The effects of collagen hydrolysate and milk protein on knee pain and skin condition among women aged ≥50 years

A thesis presented in partial fulfilment of the

requirements for the degree of

Doctor of Philosophy

in

Nutritional Science

at

Massey University, Manawatū, New Zealand.

Drew Gordon

2025

In loving memory of

Lyn Mclean: 14.07.1956 – 04.09.2025.

You were the wise donkey who never stopped loving and believing in me, even when I couldn't.

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Massey University underwent restructuring in 2023 which impacted on the project. Along with losing intellectual property through job cuts, key lab equipment for in-house analyses of biomarkers was not freely available so the samples were sent to an external laboratory. The analyses and return of results from the lab were delayed due to staff shortages and the work having lower priority than daily duties of the public health system. Overall, there was a significant delay between the time of sending the samples and receiving results for proceeding with statistical analyses and interpreting the output.

The recruitment and data collection window was also affected by Covid-19 and other unplanned absences from illnesses. Research team members with training to use specialised equipment (e.g. DXA) were either unwell so unable to attend data collection, or several research team members were unwell at the same time so participants booked for attending data collection visits were rebooked for when the research team capacity could manage. Overall, optimal numbers from recruitment were not achieved in the timeframe available for collecting the data.

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Abstract

The global population is aging, and the prevalence of age-related illness is expected to rise. Knee osteoarthritis (KOA) and deteriorating skin condition are common complaints associated with aging, and collagen breakdown is the underlying cause in both instances. Collagen is the main structural protein in humans. Its breakdown in the knee joint leads to pain and inflammation, while the skin becomes thin, dry and loses its viscoelasticity. Estrogen depletion amplifies the events underpinning collagen loss, so peri and postmenopausal women are targets for nutraceuticals such as collagen hydrolysate (CH). They are advertised as effective for joint pain relief and improving skin condition, but such claims are unconvincing from an objective standpoint. Therefore, the Collagen Hydrolysate and Milk Protein (CHAMP) study was prompted as a project.

The CHAMP study evaluated the effect of CH on knee pain (perceptions, functional performance, biomarkers) and skin condition (thickness, hydration, viscoelasticity) among women aged ≥50 years with knee pain not necessarily due to KOA. It was a randomized, double-blind controlled trial (RDBCT) of four months, and the participants consumed either an oral supplement of 15 g protein as CH (from bovine hide) or the comparators of milk protein (matched by protein and energy), or maltodextrin (matched by energy).

There was no evidence of a superior effect from CH in alleviating pain or improving functional performance. The three supplements performed similarly over time, without having any apparent impact on slowing an early stage of cartilage turnover dysregulation among the same cohort of women. The data were inconclusive about any effect on inflammation, but the CH supplement may have increased skin thickness in areas generally protected from sunlight (i.e. inner forearm). Ultimately more research is warranted before conclusions can be made about the effect of CH as a nutraceutical for skin condition or knee joint pain. Future studies would benefit by reevaluating the same outcome measures of the CHAMP study over a longer timeframe, at different doses of CH (with or without other active ingredients such as vitamin C and glucosamine), and with more participants under tightly controlled environmental conditions.

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This story begins with me reaching out to Marlena and Jane in 2022, looking to change my career path. I am eternally grateful to you both, as I am to the High Value Nutrition Challenge funding which allowed this dream to become a reality. I've always thought it was impossible, and I owe so many thanks to my supervisors, Prof. Jane Coad, Dr. Janet Weber, and Prof. Marlena Kruger, for your support and guidance which helped me develop new skills, sharpen my mind, and learn to accept that "*real problems can be overcome; only the imagined ones are unconquerable*" (*Theodore N. Vail*).

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Your unwavering love will remain by my side, wherever you may be.

I love you and dedicate this thesis to you both.

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List of abbreviations

2MWT	Two-Minute Walk Test
6MWT	Six-Minute Walk Test
30s-CST	30-second Chair Stand Test
αI	Alpha-one
αII	Alpha-two
AA	Amino acid
ACL	Anterior cruciate ligament
ACR	American College of Rheumatology
ADAMTS-4	A Disintegrin and Metalloproteinase with Thrombospondin motif 4
ADAMTS-5	A Disintegrin and Metalloproteinase with Thrombospondin motif 5
ADLs	Activities of daily living
AGEs	Advanced glycation end-products
ANOVA	Analysis of variance
APPs	Acute phase proteins
AUC	Area under a curve
BAPs	Bioactive peptides
BL	Baseline
BMI	Body mass index
BMPs	Bone morphogenic proteins
BSE	Bovine spongiform encephalopathy
CH	Collagen hydrolysate
$CH_{ChickCart}$	Collagen hydrolysate (sourced from chicken cartilage)
$CH_{ChickFeet}$	Collagen hydrolysate (sourced from chicken feet)
CH_{fish}	Collagen hydrolysate (sourced from fish)
CH_{scale}	Collagen hydrolysate (sourced from fish scale)
CH_{skin}	Collagen hydrolysate (sourced from fish skin)
$CH_{porcine}$	Collagen hydrolysate (sourced from porcine skin)
CHAMP study	Collagen hydrolysate and milk protein study
COMP	Cartilage oligomeric matrix protein
sCOMP	Serum COMP
CRP	C-reactive protein
hs-CRP	High-sensitivity CRP
CS	Chondroitin sulfate
CTX-II	Carboxyl-terminal telopeptides of Type II collagen
sCTX-II	Serum CTX-II
uCTX-II	Urinary CTX-II
DDTs	Drug development tools
DFs	Dermal fibroblasts
DT	Dermal thickness
ECH	Enzymatically hydrolyzed collagen
ECM	Extracellular matrix
EFSA	European Food Safety Authority
EH	Epidermal hydration
EM	Expectation-Maximization
EP	Endpoint
ERKs	Extracellular signal-regulated kinases
EsSKOA	Early-stage symptomatic knee osteoarthritis

FACIT	Fibril-associated collagens with interrupted triple-helices
FDA	Food and Drug Administration
FN	Fibronectin
FPWT	Fast-Paced Walk Test
GAG	Glycosaminoglycan
GALT	Gut-associated lymphoid tissue
Gly	Glycine
GRAS	Generally recognized as safe
H_20_2	Hydrogen peroxide
HA	Hyaluronic acid
HNRU	Human Nutrition Research Unit
HRT	Hormone replacement therapy
Hyl	Hydroxylysine
Hyp	Hydroxyproline
IA	Intra-articular
ICOAP	Intermittent and Constant Osteoarthritis Pain
IGF-1	insulin-like growth factor
IGD	Interglobular domain
IL-1β	Interleukin-1-beta
IL-1R1	Type I IL-1 receptor 1
IL-6	Interleukin-6
ITT	Intention-to-treat
JNKs	c-Jun N-terminal kinases
KL	Kellgren-Lawrence
KOA	Knee osteoarthritis
KOOS	Knee Injury and Osteoarthritis Outcome Score
KS	Keratin sulfate
Leu	Leucine
LISOK	Le Quesne Index of Severity for Osteoarthritis of the Knee
LMM	Linear Mixed Models
LOA	limits of agreement
LDF	Lower Dorsal Forearm
LRP1	Low-density lipoprotein receptor-related protein 1
LVF	Lower Ventral Forearm
Lys	Lysine
MAPKs	Mitogen-activated protein kinases
MCID	Minimal clinically important difference
MDC	Minimal detectable change
MDx	Maltodextrin
MetS	Metabolic syndrome
MIC	Minimally important change
MMPs	Metalloproteinases
MP	Milk protein
MW	Molecular weight
NCH	Non-enzymatically hydrolyzed collagen
NHANES	National Health and Nutrition Examination Surveys
NHLBI	National Heart Lung and Blood Institute
NSAIDs	Non-steroidal anti-inflammatory drugs
0_2^-	Superoxide
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International

