pain experienced will be minimal. You may also develop a small bruise on your arm, which can be prevented by applying pressure on the arm when the cannula has been taken out (the researcher will remind/instruct you to do this). Also, a rounded band will be attached at the site of blood sampling to prevent any contamination after completion of blood

sampling. The potential risks are small and may include the following: infection (a slight risk any time the skin is broken), bleeding of the site, hematoma (blood collected beneath the skin that usually disappear over a few days but under very rare conditions could require surgery), and bruising of the area.

What will happen to the data provided and how will my taking part in this project be kept confidential?

Throughout the study your personal information will be kept entirely confidential. Instead of your name, your data will be given an identification code, so that you will not be identifiable. Your personal data will be destroyed four years after the testing is complete. The results of this study are expected to be published in a scientific journal, but names of participants will not be published.

What will happen to the results of the research project?

The results of this study are expected to be published in a scientific journal, but names of participants will never be published.

Who is organising the study?

This study is organised by Liverpool John Moores University

Who has reviewed this study?

This study has been reviewed by, and received ethics clearance through, the Liverpool John Moores University Research Ethics Committee (18/SPS/059).

What if something goes wrong?

If you have a concern about any aspect of this study, please contact the relevant investigator who will do their best to answer your query. The researcher should acknowledge your concern within 10 working days and give you an indication of how they intend to deal with it. If you wish to make a complaint, please contact the chair of the Liverpool John Moores University Research Ethics Committee (researchethics@ljmu.ac.uk) and your communication will be re-directed to an independent person as appropriate.

Data Protection Notice

The data controller for this study will be Liverpool John Moores University (LJMU). The LJMU Data Protection Office provides oversight of LJMU activities involving the processing of personal data, and can be contacted at secretariat@ljmu.ac.uk. This means that we are responsible for looking after your information and using it properly. LJMU's Data Protection Officer can also be contacted at secretariat@ljmu.ac.uk**.** The University will process your personal data for the purpose of research. Research is a task that we perform in the public interest.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. You can find out more about how we use your information by contacting secretariat@ljmu.ac.uk.

If you are concerned about how your personal data is being processed, please contact LJMU in the first instance at secretariat@ljmu.ac.uk. If you remain unsatisfied, you may wish to contact the Information Commissioner's Office (ICO). Contact details, and details of data

subject rights, are available on the ICO website at: https://ico.org.uk/for-organisations/data-protection-reform/overview-of-the-gdpr/individuals-rights/

This study has received ethical approval from LJMU's Research Ethics Committee *(*18/SPS/059*).*

Contact for further information

Joonsung Lee (Researcher)
Research Institute for Sport & Exercise Sciences
Liverpool John Moores University
Byrom Street
Liverpool
L3 2AF
Tel
E-mail: J.Lee1@2017.ljmu.ac.uk

Dr Rob Erskine (Academic Supervisor)
Research Institute for Sport & Exercise Sciences
Liverpool John Moores University
Byrom Street
Liverpool
L3 2AF
Tel: +44 (0)151 904 62
E-mail: R.M.Erskine@ljmu.ac.uk

Thank you for reading this information sheet and for considering to take part in this study.

Note: A copy of the participant information sheet should be retained by the participant with a copy of the signed consent form.

PIS Date: 20/10/18 PIS Version No: 0.1

APPENDIX 2 – Consent form for the experimental studies in Chapter Three and Four

Informed Consent Form

Study Title:	*The dose-response of vitamin C-enriched collagen on markers of collagen synthesis in healthy young and older men and women following resistance exercise*
LJMU Ethics code:	18/SPS/059
Name of Principal Investigator:	Joonsung Lee, Chris Nulty
Faculty & School:	School of Sport and Exercise Sciences
Contact details:	J.Lee1@2017.ljmu.ac.uk

1. I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and that this will not affect my legal rights. ☐

3. I understand that any personal information collected during the study will be anonymised and remain confidential. ☐

4. I consent to my blood samples being stored securely at LJMU for the duration of this ethically approved research and used for the research purposes outlined in the Participant Information Sheet. ☐

5. I give the consent for my blood samples to be used for DNA analysis or other genetic testing as described in the Participant Information Sheet. ☐

6. I agree for my blood samples to be stored for the research purposes this ethically approved research and future projects under the regulation of the Human Tissue Act. ☐

7. I agree to take part in the above study. ☐

Name of Participant Date Signature

Name of Researcher Date Signature

Name of Person taking consent Date Signature

(If different from researcher)

APPENDIX 3 – Participant information sheet for experimental studies in Chapter Five and Six

Participant Information Sheet

Faculty of Science

LJMU's Research Ethics Committee Approval Reference: 19/SPS/054

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

Title of Study:

The Effect of Resistance Training with Vitamin C-Enriched Collagen Supplementation on Changes in Muscle and Tendon Properties in Young Healthy Women

Name and Contact Details of the Principal Investigator:

Mr. Joonsung Lee

e: J.Lee1@2017.ljmu.ac.uk

Name and Contact Details of Supervisor:

Dr. Robert Erskine

t: +44 (0)151 904 6256 e: R.M.Erskine@ljmu.ac.uk

You are being invited to take part in a research study. Before you decide it is important that you understand why the research is being done and what it involves. Please take time to

read the following information. Ask us if there is anything that is not clear or if you would like more information. Take time to decide if you want to take part or not.

Information of Covid-19 related

Due to the outbreak of this pandemic, this study will be always performed with the university guidance of Covid-19 as safety of both participants and researchers are priority. Briefly, you will be asked to

- Wear protective equipment (a face covering, protective goggles and disposable apron) all the times when you are inside such as a laboratory
- Measure your body temperature
- Tell researchers if you have symptoms of Covid-19 (a high temperature, new and continuous cough loss and/or change to your sense of smell or taste) BEFORE coming to the university.

Those will be applied to researchers as well. Detailed information is on the LJMU website (https://www.ljmu.ac.uk/microsites/moving-forward/information-for-students).

What is the purpose of the study?

Tendons, which attach muscle to bone play an important role in body movement by transmitting the force produced by skeletal muscles and collagen is the most abundant protein in tendons. Like skeletal muscles, tendons adapt to resistance exercise and nutrition. Recent evidence suggests that collagen supplementation with vitamin C increases collagen synthesis following exercise. Young women are at a greater risk of sustaining sports injuries in collagen-rich tissues, such as ligament and tendon, than young men and the incidence of anterior cruciate ligament injuries is 2 to 5 times higher in women due to different level of circulating oestrogen in the body between men and women. Therefore, resistance exercise with collagen supplementation might have a synergetic effect on tendons, which is linked

to better athletic performance and quality of life. However, the combined effect of resistance exercise and collagen supplementation over a period of time is not known. Our aim is to investigate how 10-week resistance training with or without collagen supplementation have the synergetic effect on tendon health in young women.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form – if applicable. You can withdraw at any time by informing the investigators without giving a reason and without it affecting your rights/any future treatment/service you receive.

May I be excluded from the study?

Yes. You can be excluded from the study at any time point if you do not adhere to the study conditions.

Why have I been invited to participate?

You are likely to be eligible for this study if you fulfil the following criteria.

- Healthy young woman
- Age 16-39 years
- No history of patellar and tendon injuries in the past 6 months
- No history of lower limb musculoskeletal injuries in the past 6 months
- Non-smokers (including e-cigarettes)
- Free from cardiovascular and metabolic diseases

- Nullipara (a woman who has never given birth)

You MUST NOT take part in this study if:

- You are younger than 16 or older than 39 years
- Vegan and vegetarian (collagen is derived from mammals)
- You consume any purported muscle building (e.g. protein/amino acids powder) or antioxidant (e.g. vitamin C) supplements
- You are a smoker (including e-cigarettes)
- Having a history or family history of kidney stones
- Having a meat allergy
- BMI over 30 kg/m^2

What will happen to me if I take part?

You will be asked to visit laboratories located in Liverpool John Moores University for two separate visits (please see Figure 1).

Baseline Test	**Training Intervention**	**Post-training Test**
1st Visit		2nd Visit
1 day	10 weeks	1 day
(Week 1)	(3d/wk)	(Week 12)
% Bodyfat	Regular Resistance Training with Collagen Supplementation	The Same as 'Baseline Testing'
MTU Measurement		
H:Q ratio test		

Figure 1. An overview of the study intervention periods and measurements. MTU, muscle-tendon unit assessment; %Bodyfat, percentage of body fat measurement; H:Q ratio, the hamstring-to-quadriceps muscle strength test.

Baseline Test (1 hour)

The baseline testing session comprises three tests (muscle and tendon measurements and body fat percentage, all assessed via ultrasound), and the completion of three questionnaires. None of these assessments will be invasive or painful in any way.

1) Filling in questionnaires

- The Readness to Exercise Screening questionnaire (to screen your health statues and injury history)

- The Physical Activity Readiness Questionnaire (to measure your physical activity level)

- The low energy availability in females questionnaire (to identifiy whether a woman is at the risk of impaired bone health and/or having an eating disorder)

2) Muscle-tendon assessment

You will be required to sit on an isokinetic dynamometer (specialized eqiupment for measuring maximal strength) and perform a few maximal muscle contractions such as knee extension (kicking forward) and flexion (pulling back). While you perform the aforementioned muscle contractions, an ultrasound probe will be placed below your kneecap to measure patellar and tendon properties and electromyography electrodes will be attached to your back thigh in order to measure the electical activity in your muscles during muscle

contractions. Only the dominant leg will be assessed. Also, you'll be asked to lie on a massage bed in a rested condition. The ultrasound probe will be placed on your upper thigh muslce to assess muscle architecture, which is associated with force production.

Isokinetic Dynamometer

Ultrasound Machine

Electromyography Electrode

3) Percantage of body fat measurement

By using the ultrasound machine, fat thichkness will be measured at 2 differnent sites for men (the belly and fornt thigh) and women (the belly and calf). This estimates the total percentage of body fat on your body.

4) The hamstring-to-quadriceps muscle strength test

This test has been used to detect muscle imbalance, and if the strength of the quadriceps muscle is much greater than the hamstrings, this increases the risk of hamstring strain injury. You will be required to sit on the isokinetic dynamometer and perform a few maximum muscle contractions: concentric knee extension (kicking your leg out) and eccentric knee

flexion (this type of muscle contraction is similar to the Nordic hamstring exercise or lowering the weight during the deadlift).

5) Filling in the food and drink diary

End of baseline test, a food and drink diary will be provided in order to measure your energy intake and assess relationship between energy intake and body fat percentage. You will be asked to record the diary for three days (Thursday, Friday and Saturday) at the start and end of the day. You will be instructed how to complete the diary by the researcher and detailed instruction is on the first page of the diary.

Resistance training with supplement intake

You will perform regular resistance training and the researcher will provide you with a supplement drink (containing collagen or maltodextrin) immediately before each training session. You will be assigned to one of two groups: collagen or control. The collagen group will be given a drink containing hydrolyzed collagen, while the control group's drink will contain maltodextrin and no collagen. **This is a blinded study** (i.e. you will not know which nutritional supplementation you will be given) but you will receive individual verbal debriefing at the end of the study about which group you were in.

Nutritional Supplementation

All nutritional supplements in this study have been certified by Informed-Sport, which means all the supplements have been tested for a wide variety of WADA banned substances and thus they are safer for athletes. During the training period, you will be given a supplement to be consumed with each resistance training session. The supplements used in this

study will comprise either **30 g hydrolysed collagen** (HC, derived from bovine skin, so vegans, vegetarians and anyone who suffers from a meat allergy will not be able to participate in this study) or **49.3 g maltodextrin** (a commonly used high-glycaemic food additive derived from starch in potato, rice, and corn), with the latter being consumed by the control group. Both HC and maltodextrin supplements will each be mixed in 300 mL water. Also, **a 500 mg chewable vitamin C tablet** will be given to both groups to consume with their respective supplement. Both the HC and maltodextrin doses are considered to be low, with 30 g HC constituting 20-25% of the recommended daily intake of protein and 49.3 g maltodextrin comprising 17% of the recommended daily amount of carbohydrate in adults. The vitamin C dose is low and comes with no known adverse risks or side-effects. Although there is no documented evidence of a deleterious effect from the ingestion of collagen, a feeling of heaviness in the stomach, mild diarrhoea or temporary rashes might occur. If any of these transpire, please inform the researchers immediately. The low dose of maltodextrin carries minimal risk or adverse effects, although the high-glycaemic index of this food additive can cause an increase in blood sugar levels, so diabetics should be aware of this. However, due to the exercise-induced transportation of blood glucose into the muscle, the insulin response to the increase in blood glucose following maltodextrin ingestion is likely to be blunted. Much larger doses of maltodextrin than that used in this study have been associated with bloating, flatulence and in severe cases, diarrhoea. However, this risk is very low with the dose used in this study.