OMERACT	Outcome Measures in Rheumatology
PEPT1	Peptide Transporter 1
PIICP (CPII)	Carboxyl terminus propeptide of Type II collagen
PIINP	Amino terminus propeptide of Type II collagen
PIIANP	Alpha amino terminus propeptide of Type II collagen
PIIBNP	Beta amino terminus propeptide of Type II collagen
PASS	Patient acceptable symptom score
PEPT1	Peptide Transporter 1 (PEPT1)
PGs	Proteoglycans
PP	Per protocol
PRG4	Proteoglycan 4
PRIMOS	Phase-shift rapid in vivo measurement of skin
Pro	Proline
Pro-Hyp	Prolyl-hydroxyproline
PROMs	Patient reported outcome measures
QoL	Quality of life
RA	Rheumatoid arthritis
RDBCT	Randomized double-blind clinical trial
RH	Relative humidity
RoB	Risk of bias
ROC	Receiver operating characteristic)
ROM	Range-of-motion
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
SCFA	Short-chain fatty acids
SCT	Stair Climb Test
SEM	Standard error of measurement
SF	Synovial fluid
Sox9	SRY-box 9 protein transcription factor
SPF	Sun protection factor
STZ	Superficial tangential zone
T_d	Denaturation temperature
TEWLL	Transepidermal water loss
TGF-β	Transforming growth factor-beta
TIMPs	Tissue Inhibitors of Metalloproteinases
TNF-α	Tumor Necrosis Factor-Alpha (TNF-α)
TUG	Timed Up and Go
UC	Undenatured collagen
UC-I	Type I undenatured collagen (UC)
UC-II	Type II undenatured collagen (UC)
UDF	Upper Dorsal Forearm
UVF	Upper Ventral Forearm
VAS	Visual Analogue Scale / Score
VE	Viscoelasticity
VF	Ventral Forearm
WOMAC	Western Ontario and McMaster Universities Arthritis Index

Chapter 1: Introduction

This chapter provides a background to the overall Collagen Hydrolysate and Milk Protein (CHAMP) study. It also justifies the research focus on hydrolyzed collagen in the ensuing chapters.

1.1 Background

The global population is aging.[1,2] Between 2015 and 2050, the World Health Organization (WHO) predicts the proportion of adults aged ≥60 years worldwide will have increased from 12 to 22%.[2,3] Over that time, the prevalence of chronic disease is also expected to increase,[1,4] so the WHO have designated 2021-2030 a decade of healthy aging. This entails a quality of life (QoL) despite one's increasing odds of suffering age-related ailments such as osteoarthritis (OA) and deteriorating skin condition.[5]

Osteoarthritis (OA) is a degenerative condition of the joints. It commonly occurs in the knee (KOA), and is characterized by cartilage breakdown causing pain, stiffness and inflammation.[6] Collagen fibers are the main organic component of cartilage, and they provide it with structural integrity for supporting smooth joint articulation and absorption of mechanical force.[7-9] However, collagen fibers in cartilage are sensitive to the effects of chronological (intrinsic) aging as much as any other body tissue such as the skin.

Skin is the largest organ of the human body.[10] It is comprised of three layers (epidermis, dermis and hypodermis), which altogether are the first line of defense from pathogens, mechanical injury and environmental stressors.[10] Most of the skin's dry weight is accounted for by collagen fibers in the dermal layer,[10] and they give skin a viscoelastic property for its optimal function and condition.[10,11] However, skin tends to show the first signs of intrinsic aging due to its large surface area and constant contact with the environment.[12,13]

Intrinsic aging is a natural phenomenon of cellular senescence and oxidative stress.[11,14,15] It is amplified by extrinsic aging to a variable extent depending on one's exposure to environmental factors including ultraviolet (UV) radiation (i.e. sunlight), pollution (e.g. tobacco smoke), and poor nutrition.[16-18] Chondrocytes within articular cartilage and fibroblasts within the dermal layer of skin are adversely affected by the effects of aging (intrinsic and extrinsic), leading to cartilage degradation, and thin, dry or wrinkly skin.[11,18-20] Collagen fragmentation (i.e. net collagen loss) is the underlying cause,[12] due to a cascade of events involving an upregulation of proteases (i.e. aggrecanases and collagenases) and

proinflammatory cytokines (e.g. IL-1β, TNF-α).[11,15,18] Their deleterious effects on cartilage and skin are exacerbated by estrogen depletion,[16] if not explained by other sex-related differences which are yet to be fully elucidated.[21] Therefore, peri- and postmenopausal women are prime targets for the marketing of "anti-aging" products for their knee joints and skin, especially oral supplements which may be more effective than topical applications.[16]

Collagen hydrolysates (CHs) are advertised as effective for relieving joint pain and improving skin condition.[22-25] Also known as collagen peptides, the fragments are derived from undenatured collagen (UC) which is the main structural protein of vertebrates.[26,27] It is highly concentrated in prolyl-hydroxyproline (Pro-Hyp) which is purported as a bioactive peptide (BAP) for alleviating KOA symptoms and improving skin condition.[26,28,29] However, research underlying the health claims of Pro-Hyp is unconvincing from a scientific standpoint.[25,28-31] Results from preclinical studies are mixed,[32-35] and the majority of human studies which have reported a positive effect from CH compared to a placebo were based solely on subjective measures.[36,37] A minority of clinical trials have included objective outcomes, but they found no difference between CH and placebo with their data on joint pain relief or skin condition.[38-40] Furthermore, the placebos were matched by weight rather than energy, and they lacked protein so any observed effects are not necessarily attributable to CH.[41-43] Therefore, the Collagen Hydrolysate and Milk Protein (CHAMP) study was planned as a research project to address this existing gap in research on the BAP potential of CH among women aged ≥50 years with self-reported knee pain and signs of aging skin (Appendix 1).

The CHAMP study was funded by the High Value Nutrition (HVN) Project (grant number HVN1943). It was one of the 11 National Sciences Challenges established and administered by the Ministry of Business, Innovation and Employment (MBIE) in New Zealand. It was sponsored by Southern Pastures (NZ) Ltd. (New Zealand Business Number 9429031978873) (https://southernpastures.co.nz/) and Ovation NZ Ltd. (https://www.ovation.co.nz/) who provided the bovine hide from which the collagen was extracted and hydrolyzed with their funding. The prototype spray-dried enzymatic CH was prepared conventionally from frozen, fresh dehaired cow

hide in the FoodPilot, Massey University, Palmerston North, New Zealand. Neither the funder nor sponsor had any role in the study design, data collection and interpretation or writing of this thesis, nor were they involved in the decision to submit any manuscripts for publication.

1.2 Importance of research

Type I and II collagen fibrils are the primary components of the skin and cartilage in mammals.[7] They have a virtually mutual structure and composition,[44,45] which is important for recognizing Type I undenatured collagen (UC-I) is a byproduct of the livestock industry with potential to be a nutraceutical for humans as CH (i.e. after extraction of UC-I and hydrolysis).[26,46,47] Therefore, several stakeholders may benefit from this research project.

The CH market grew from an estimated $797.43m (USD) in 2023 to $843.28m in 2024.[48] By 2030, it is projected to be worth more than $1 billion,[48] even though consumers may be spending their money without receiving measurable improvements in knee function or skin condition based on the existing evidence.[25] As such, the CHAMP study will provide objective data for the scientific community, and regardless of finding positive effects or not, the results may allow consumers more insight or informed choice when seeking products for joint pain relief or anti-aging effects for their skin. The NZ economy may also benefit considering its primary industries contributed 5.7% towards the gross domestic product (GDP) in 2023.[49] Beef cattle farming (combined with sheep and grain farming) made up 1.0% of that figure, and NZ exports more than $5 billion a year of beef and lamb.[50] Over 2.5 million cattle were slaughtered for export in the 2023-2024 season alone,[51] so there is value adding potential from reduced disposal costs of bovine hide if it proves useful as a source of UC-I for extraction and hydrolysis.[26,46,47,52]

1.3 Research aim and objectives

The CHAMP study was a randomized double-blind controlled trial (RDBCT). The aim of the project was to evaluate the effect of 15 g protein as CH (from bovine hide) on knee pain (perceptions, functional performance, biomarkers) and skin condition (thickness, hydration, viscoelasticity) among women aged ≥50 years. Its primary focus was on the subjective measure of knee pain while the

objective measures of functional performance, biochemical markers (biomarkers) of cartilage turnover and inflammation, and parameters of skin condition were secondary outcomes. Overall, the study had four objectives, and they were to investigate the effect of 15 g protein as collagen hydrolysate (CH) consumed orally each day for four months by women aged ≥50 years on their:

1. Perceptions of pain and impairment from symptoms using patient reported outcome measures (PROMs) as assessed with a validated multidimensional index (i.e. the Knee Osteoarthritis Outcome Score, KOOS);
2. Physical performance in activities (30s chair stand, 40m fast walk, stair climb, timed up and go, two-minute walk) endorsed by the Osteoarthritis Research Society International (OARSI);
3. Biomarkers of cartilage turnover (synthesis and breakdown) and inflammation by measuring plasma or serum samples of PIIANP, COMP, IL-1β, TNF-α and urinary CTX-II;
4. Skin condition (thickness, hydration, viscoelasticity) using objective measures from the Dermalab Combo device and subjective patient reported outcome measures (PROMs).

1.4 Thesis outline

The thesis is composed of eight chapters. Five of these are prepared as manuscripts which have been or will be submitted to top-ranking peer-reviewed journals for publication consideration. They were written in alignment with specifications for the journals they were targeted at, but reference styles, spelling and text formatting are amended for consistent flow to the thesis. Given the nature of the topic and structure of a thesis by publications, there is inevitably some repetition between chapters which is kept to a minimum as best possible.

Chapter Two is a review of the literature on collagen and a general overview of knee osteoarthritis (KOA) considering its interrelationship with pain. The pathophysiology of KOA, its burden of disease and risk factors are outlined before CH as a nutraceutical ingredient is discussed (i.e. bioactivity and bioavailability). The origins of CH are explained (i.e. animal sources, extraction methods and hydrolysis agents) to justify the research interest in CH derived from terrestrial mammals. Finally, fundamentals for planning and evaluating clinical trials are discussed so the existing

literature investigating the effect of CH from terrestrial mammals on knee pain can be critiqued in Chapter Three. It is a systematic review which gives reason for the research in Chapters Four and Five.

Chapter Four reports on the effect of CH on subjective PROMs (i.e. knee pain) and objective functional performance outcomes. An abstract was accepted for an oral presentation in April 2025 at the Osteoarthritis Research Society International (OARSI) World Congress on Osteoarthritis, and it was published in *Osteoarthritis and Cartilage*.[53] Chapter Five reports on the effect of CH on a set of biomarkers for cartilage turnover and inflammation in comparison to the isoproteic and isoenergetic comparators consumed by the same cohort of women aged ≥50 years with self-reported knee pain.

Chapter Six is a systematic scoping review with a primary objective to assess the internal validity of evidence from studies investigating the effect of CH from terrestrial mammals on skin condition (i.e. thickness, hydration, and viscoelasticity). Its secondary objectives were to explore and appraise the key features of each study's design (i.e. participants' characteristics, intervention regimen, environmental conditions), including the methods of data collection (i.e. which outcome parameters were evaluated, and from what areas on the body they were taken), and to ascertain the main findings for those outcome measures. The overarching goal of the review was to identify opportunities for assisting the design of future studies to ultimately improve the quality of evidence on CH from terrestrial mammals and skin condition. It also enabled comparisons with data derived from the CHAMP study on skin condition in Chapter Seven.

Chapter Seven presents and discusses data on the effect of CH on skin condition for the fourth objective of the CHAMP project. It is a report on secondary outcomes of the study which identifies future research opportunities for the topic as a primary focus.

To conclude, Chapter Eight is a synthesis of the results reported in Chapters Four, Five and Seven. The findings are discussed in relation to existing literature, limitations are acknowledged, and future research opportunities are explored before an overall conclusion about the efficacy of CH as a nutraceutical for joint pain and/or skin condition based on the CHAMP study data. Overall, the chapter explains how this thesis contributes meaningfully to the scientific community.

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Chapter 2: Literature review

This chapter has three components:

1. *Part one is an overview of articular cartilage and collagen to understand how Type II collagen degradation is associated with the pathogenesis of knee osteoarthritis (KOA). A comprehensive review of KOA was outside the scope of this thesis, but its burden of disease and risk factors are outlined to explain why an effective strategy for symptom relief is pertinent to investigate.*
2. *Part two overviews the literature on collagen hydrolysate (CH) as a potential nutraceutical ingredient. It shows how animal sources of collagen impact on its bioactivity and bioavailability.*
3. *Part three concludes the chapter by exploring key aspects of study design so the research on CH sourced from terrestrial mammals can be critiqued.*

2.1 Introduction

Collagen derives from the Greek terms 'kolla' and 'gen.'[1] They translate to 'glue-producing' in English and allude to functional properties of significance for humans. For example, collagen is the main structural protein of all vertebrates, and it accounts for ≈30% of total body protein in humans.[2,3] It is a superfamily of eight sub-groups (families), further defined by type (sub-family) to distinguish their various assemblies within the body.[2] There are 28 types of collagen known, and they have been assigned roman numerals in the order of their discovery.[4] Type I collagen is predominantly found in the skin (and bone) while Type II is most abundant in articular cartilage (Table 2-1).