Post-training tests (1 hour)

All testing procedures are the same as ‘Baseline Test’.

Are there any risks/benefits involved?

Benefits

In participating in this study, it is expected that your strength and muscle size will be increased and your tendon health improved. Also, if a strength imbalance between front (quadriceps) and back (hamstrings) muscles is identified via the hamstring-to-quadriceps muscle strength test, the researcher will inform the fitness coaches of this result in order to minimize hamstring strain injuries.

Possible risks

Risk of muscle pain caused by resistance exercise

Injuries during resistance training are unlikely to occur as training will be supervised by the strength and conditioning coaches and complete a well-structured warm-up beforehand.

What will happen to the data provided and how will my taking part in this project be kept confidential?

The information you provide as part of the study is the **study data**. Any study data from which you can be identified (e.g. from identifiers such as your name, date of birth, audio recording etc.), is known as **personal data**. This includes more sensitive categories of personal data (**sensitive data**) such as your race; ethnic origin; politics; religion; trade union membership; genetics; biometrics (where used for ID purposes); health; sex life; or sexual orientation. Throughout the study your personal information will be kept entirely confidential. Instead of your name, your data will be given an identification code, so that you will

not be identifiable. Your personal data will be destroyed four years after the testing is complete. The results of this study are expected to be published in a scientific journal, but names of participants will not be published. In addition, responsible members of Liverpool John Moores University may be given access to personal data for monitoring and/or audit of the study to ensure that the study is complying with applicable regulations.

What will happen to the results of the research project?

The results of this study are expected to be published in a scientific journal, but names of participants will never be published.

Who is organising the study?

This study is organised by Liverpool John Moores University

Who has reviewed this study?

This study has been reviewed by, and received ethics clearance through, the Liverpool John Moores University Research Ethics Committee (19/SPS/054).

What if something goes wrong?

If you have a concern about any aspect of this study, please contact the relevant investigator who will do their best to answer your query. The researcher should acknowledge your concern within 10 working days and give you an indication of how they intend to deal with it. If you wish to make a complaint, please contact the chair of the Liverpool John Moores University Research Ethics Committee (researchethics@ljmu.ac.uk) and your communication will be re-directed to an independent person as appropriate.

Data Protection Notice

The data controller for this study will be Liverpool John Moores University (LJMU). The LJMU Data Protection Office provides oversight of LJMU activities involving the processing of personal data, and can be contacted at secretariat@ljmu.ac.uk. This means that we are responsible for looking after your information and using it properly. LJMU's Data Protection Officer can also be contacted at secretariat@ljmu.ac.uk**.** The University will process your personal data for the purpose of research. Research is a task that we perform in the public interest. Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. You can find out more about how we use your information by contacting secretariat@ljmu.ac.uk. If you are concerned about how your personal data is being processed, please contact LJMU in the first instance at secretariat@ljmu.ac.uk. If you remain unsatisfied, you may wish to contact the Information Commissioner's Office (ICO). Contact details, and details of data subject rights, are available on the ICO website at: https://ico.org.uk/for-organisations/data-protection-reform/overview-of-the-gdpr/individuals-rights/

This study has received ethical approval from LJMU's Research Ethics Committee (19/SPS/054).

Contact for further information

Joonsung Lee (Researcher)
Research Institute for Sport & Exercise

Dr Rob Erskine (Academic Supervisor)
Research Institute for Sport & Exercise

Sciences

Liverpool John Moores University

Byrom Street

Liverpool

L3 2AF

Tel

E-mail: J.Lee1@2017.ljmu.ac.uk

Sciences

Liverpool John Moores University

Byrom Street

Liverpool

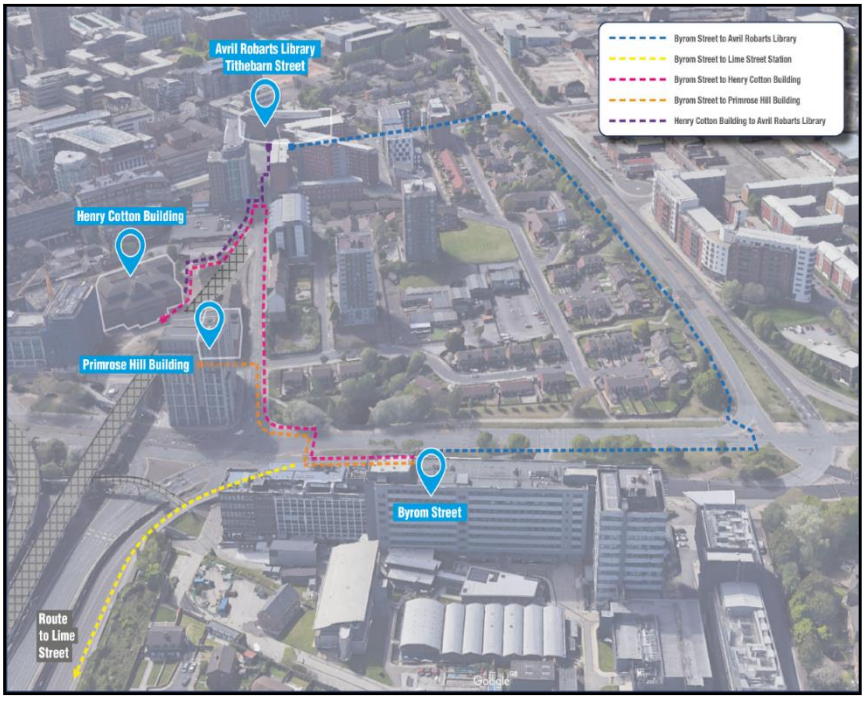
L3 2AF

Tel: +44 (0)151 904 62

E-mail: R.M.Erskine@ljmu.ac.uk

Location

Address: Tom Reilly Building, Byrom Street, Liverpool L3 5AF



Thank you for reading this information sheet and for considering to take part in this study.