Table 2-1 The 28 types of collagens in the human body (adopted with permission from Gelse et al.[5]).

Family	Type (Sub-Family)	Body Tissue(s)
Fibril-forming	I	Bone, cornea, ligaments, skin, tendons
	II	Cartilage, eyeball fluid, intervertebral disc fluid
	III	Skin, vessel wall, reticular fibers (lungs, liver, spleen)
	V	Lungs, cornea, bone, fetal membranes
	XI	Cartilage, vitreous body
Basement membrane	IV	Basement membranes
Microfibrillar	VI	Widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils	VII	Skin, dermal– epidermal junctions; oral mucosa, cervix
Hexagonal network-forming	VIII	Endothelial cells, Descemet's membrane
	X	Hypertrophic cartilage
Fibril-associated collagens with interrupted triple-helices (FACIT)	IX	Cartilage, vitreous humor, cornea
	XII	Perichondrium, ligaments, tendon
	XIV	Skin, tendon, vessel wall, placenta, lungs, liver
	XIX	Human rhabdomyosarcoma
	XX	Corneal epithelium, embryonic skin, sternal cartilage, tendon
	XXI	Blood vessel wall
Transmembrane	XIII	Skin, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
	XVII	Dermal– epidermal junctions
Multiplexins	XV	Fibroblasts, smooth muscle cells, kidney, pancreas
	XVI	Fibroblasts, amnion, keratinocytes
	XVIII	Lungs, liver

2.2 Articular cartilage and collagen

Articular cartilage is an extracellular matrix (ECM) on the surface of subchondral bone. Otherwise known as hyaline cartilage, it primarily consists of collagen fibers, glycoproteins and water dispersed across three distinct zones (Figure 2-1). Glycoproteins are proteoglycans (PGs) with glycosaminoglycan (GAG) chains attached. The main GAGs in cartilage are chondroitin sulfate (CS) and keratin sulfate (KS) attached to a hyaluronan/hyaluronic acid (HA) backbone.[6-8] They are encased by collagen fibers positioned horizontally in the superficial tangential zone (STZ), obliquely in the middle, and vertically in the deep zone nearest to the bone (Figure 2-1). The collagen fibers are anchored to the bone by a fourth layer of the ECM called the calcified zone.[4] Overall, the ECM enables smooth joint articulation at the STZ and absorption of mechanical force in the middle and deep layers.[9]

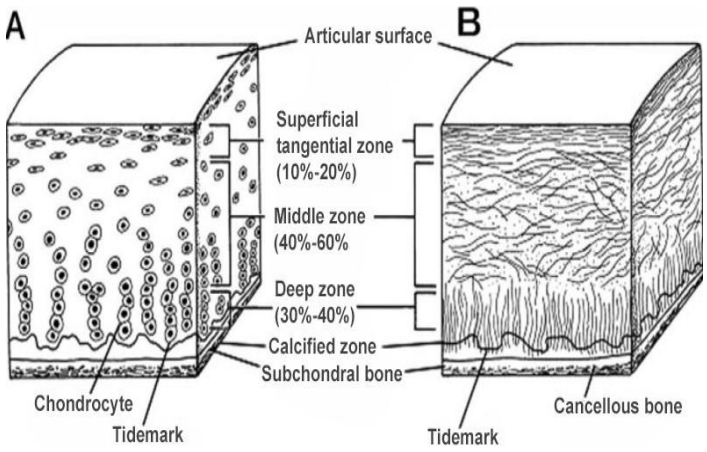

Figure 2-1. The three zones of articular cartilage showing the dispersion of chondrocytes within each layer (A). The figure shows how collagen fibers are arranged within each layer (B). The figure was adopted with permission from Buckwalter et al.[10]

2.2.1 Chondrocytes

The cartilage ECM is synthesized by chondrocytes.[11] They are the only cells in cartilage; accounting for ≈5% of its volume.[12,13] The chondrocytes are most dense and active at the outer layers (i.e. STZ and middle zone), and least dense but largest in the deep zone sitting terminally differentiated.[9] The ECM also contains cartilage oligomeric matrix protein (COMP), fibronectin (FN), integrins on chondrocytes and link protein (Figure 2-2). These non-collagenous proteins help secure chondrocytes within the ECM as it develops,[14-18] although chondrocytes are not metabolically stable in the ECM during cartilage turnover.[19,20]

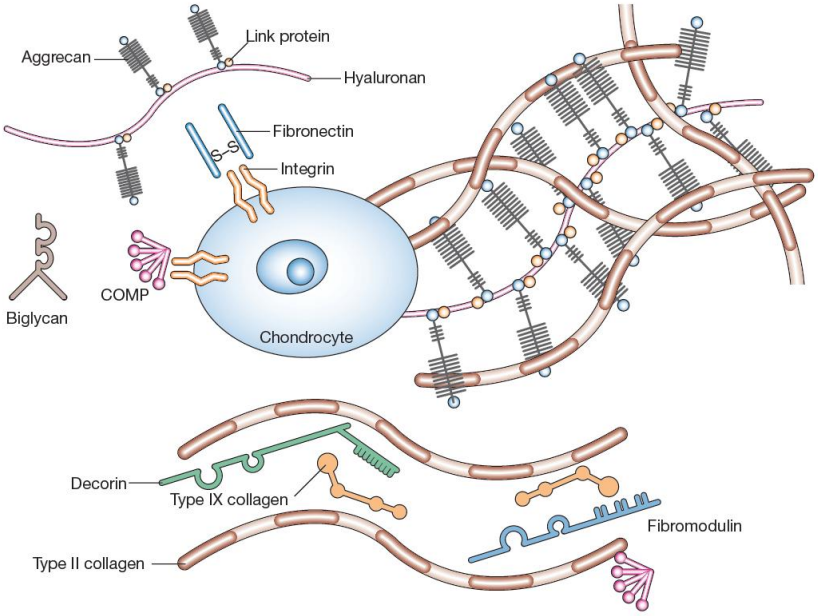

Figure 2-2. Major and minor constituents in the cartilage extra cellular matrix (ECM) (adopted with permission from Chen et al.[21]).

Cartilage turnover describes a dynamic balance between synthesis and breakdown. Up until maturity, the rate of ECM synthesis exceeds the rate of breakdown as immature chondrocytes (chondroblasts) are formed from mesenchymal (progenitor) cells in the STZ layer.[12,22] The chondroblasts are narrow in shape and densely packed together without a matrix around them in the STZ (Figure 2-3). They secrete collagen and lubricin which is a glycoprotein with proteoglycan4 (PRG4) attached.[23,24] Lubricin has an extremely low friction coefficient in synovial fluid (SF), enabling the horizontally aligned collagen fibers of opposing joints to glide smoothly during movement.[25]

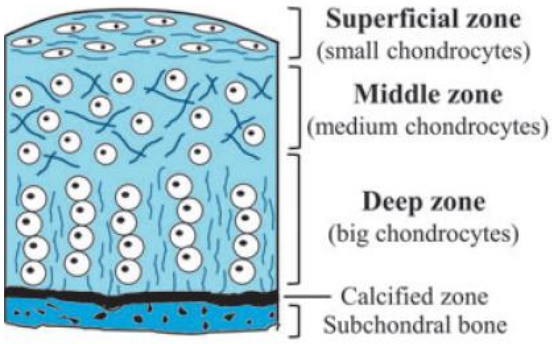

Figure 2-3. The morphology and arrangement of chondrocytes at each ECM layer (adopted with permission from Tee et al.[24]).

Chondroblasts naturally mature (differentiate) into chondrocytes during the development phase of the ECM.[26] They essentially migrate into the mid layer as the ECM expands, and some go on to establish the deep zone of hypertrophic chondrocytes as new chondroblasts arise in the STZ layer.[20,27] The proliferation is facilitated by SRY-box 9 (Sox9) protein transcription factor through the coordinated release of growth factors including insulin-like growth factor (IGF-1) and the transforming growth families-beta (TGF-β) which include a superfamily of bone morphogenic proteins (BMPs).[28] The BMPs jointly promote chondrogenesis (cartilage formation) and ossification (bone formation) whilst inhibiting excessive hypertrophy and mineralization as the ECM matures into its four layers.[12,26,27,29]

Mature chondrocytes in humans are larger, rounder and more randomly dispersed within the mid-layer of the ECM compared to chondroblasts in the STZ. The matrix surrounding each cell expands more than eight-fold between birth and two months of age.[27] The ECM continues to develop, but at a slower rate and by early adulthood it is in a steady state as the mid-layer chondrocytes become quiescent.[22,30,31] In addition to their changed morphology, quiescent chondrocytes have a different phenotype compared to chondroblasts[13]; they retain the ability of producing collagen, but secrete aggrecan instead of lubricin.[22]

2.2.2 Aggrecan

Aggrecan is the predominant PG of the ECM with a half-life of ≈20-25 years or less.[6,32,33] It consists of three globular domains with a large area between the second and third domain for the attachment of hydrophilic GAGs (Figure 2-4). The first domain interacts with HA and link protein to stabilize each PG aggregate,[6,33] and the interglobular domain (IGD), between the first and second domain, is the cleavage site for proteases.[16,32,33]

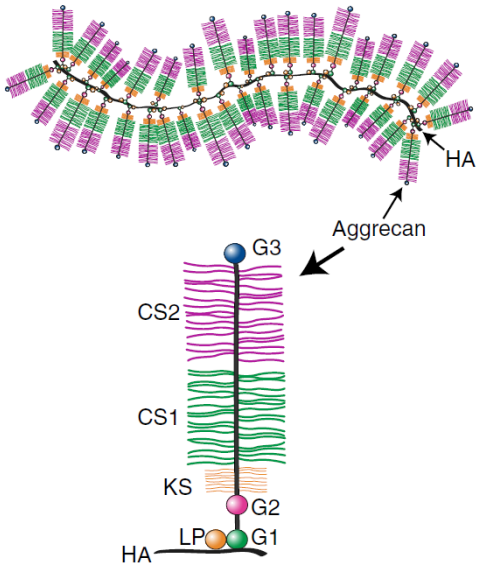

Figure 2-4. An aggrecan filament showing hydrophilic keratin sulfate (KS) and chondroitin sulfate (CS) between globular domains (G2) and (G3). The filament is attached to a hyaluronan (HA) backbone by link protein (LP) at globular domain 1 (G1) (Adopted with permission from Roughley and Mort[33]).

Aggrecan imparts cartilage with its function of cushioning mechanical force in the middle and deep layers.[6,8,33] Water, toxins and degraded products exude out of the ECM when it is compressed by a load (Figure 2-5), then water and nutrients return by osmosis once that force is dissipated.[31,33] The tensile strength, elasticity and porosity of collagen allows for this flux whilst restraining the imbibing (swelling) effect of aggrecan.[24,33] Therefore, collagen is fundamental for the structural integrity of mature cartilage.

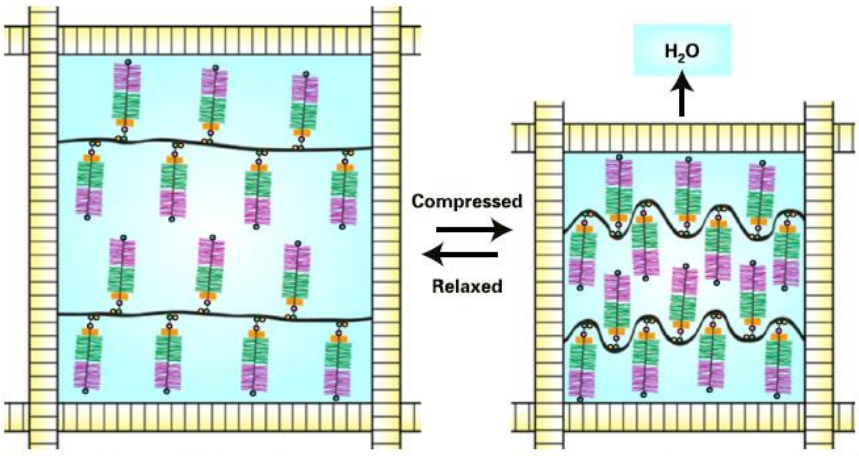

Figure 2-5. The movement of water to and from the extra cellular matrix (ECM) to enable cushioning of mechanical force (adopted with permission from Roughley and Mort [33]).

2.2.3 Collagen

Collagen is the main organic component of articular cartilage.[2] It accounts for ≈60% of its dry weight, mainly as Type II fibrils (≈95%) which coexist with Type IX collagen from the FACIT (fibril-associated collagens with interrupted triple-helices) family and Type XI from the fibril forming family.[2,4,34] Altogether, they form collagen fibers in a highly organized manner.

2.2.3.1 Procollagen

Type II fibrils originate as procollagen. Individual polymers are tightly coiled into right-handed (clockwise) triple helices within the endoplasmic reticulum and Golgi apparatus of chondrocytes.[35,36] The helices are homotrimers of Type II, alpha-one (αI) chains ($[\alpha I(II)]_3$), and they contain ≈1,000 amino or imino acids in repeating sequences of glycine (Gly)-X-Y.[2] Proline (Pro) and hydroxyproline (Hyp) are imino acids which often occur at the X and Y positions,[5,35,37,38] and they differ from amino acids as they have a secondary amine group rather than a primary group.[39] Gly has the smallest number of side chains of all amino acids (i.e. one hydrogen side chain), so the kinked arrangement of Gly-Pro-Hyp allows for tighter coiling of the triple helix which is strengthened by intramolecular hydrogen bonds from post-translational hydroxylation of Pro.[3,5,40] However, the host must be nutritionally replete in ascorbic acid and iron in its reduced form (i.e. ferrous, Fe^{2+}) to support the hydroxylation process (Figure 2-6).