Note: A copy of the participant information sheet should be retained by the participant with a copy of the signed consent form.

PIS Date: 11/12/20 PIS Version No: 0.3

Informed Consent Form

Study Title:	The effect of resistance training with vitamin C-enriched collagen supplementation on changes in muscle and tendon properties in young healthy men and women
LJMU Ethics code:	19/SPS/054
Name of Principal Investigator:	Joonsung Lee
Faculty & School:	School of Sport and Exercise Sciences
Contact details:	J.Lee1@2017.ljmu.ac.uk

Please initial

1. I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and that this will not affect my legal rights. ☐
3. I understand that I am consenting to be part of this study, which involves questionnaires and for my data to be used as described in the information sheet provided. ☐
4. I understand that any personal information collected during the study will be anonymised and remain confidential. ☐
5. I agree to take part in the above study. ☐

Name of Participant Date Signature

Name of Researcher Date Signature

Name of Person taking consent Date Signature

(If different from researcher)

APPENDIX 5 – Participant information sheet for experimental study in Chapter Seven

Participant Information Sheet

Faculty of Science

LJMU's Research Ethics Committee Approval

Reference: 19/SPS/054

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

Title of Study:

The Effect of Resistance Training with Vitamin C-Enriched Collagen Supplementation on Changes in Muscle and Tendon Properties in Healthy Young Men

Name and Contact Details of the Principal Investigator:

Mr. Joonsung Lee

e: J.Lee1@2017.ljmu.ac.uk

Name and Contact Details of Supervisor:

Dr. Robert Erskine

t: +44 (0)151 904 6256 e: R.M.Erskine@ljmu.ac.uk

You are being invited to take part in a research study. Before you decide it is important that you understand why the research is being done and what it involves. Please take time to read the following information. Ask us if there is anything that is not clear or if you would like more information. Take time to decide if you want to take part or not.

Information of Covid-19 related

Due to the outbreak of this pandemic, this study will be always performed with the university guidance of Covid-19 as safety of both participants and researchers are priority. Briefly, you will be asked to

- Wear protective equipment (a face covering, protective goggles and disposable apron) all the times when you are inside such as a laboratory
- Measure your body temperature
- Tell researchers if you have symptoms of Covid-19 (a high temperature, new and continuous cough loss and/or change to your sense of smell or taste) BEFORE coming to the university of gym.

Those will be applied to researchers as well. Detailed information is on the LJMU website (https://www.ljmu.ac.uk/microsites/moving-forward/information-for-students).

What is the purpose of the study?

Tendons, which attach muscle to bone play an important role in body movement by transmitting the force produced by skeletal muscles and collagen is abundant in tendons. Like skeletal muscles, tendons adapt to resistance exercise and nutrition. Recent evidence suggests that collagen supplementation with vitamin C increases collagen synthesis following

exercise. Therefore, Resistance exercise with collagen supplementation might have a synergetic effect on tendons, which is linked to better athletic performance and quality of life for both men. However, the combined effect of resistance exercise and collagen supplementation over a period of time is not known. Our aim is to investigate how 6-week resistance training with or without collagen supplementation have the synergetic effect on tendon health in young men.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form – if applicable. You can withdraw at any time by informing the investigators without giving a reason and without it affecting your rights/any future treatment/service you receive.

May I be excluded from the study?

Yes. You can be excluded from the study at any time point if you do not adhere to the study conditions.

Why have I been invited to participate?

You are likely to be eligible for this study if you fulfil the following criteria.

- Healthy young man
- Age 18-39 years
- No history of patellar tendon injuries in the past 6 months
- No history of lower limb musculoskeletal injuries in the past 6 months
- Non-smokers (including e-cigarettes)
- Free from cardiovascular and metabolic diseases

You MUST NOT take part in this study if:

- You are younger than 18 or older than 39 years
- Vegan and vegetarian (collagen is derived from mammals and fish)
- You consume any muscle building (e.g. protein/amino acids powder) or antioxidant (e.g. vitamin C) supplements
- You are a smoker (including e-cigarettes)
- Having a history or family history of kidney stones
- Having a meat allergy
- BMI over 30 kg/m^2

What will happen to me if I take part?

You will be asked to visit laboratories located in Liverpool John Moores University for two separate visits (please see Figure 1).

Baseline Test	**Training Intervention**	**Post-training Test**
1st Visit		2nd Visit
1 day	6 weeks	1 day
(Week 1)	(2d/wk)	(Week 7)
% Bodyfat	Regular Resistance Training with Collagen Supplementation	The Same as 'Baseline Testing'
MTU Measurement		
H:Q ratio test		

Figure 1. An overview of the study intervention periods and measurements. MTU, muscle-tendon unit assessment; %Bodyfat, percentage of body fat measurement; H:Q ratio, the hamstring-to-quadriceps muscle strength test.

Baseline Test

The baseline testing session comprises three tests (muscle and tendon measurements and body fat percentage, all assessed via ultrasound), and the completion of three questionnaires. None of these assessments will be invasive or painful in any way.

1) Filling in questionnaires

- The Readness to Exercise Screening questionnaire (to screen your health statues and injury history)

- The Physical Activity Readiness Questionnaire (to measure your physical activity level)

2) Muscle-tendon assessment

You will be required to sit on an isokinetic dynamometer (specialized eqiupment for measuring maximal strength) and perform a few maximal muscle contractions such as knee extension (kicking forward) and flexion (pulling back). While you perform the aforementioned muscle contractions, an ultrasound probe will be placed below your kneecap to measure patellar and tendon properties and electromyography electrodes will be attached to your thigh in order to measure the electical activity in your muscles during muscle contractions. Only the dominant leg will be assessed. Also, you will be asked to lie on a massage bed in a rested condition. The ultrasound probe will be placed on your upper thigh muslce to assess muscle architecture, which is associated with force production.

Isokinetic Dynamometer

Ultrasound Machine

Electromyography Electrode

3) Percantage of body fat measurement

By using the ultrasound machine, fat thichkness will be measured at 2 differnent sites for men (the belly and fornt thigh). This estimates the total percentage of body fat on your body.