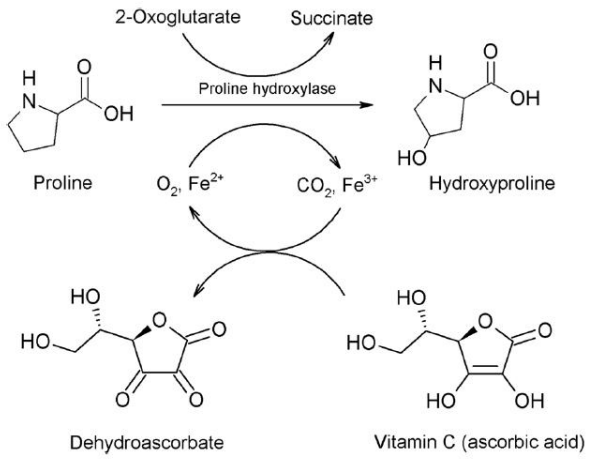

Figure 2-6. The hydroxylation of proline (Pro) converts ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) which means vitamin C is needed to reduce Fe^{3+} back into Fe^{2+} for the cycle to continue (adopted with permission from van Huizen et al.[36]).

2.2.3.2 Tropocollagen

Procollagen develops into tropocollagen monomers. The procollagen helices are coiled in a zipper-like fashion starting from the C-termini and ending at the N-termini called propeptides (Figure 2-7). These propeptides are short, non-collagenous globular domains positioned alongside non-helical C- and N-telopeptides containing 27 and 19 amino acid residues respectively.[3,5,31] They are cleaved by C- and N-proteinases once the triple helix is formed and has been secreted into the ECM.[5,36,41] Thereafter, the C- and N-termini (telopeptides) of newly created tropocollagen monomers facilitate fibril formation.[36]

Figure 2-7. The coiling of procollagen helices in a zipper fashion beginning at the C-terminal. The C- and N-terminal propeptides are cleaved once the triple helix is formed to create tropocollagen (adopted with permission from Sharma et al.[42]).

2.2.3.3 Fibril formation

Tropocollagen monomers progressively organize into microfibrils then fibrils within the ECM.[5,31,36] They self-assemble laterally and longitudinally for covalent crosslinking of their telopeptides (Figure 2-8); achieved through enzymatic oxidation of lysine (Lys) into hydroxylysine (Hyl).[36,43,44] The crosslinking is enhanced by non-enzymatic glycation over time, causing a buildup of advanced glycation end-products (AGEs) which have a stiffening effect on the collagen fibers later in life.[45-47]

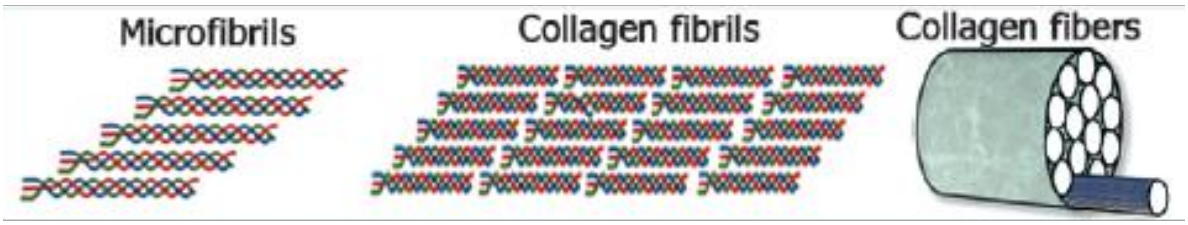

Figure 2-8. Collagen fiber formation from lateral and longitudinal assembly of microfibrils into fibrils (adopted with permission from Singh et al.[48]).

2.2.3.4 Collagen fibers

Mature collagen fibers are supramolecular structures of individual fibrils. The fibrils bunch together under the influence of PGs such as decorin, versican, and biglycan,[3,5,36,49] and they increase in thickness until reaching maturity.[31,50] For example, the proportion of Type II to Types XI and IX changes from ≈80:10:10 in fetal cartilage to 96:3:1 at maturity.[31,50] Type XI fibrils are centrally positioned within the fiber (Figure 2-9); and their N-termini propeptides extend to the outer surface to regulate the diameter of each fiber.[31,51] Meanwhile, Type IX collagen acts as a glue.[4,52] It covalently binds to the outer surface of the fiber with its globular non-collagenous N-terminal protruding outward,[17] thus preventing the addition of more fibrils whilst securing the components which the collagen fiber is encasing (i.e. chondrocytes, PGs and COMP).[17,36,52]

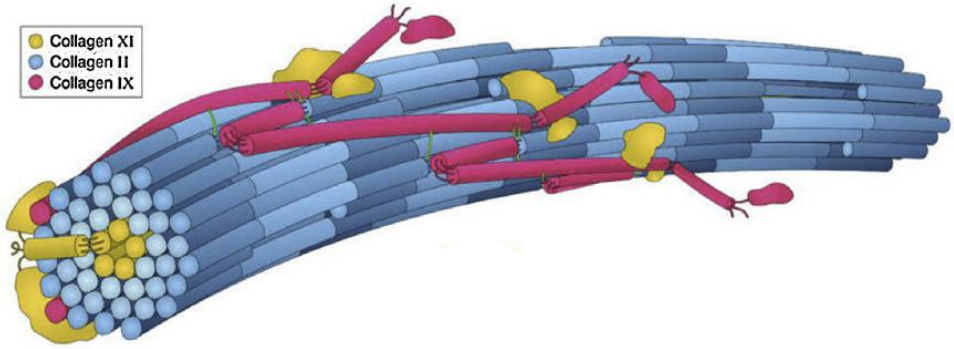

Figure 2-9. Demonstration of collagen fiber formation from the co-assembly of Type II fibrils with Types IX and XI (adopted with permission from Tiku and Madhan.[31]).