4) Filling in the food and drink diary

End of baseline test, a food and drink diary will be provided in order to measure your energy intake and assess relationship between energy intake and body fat percentage. You will be asked to record the diary for three days (Thursday, Friday and Saturday) at the start and end of the day. You will be instructed how to complete the diary by the researcher and detailed instruction is on the first page of the diary.

Resistance training with collagen intake

You will perform 6-week resistance training and the researcher will give collagen or maltodextrin immediately before each training session. The training intensity will be 5 sets of 10 reps at 10-repetition maximum load (the load you can lift for 10 repetitions) and the intensity will be increased in a weekly basis. You will perform three resistance exercise targeting the thigh muscles (e.g. leg press and leg extension). You will be assigned one of two groups:

collagen or control. The collagen group will be given a drink containing hydrolyzed collagen, while the control group's drink will contain maltodextrin and no collagen. This is a blinded study (you will not know which nutritional supplementation you will be given) but you will receive individual verbal debriefing at the end of the study about which group you were in.

Nutritional Supplementation

During the training period, you will be given a supplement to be consumed with each resistance training session (2 times per week). The supplements used in this study will comprise either 30 g **hyrolysed collagen** (HC, derived from bovine skin, so vegans, vegetarians and anyone who suffers from a meat allergy will not be able to participate in this study) or 30.5 g **maltodextrin** (a commonly used high-glycaemic food additive derived from starch in potato, rice, and corn), with the latter being consumed by the control group. Both HC and maltodextrin supplements will each be mixed with 50 mg **vitamin C** and 3 g non-calorie **sweetener** (comprising a food additive derived from the stevia leaf) in 300 mL water. Both the HC and maltodextrin doses are considered to be low, with 30 g HC constituting 20-25% of the recommended daily intake of protein and 30.5 g maltodextrin comprising 10% of the recommended daily amount of carbohydrate in adults. The vitamin C and sweetener doses are extremely low and come with no known adverse risks or side-effects at such low doses. Although there is no documented evidence of a deleterious effect from the ingestion of collagen, a rare allergy, sensation of unpleasant taste or feeling of heaviness in the stomach might occur. If any of these transpire, please inform the researchers immediately. The low dose of maltodextrin carries minimal risk or adverse effects, although the high-glycaemic index of this food additive can cause an increase in blood sugar levels, **so**

diabetics should be aware of this. However, due to the exercise-induced transportation of blood glucose into the muscle, the insulin response to the increase in blood glucose following maltodextrin ingestion is likely to be blunted. Much larger doses of maltodextrin than that used in this study have been associated with bloating, flatulence and in severe cases, diarrhoea. However, this risk is very low with the highest dose used in this study.

Post-training tests

All testing procedures are the same as 'Baseline Test'.

Are there any risks/benefits involved?

Benefits

In participating in this study, it is expected that your strength and muscle size will be increased and your tendon health improved.

Possible risks

Risk of muscle pain caused by resistance exercise

Injuries during resistance training are unlikely to occur as training will be supervised by the researcher, and you will learn the proper techniques for each resistance exercise, and complete a well-structured warm-up beforehand.

What will happen to the data provided and how will my taking part in this project be kept confidential?

The information you provide as part of the study is the **study data**. Any study data from which you can be identified (e.g. from identifiers such as your name, date of birth, audio recording etc.), is known as **personal data**. This includes more sensitive categories of personal data (**sensitive data**) such as your race; ethnic origin; politics; religion; trade union membership; genetics; biometrics (where used for ID purposes); health; sex life; or sexual orientation. Throughout the study your personal information will be kept entirely confidential. Instead of your name, your data will be given an identification code, so that you will not be identifiable. Your personal data will be destroyed four years after the testing is complete. The results of this study are expected to be published in a scientific journal, but names of participants will not be published. In addition, responsible members of Liverpool John Moores University may be given access to personal data for monitoring and/or audit of the study to ensure that the study is complying with applicable regulations.

What will happen to the results of the research project?

The results of this study are expected to be published in a scientific journal, but names of participants will never be published.

Who is organising the study?

This study is organised by Liverpool John Moores University

Who has reviewed this study?

This study has been reviewed by, and received ethics clearance through, the Liverpool John Moores University Research Ethics Committee (19/SPS/054).

What if something goes wrong?

If you have a concern about any aspect of this study, please contact the relevant investigator who will do their best to answer your query. The researcher should acknowledge your concern within 10 working days and give you an indication of how they intend to deal with it. If you wish to make a complaint, please contact the chair of the Liverpool John Moores University Research Ethics Committee (researchethics@ljmu.ac.uk) and your communication will be re-directed to an independent person as appropriate.

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This study has received ethical approval from LJMU's Research Ethics Committee (19/SPS/054).

1. Contact for further information

Joonsung Lee (Researcher)
Research Institute for Sport & Exercise Sciences
Liverpool John Moores University
Byrom Street
Liverpool
L3 2AF
Tel
E-mail: J.Lee1@2017.ljmu.ac.uk

Dr Rob Erskine (Academic Supervisor)
Research Institute for Sport & Exercise Sciences
Liverpool John Moores University
Byrom Street
Liverpool
L3 2AF
Tel: +44 (0)151 904 62
E-mail: R.M.Erskine@ljmu.ac.uk

Thank you for reading this information sheet and for considering to take part in this study.

Note: A copy of the participant information sheet should be retained by the participant with a copy of the signed consent form.

PIS Date: 08/12/20 PIS Version No: 0.3

APPENDIX 6 – Consent form for the experimental study in Chapter Seven

Informed Consent Form

Study Title:	The effect of resistance training with vitamin C-enriched collagen supplementation on changes in muscle and tendon properties in young healthy men and women
LJMU Ethics code:	19/SPS/054
Name of Principal Investigator:	Joonsung Lee
Faculty & School:	School of Sport and Exercise Sciences
Contact details:	J.Lee1@2017.ljmu.ac.uk

1. I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and that this will not affect my legal rights. ☐
3. I understand that any personal information collected during the study will be anonymised and remain confidential. ☐
4. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Researcher	Date	Signature
Name of Person taking consent	Date	Signature

(If different from researcher)

APPENDIX 7 – The Low Energy Availability in Females Questionnaire

The LEAF-Q: Questionnaire for Female Athletes

1. Injuries - Please mark the response that most accurately describes your situation

A: Have you had absences from your training, or participation in competitions during the last year due to injuries?