Mature collagen fibers vary in thickness depending on their location in the ECM.[31,36] They are the thinnest at the STZ layer and thickest in the deep zone to support the functional requirements of these layers.[9,31] For example, collagen fibers in the deeper zones have a greater need for tensile strength to absorb mechanical force.[9,31,34] Consequently, the STZ layer is more sensitive to dysregulation in cartilage turnover, especially in its 'weaker' areas without Pro-Hyp.[3,53,54]

Normally, the turnover of mature cartilage is low.[22,45,55] Chondrocytes are encased in a pericellular matrix (chondron) without much oxygen so they are generally protected from interactions with components in the interterritorial region of the ECM.[56-58] However, homeostasis is interrupted when transmembrane integrins on quiescent chondrocytes detect runt-related transcription factor 2

(Runx2). It is one of many factors which trigger apoptosis of chondrocytes in the mid layer of the ECM, if not promote premature senescence or terminal differentiation (i.e. hypertrophy).[11,20,22]

Hypertrophic chondrocytes in the ECM deep layer are larger in size and volume than quiescent chondrocytes.[59] They produce Runx2, and secrete Type X collagen instead of Type II.[22,26,59] Type X collagen is a core component of bone development during embryogenesis and fetal development, but it is a marker of osteophyte formation and bone sclerosis (thickening) in mature cartilage.[20,26,29,59] The hypertrophic chondrocytes also upregulate their secretion of collagenases (i.e. collagenase-1, -2 and -3) which are also produced by quiescent chondrocytes and fibroblasts in skin.[22,60] Otherwise called metalloproteinases (MMPs), and aggrecanases 1 and 2 which are known as ADAMTS-4 and -5 (A Disintegrin and Metalloproteinase with Thrombospondin motif),[22,26,59] these proteases are secreted into the ECM as pro-enzymes for activation.[61,62] Collagenases-1, -2 and -3 (i.e. MMP-1, MMP-8, and MMP-13) cleave collagen fibers in areas without Gly-Pro-Hyp residues (e.g. between Gly_{775} and Leucine$(Leu)_{776}$ or Gly_{794} and Leu_{795}),[54,61,63] and they can cleave aggrecan in the IGD where ADAMTS-4 and -5 are active too. However, the aggrecanases cannot reciprocate and cleave collagen,[12,19,59] thus MMPs are particularly deleterious to mature collagen fibers if they are in the ECM for a prolonged period (especially MMP-13 which has a higher affinity for Type II collagen).[2,64,65]

Ordinarily, the low levels of ECM proteases (i.e. collagenases and aggrecanases) produced by quiescent chondrocytes are rapidly suppressed by a family of Tissue Inhibitors of Metalloproteinases (TIMPs).[31,65,66] The deactivated proteases bind to low-density lipoprotein receptor-related protein 1 (LRP1) on chondrocytes for endocytosis,[61,62] so they are eliminated before they can cleave collagen which has a usual half-life of more than 100 years in the ECM.[31,67] However, the capability of metabolically active chondrocytes to maintain a balance in cartilage turnover is increasingly overwhelmed in states of injury or disease such as osteoarthritis.[11,13,19]

2.3 Osteoarthritis

Osteoarthritis (OA) is a complicated whole joint disease which often occurs in the knee (KOA). It is different to rheumatoid arthritis (RA) which is an autoimmune disease and less common.[68,69] Without one single initiating cause,[70,71] KOA can involve subchondral bone sclerosis, joint space narrowing, and osteophyte (bony spur) formation (Figure 2-10), but not in all cases and only after the disease has progressed from molecular level changes.[72-74] Cartilage degradation and low-grade inflammation are hallmark clinical signs of KOA,[22,69,75] but it is a heterogeneous disease with several phenotypes (Figure 2-11).

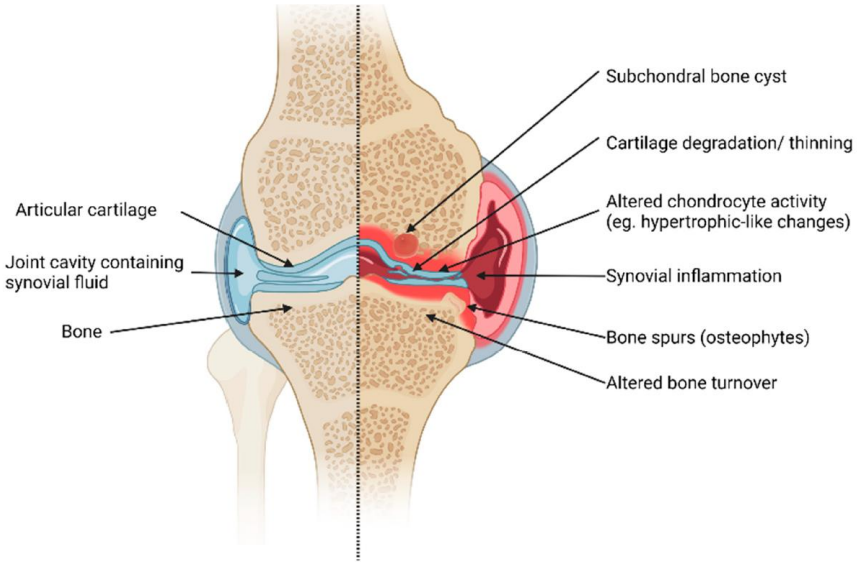

Figure 2-10. A healthy knee joint (left) and a knee joint showing signs of knee osteoarthritis (right) with synovial inflammation, narrowing of the joint space, formation of osteophytes and thinning of articular cartilage (adopted with permission from Larder et al.[76]).

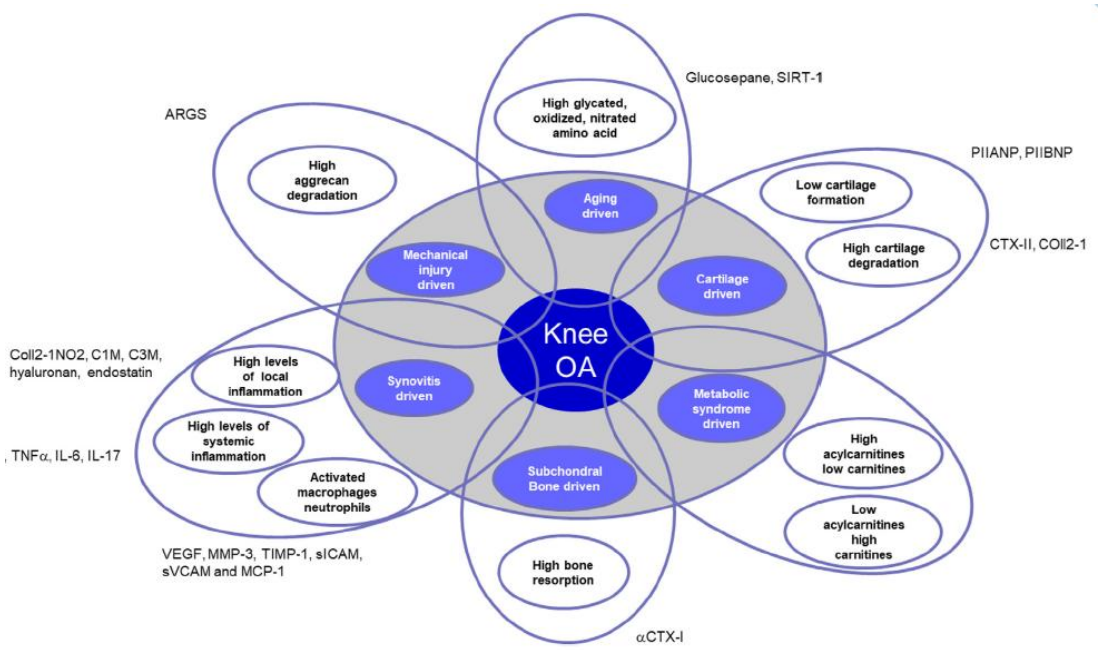

Figure 2-11. The known phenotypes and endotypes for knee osteoarthritis (with permission from Henrotin[77]).