☐ No, not at all ☐ Yes, once or twice

☐ Yes, three or four times ☐ Yes, five times or more

A1: If yes, for how many days absence from training or participation in competition due to injuries have you had in the last year?

☐ 1-7 days ☐ 8-14 days

☐ 15-21 days ☐ 22 days or more

A2: If yes, what kind of injuries have you had in the last year?

_____

Comments or further information regarding injuries: _____

---

2. Gastrointestinal function

A: Do you feel gaseous or bloated in the abdomen, even when you do not have your period?

☐ Rarely or never ☐ Yes, several times a day

☐ Yes, several times a week ☐ Yes, once or twice a week or more seldom

B: Do you get cramps or stomach ache, which cannot be related to your menstruation?

☐ Rarely or never ☐ Yes, several times a day

☐ Yes, several times a week ☐ Yes, once or twice a week or more seldom

C: How often do you have bowel movements (an act of stool) on average?

☐ Several times a day ☐ Once a day ☐ Every second day

☐ Twice a week ☐ Once a week or more rarely

D: How would you describe your normal stool?

☐ Normal (soft) ☐ Diarrhoea-like (watery)

☐ Hard and dry

---

3. Menstrual function and use of hormonal contraception

3.1 Contraceptives – Please mark the response that most accurately describes your situation

A: Do you use oral contraceptives?

☐ Yes (**Go to question A1**) | ☐ No **(Go to question A3**)

A1-1: If yes, what is the name and type of oral contraceptive pills you use?

☐ Combined pill

☐ Progesterone-only pill

Name of pills (e.g., Microgynon, Rigevidon, and Yasmin are examples of combined pills)

(Please see the list of oral contraceptive pills on page 6.)

:_____

A1-2: How long have you used oral contraceptive pills?

Year(s) Month(s)

A2: If yes, why do you use oral contraceptives?

☐ Contraception ☐ Reduction of menstruation pains ☐ Reduction of bleeding

☐ To regulate the menstrual cycle in relation to performances etc.

☐ Otherwise menstruation stops

☐ Other

A3: If no, have you used oral contraceptives previously?

☐ Yes | ☐ No

A3-1: If yes, for how long?

Year(s) Month(s)

B: Do you use any other kind of hormonal contraception? (e.g., hormonal implant or coil)

☐ Yes | ☐ No

B1: If yes, what kind?

☐ Hormonal patches ☐ Hormonal ring

☐ Hormonal implant ☐ Hormonal coil (or copper coil)

☐ Intrauterine system (or Levonorgestrel Intrauterine System)

☐ Other

B2: If yes, what is the name of hormonal contraception you use?

(For example, ‘NEXPLANON’ is contraceptive implant, and ‘T-Safe 380’ is intrauterine device)

_____

B3: If yes, for how long?

Year(s) Month(s)

3.2 Menstrual function. Please mark the response that most accurately describes your situation

A: How old were when you had your first period?

☐ 11 years or younger ☐ 12-14 years ☐ 15 years or older

☐ I don't remember ☐ I have never menstruated *

If you have answered "I have never menstruated" there are no further questions to answer.

B: Did your first menstruation come naturally (by itself)?

☐ Yes ☐ No ☐ I don't remember

B1: If no, what kind of treatment was used to start your menstrual cycle?

☐ Hormonal treatment ☐ Weight gain

☐ Reduced amount of exercise ☐ Other

C: Do you have normal menstruation?

☐ Yes ☐ No **(go to question C6)** ☐ I don't remember **(go to question C6)***

*** If your bleeding pattern is irregular because of contraceptive method you use, tick 'I don't remember.'**

C1: If yes, are your periods regular? (every 28th to 34th day)

☐ Yes, most of the time ☐ No, mostly not

C1-1: If yes, what is the length of your menstrual cycle?

(Menstrual cycle includes menstruation (bleeding) + follicular phase + ovulation + luteal phase so not just the bleeding period. This refers to the menstrual cycle that generally lasts between 28 and 34 days.)

_____

C2: If yes, when was your last period?

☐ 0-4 weeks ago ☐ 1-2 months ago

☐ 3-4 months ago ☐ 5 months ago or more

C2-11 If yes, on what date did your last menstrual cycle begin? (Answer this question only if you ticked '☐ 0-4 weeks ago' above question.

Menstrual cycle begins on the first day you have regular bleeding and for example, if the first day of bleeding is 7th of this January, please specify that date.

C3: If yes, for how many days do you normally bleed?

☐ 1-2 days ☐ 3-4 days ☐ 5-6 days

☐ 7-8 days ☐ 9 days or more

C4: If yes, have you ever had problems with heavy menstrual bleeding?

☐ Yes | ☐ No

C5: If yes, how many periods have you had during the last year?

☐ 12 or more ☐ 9-11 ☐ 6-8

☐ 3-5 ☐ 0-2

C6: If no or “I don’t remember”, when did you have your last period?

☐ 2-3 months ago ☐ 4-5 months ago

☐ 6 months ago ☐ I’m pregnant and therefore do not menstruate.

D: Have your periods ever stopped for 3 consecutive months or longer (besides pregnancy)?

☐ No, never ☐ Yes, it has happened before ☐ Yes, that’s the situation now

---

E: Do you experience any changes with your menstruation when you increase your exercise intensity, frequency or duration?

☐ Yes | ☐ No

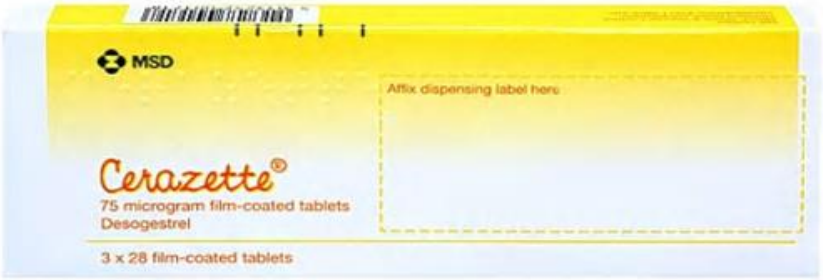
E1: If yes, in what way(s)? (Check one or more options)

☐ I bleed less ☐ I bleed fewer days ☐ My menstruations stops

☐ I bleed more ☐ I bleed more days


Microgynon® 30
levonorgestrel
ethinylestradiol
— 3 x 21 tabs.
— oral

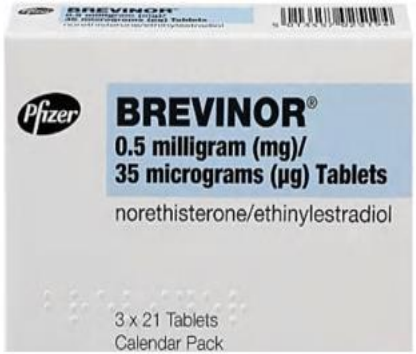

yasmin®
0.03 mg/3 mg film-coated tablets
Ethinylestradiol/Drospirenone
— 3 x 21 film-coated tablets
— oral use


MSD
Affix dispensing label here
Cerazette®
75 microgram film-coated tablets
Desogestrel
3 x 28 film-coated tablets


Cilest®
3
35/250 micrograms film-coated tablets
ethinylestradiol, norgestimate
janssen


CERELLE®
75 micrograms film-coated tablets
desogestrel
3 x 28 film-coated tablets


MSD
Marvelon®
desogestrel/ethinylestradiol
3 x 21 tablets


Pfizer
BREVINOR®
0.5 milligram (mg)/
35 micrograms (µg) Tablets
norethisterone/ethinylestradiol
3 x 21 Tablets
Calendar Pack


BAYER
Femodene®
gestodene
ethinylestradiol
3 x 21 tabs.
oral


Femodette®
gestodene
ethinylestradiol
BAYER
3 x 21 tablets
oral


RIGEVIDON®
150 micrograms/30 micrograms
coated tablets
levonorgestrel / ethinylestradiol
3 x 21 coated tablets


CILIQUE®
250/ 35 microgram tablets
norgestimate and ethinylestradiol
3 x 21 tablets


MSD
Mercilon®
desogestrel/ethinylestradiol
3 x 21 tablets


GEDAREL® 30/150
microgram film-coated tablets
ethinylestradiol / desogestrel
3 x 21 film-coated tablets


Loestrin 20


Katya 30/75 Coated Tablets
ETHINYLESTRADIOL / GESTODENE
STRAGEN UK
3 x 21 tablets


Levest 150/30 Coated Tablets
Levonorgestrel 150 micrograms &
Ethinylestradiol 30 micrograms
FOR ORAL USE
63 Tablets


TRIREGOL® coated tablets
ethinylestradiol / levonorgestrel
3 x 21 coated tablets


MILLINETTE® 20/75
microgram coated tablets
ethinylestradiol / gestodene
3 x 21 coated tablets


Pfizer
Norinyl®-1
3 x 21 Tablets


3x21
tablets
Ovranette®
150/30 micrograms
Coated Tablets


BAYER
Norgeston®
Levonorgestrel
30 micrograms
35 tabs.
oral


Pfizer
Noriday®
350 microgram tablets
Norethisterone
3 x 28 Tablets
Calendar Pack


Logynon®
coated tablets
BAYER
3 x 21 tablets
oral


Pfizer
Norimin®
1 mg/0.035 mg tablets
norethisterone &
ethinylestradiol
3 x 21 tablets

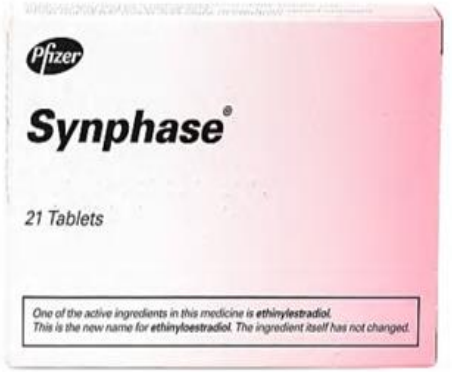

Triadene
BAYER
Triadene®
gestodene
ethinylestradiol
— 3 x 21 tablets
— oral


zoely® 2.5 mg/1.5 mg
Film-coated tablets
nomegestrol acetate/estradiol
84 Film-coated tablets | Oral use
Read the package leaflet before use.


LUCETTE®
0.03 mg/3 mg film-coated tablets
ethinylestradiol and drospirenone
3 x 21 film-coated tablets


Qlaira®
BAYER
film-coated tablets
Estradiol valerate/Dienogest
— 28 film-coated tablets
— oral use


Sunya 20/75 Coated Tablets
ETHINYLESTRADIOL / GESTODENE
STRAGEN UK
3 x 21 tablets


Pfizer
Synphase®
21 Tablets
One of the active ingredients in this medicine is ethinylestradiol.
This is the new name for ethinyloestradiol. The ingredient itself has not changed.

APPENDIX 8 – Readiness to exercise screening questionnaire

Readiness to exercise screening questionnaire

Participant ID:			Date
DOB	Age	Home Phone	Work Phone

Regular exercise is associated with many health benefits. Increasing physical activity is safe for most people. However, some individuals should check with a physician before they become more physically active. Completion of this questionnaire is a first step when planning to increase the amount of physical activity in your life. Please read each question carefully and answer every question honestly:

Yes	No	1) Has a physician ever diagnosed you with a heart condition **and** indicated you should restrict your physical activity?
Yes	No	2) When you perform physical activity, do you feel pain in your chest?
Yes	No	3) When you were not engaging in physical activity, have you experienced chest pain in the past month?
Yes	No	4) Do you ever faint or get dizzy and lose your balance?

Yes	No	5) Do you have an injury or orthopaedic condition (such as a back, hip, or knee problem) that may worsen due to a change in your physical activity?
Yes	No	6) Do you have high blood pressure or a heart condition in which a physician is currently prescribing a medication?
Yes	No	7) Are you pregnant?
Yes	No	8) Do you have insulin dependent diabetes?
Yes	No	9) Are you 69 years of age or older **and** not used to being very active?
Yes	No	10) Do you know of any other reason you should not exercise or increase your physical activity?
Yes	No	11) Are you currently on a medically prescribed diet? Explain
Yes	No	12) Do you currently follow on specific diet restrictions (e.g. gluten free, vegetarian)? Explain

Yes	No	13) Do you take any dietary supplements? If yes, please state what and the frequency

If you answered yes to any of the above questions, talk with your doctor **before** you become more physically active. Tell your doctor your plan to exercise and to which questions you answer yes.

If you honestly answered no to all questions you can be reasonably certain you can safely increase your level of physical activity **gradually**.

If your health changes so you then answer yes to any of the above questions, seek guidance from a physician. I have read, fully understood and completed this questionnaire. The answers I have given are accurate to the best of my knowledge.

Participant signature	Date

APPENDIX 9 – Food and drink diary

Research Institute for Sport & Exercise Science

FOOD AND DRINK DIARY

Name:

How to complete the food record booklet

1. The purpose of asking you to record your dietary intake is to assess the relationship between habitual diet and body composition.

2. Please record everything you eat and drink each day for three consecutive days (e.g. **Thursday, Friday and Saturday**). It's important that you do not change your eating habits in any way due to the dietary analysis, so please be as honest as you can and eat the foods that you normally eat.

3. Please give us as much detail as possible about what you eat and drink, i.e. description (e.g. wholemeal or white bread), portion size, packaging, etc., and what time you eat and drink.

4. Please state the method of cooking e.g. boiled, grilled, fried.

5. Please state the amount of food eaten (small, medium and large portion) and drink consumed, e.g. 300 mL mug of tea, ½ or full pint beer, small/large (175/250 mL) glass wine.

6. Please state **the brand of food** wherever possible, e.g. Heinz Cream of Tomato Soup, McVities Digestive Biscuits.

7. If two items are eaten together, please state the individual amounts, e.g. apple and custard: fist-size helping of stewed apple and half a 350 mL can Ambrosia Custard.

8. For items regularly consumed (e.g. cup of tea), please state the components, (i.e. water, milk, sugar) once. We will then assume all mugs of tea are the same thereafter.

9. For the milk- please also specify skimmed, semi-skimmed, full-fat; and whether cow, soya, goat, rice source

10. For the sugar- please also specify whether brown, white, cane etc.

11. For the tea itself, please specify which brand and leaf-type.

12. Please remember to record all snacks and drinks.

13. If you write this dairy while you participate the LJMU research, please write the drink you are given as 'Collagen drink.' (you do not need to put the amount)

14. Once you completed filling in this diary, please simply return it to Josh during training sessions.

- If you do not know the exact quantity of food (in grams) or drink (in mL), please use the following tips to help you estimate these quantities:

1. **Household measures:** use cups, teaspoons, tablespoons.
2. **Visually:**

- The Palm of your hand = 85g of meat or to describe the size of potatoes

- Your fist = the same size as a cup. Use it to indicate amounts of Pasta, rice, cereal and fruit/veg

3. The tip of your thumb is a teaspoon – indicate sugar, marg, oils etc.

An example of a daily record of nutritional intake:

DAY ONE		DATE 16.06.2011			OFFICIAL USE ONLY	
Meal	**Time**	**Food & Drink**	**Amount**	**Left-overs?**	**Food Code**	**Amount (g)**
Early am	7am	Mug of tea, strong, milky, made with skimmed milk	300ml (30ml milk)			
Break-fast	7.30 am	Bowl of Kellogg's branflakes, Semi-skimmed milk. Sliced banana. White sugar. Orange juice. Mug tea, made as above.	Medium bowl. ½ pt milk. Large 2 teaspoons ¼ pt. 300ml			
Mid am	10am	Tesco finest white toast. Flora margarine 2 glasses water.	2 slices Thinly applied ½ pt in total			

		Mug of filter coffee, black	300ml			
Midday Meal	1pm	2 crusty rolls. Flora marg. Mature Cheddar cheese 2 Jordan's cereal bar-fruit and nut. 1 can of Coca-cola. 1 glass water.	2 medium-sized rolls. Thinly applied Thick chunks 2 x 60g bar. 330ml. ½ pt glass			
Mid pm	3pm	1 mug of filter coffee, as above. 3 custard cream biscuits (nutritional info attached).	300ml. 3 biscuits.			
Evening Meal	6.30 pm	New potatoes steamed in skins, Steamed broccoli, Grilled fillet of salmon with paprika.	7 small pota-toes. Handful. 130g (uncooked). Pinch.	3 pota-toes, Salmon skin		

		2 low fat Ski strawberry yoghurt. 2 large glasses of white wine.	2 x 120g 2 x 250ml			
Evening Snack	9pm to 10.30pm	2 mugs of tea 1 chocolate brownie (nutritional info attached) 1 large glass of Tesco's blackcurrant squash (no added sugar).	2 x 300ml. large 1 pt			
Extras		Mars Bar	1 standard size			

Your daily record of nutritional intake:

DAY ONE *Thursday*		DATE			OFFICIAL USE ONLY	
Meal	**Time**	**Food & Drink**	**Amount**	**Left-overs ?**	**Food Code**	**Amount (g)**
Early am						
Breakfast						
Mid am						

Midday Meal						
Mid pm						
Evening Meal						

Evening Snack						
Extras						

NOTES:

Please record anything else which you may feel is relevant, e.g. illness

DAY TWO *Friday*		**DATE**			**OFFICIAL USE ONLY**	
Meal	**Time**	**Food & Drink**	**Amount**	**Left-overs ?**	**Food Code**	**Amount (g)**

Early am						
Breakfast						
Mid am						
Midday Meal						

Mid pm						
Evening Meal						
Evening Snack						

Extras						

NOTES:

Please record anything else which you may feel is relevant, e.g. illness

DAY THREE *Saturday*		DATE			OFFICIAL USE ONLY	
Meal	**Time**	**Food & Drink**	**Amount**	**Left-overs ?**	**Food Code**	**Amount (g)**
Early am						
Breakfast						
Mid am						

Midday Meal						
Mid pm						
Evening Meal						

Evening Snack						
Extras						

NOTES:

Please record anything else which you may feel is relevant, e.g. illness