2.3.1 Burden of disease

KOA impacts on the quality of life (QoL) of sufferers worldwide.[78] In 2020, the global prevalence of medically diagnosed KOA was estimated as 368 million cases, translating to an age standardized rate of 4,307.4 per 100,000.[79] The prevalence in New Zealand at that time was estimated as 362,000 cases, reflecting a higher age standardized rate of 4,637.8 per 100,000.[79] Moreover, the population-based quality-adjusted life year losses from KOA in NZ was estimated as 467,240 by Abbot et al.,[80] equating to 3.44 life year losses per person with KOA.

The projected prevalence of KOA in 2050 for males and females combined globally is 642 million (at an age standardized rate of 4,330 cases per 100,000). The projection for Australasia is 3.48 million, which is a higher age standardized prevalence of 4,910 cases per 100,000.[79] However, these figures are underestimates according to experts of the Osteoarthritis Research Society International (OARSI) community, due in part to the lack of accurate data they were based on, and that KOA is already established by the time a diagnosis is made.[73,81]

2.3.2 Diagnosis

Strictly speaking, medical diagnoses of KOA only occur after physical examination and radiographic imaging (i.e. X-ray). In reference to the Kellgren-Lawrence (KL) scale (Table 2-2), KOA is confirmed when structural changes are evident (i.e. KL Grade II: at least one osteophyte has formed with or without any joint space narrowing).[79,82] However, radiographic changes are often asymptomatic,[83,84] and a correlation between changes detected by radiography and reported symptoms (i.e. pain) is weak.[85,86] Meanwhile, the American College of Rheumatology (ACR) criteria are used in research, and KOA can be classified with clinical examination, with or without reference to imaging or laboratory data.[83] Consequently, the ACR criteria are helping OARSI establish a definition of early-stage symptomatic KOA (EsSKOA), and doing so may address the sensitivity/specificity issues with existing clinical trials due to the presumed inclusion of several phenotypes in one study.[81,83,87,88] Overall, understanding the risk factors for KOA can help identify cohorts for targeting research towards to lower the risk of its onset and progression.[89]

Table 2-2 Classification of osteoarthritis severity for a medical diagnosis (adapted with permission from Kellgren & Lawrence[90] and Kohn et al.[82]).

Grade	Definition
0	Unlikely: *Absence of X-ray evidence.*
1	Possible: *The formation of an osteophyte.*
2	Mild: *Definite osteophytes with or without minimal joint space narrowing.*
3	Moderate: *Definite joint space narrowing with subchondral bone sclerosis.*
4	Severe: *Pseudocystic areas with sclerotic walls in subchondral bone*

2.3.3 Risk factors

KOA has a complicated etiology with a myriad of interacting risk factors involved in its onset or progression (Figure 2-12). Some of these are fixed (i.e. non-modifiable), others are modifiable, and they all involve a low-grade inflammatory response to variable extents.[69-71,91]

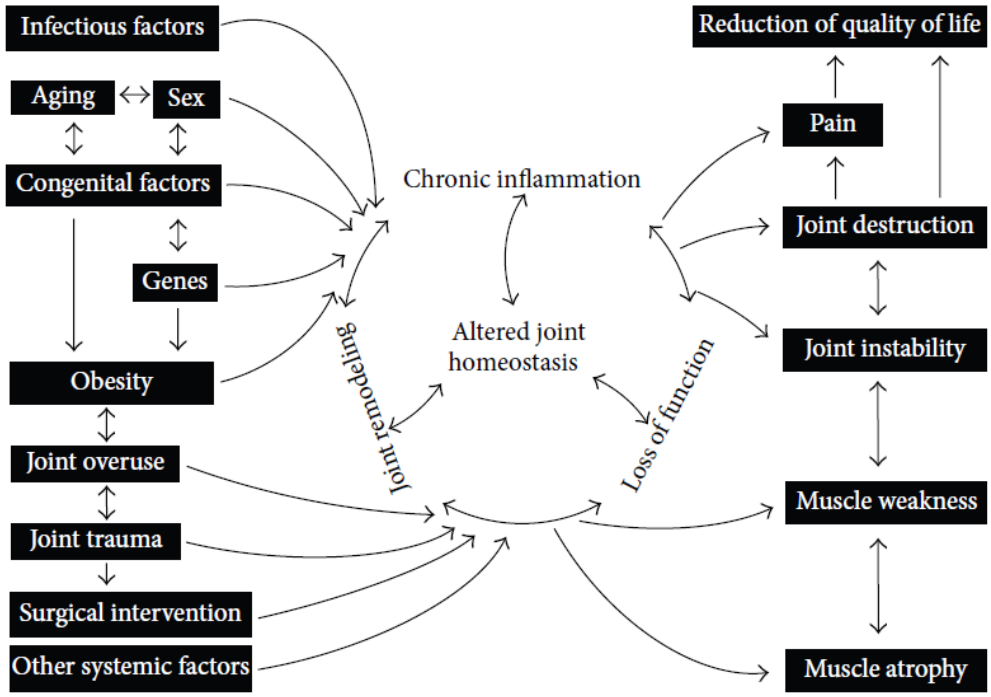

Figure 2-12. The interacting risk factors for osteoarthritis (adopted with permission from Wojdasiewicz et al.[92]).

2.3.3.1 Non-modifiable risk factors

Chronological age

Chronological (intrinsic) aging is the most significant of all risk factors for KOA.[93] Its median age of diagnosis is 55 years,[94] although articular cartilage starts losing function once it matures, especially after the age of 40.[95] In fact, over half of all new KOA cases in 2019 were under 55 years according to Weng et al.[96] This indicates the increasing prevalence of early-onset KOA due to the exacerbating effects of modifiable risk factors, such as body composition, on the changes already occurring naturally. For instance, there is an accumulation of advanced glycation end products (AGEs) over time which makes the ECM brittle (as discussed in Section 2.2.3.3), and mutations in mitochondrial DNA occur more often as the chondrocytes get older, thus their production of reactive oxygen species (ROS) progressively outnumber antioxidant enzymes in the ECM.[97] Ultimately, oxidative stress on chondrocytes adversely affects their capability of producing collagen and PGs (i.e. aggrecan) at an equivalent rate of breakdown during cartilage turnover.[22,46]

Sex

Postmenopausal women are expected to be over-represented in the burgeoning prevalence of KOA worldwide.[98] In 2020, its prevalence was 3% among males and females aged 25-49-years, 23% for the 50-69-year age bracket, and 38% for the 70+ age-group.[79] The prevalence of total OA (i.e. all joints) among women of all ages was 8.06% compared to 5.78% for men.[79] Overall, women are disproportionately affected, especially after menopause, indicating an effect from estrogen depletion, if not from other sex-related differences which are yet to be fully elucidated.[99-103]

2.3.3.2 Modifiable risk factors

Body composition

Body composition is the most prominent modifiable risk factor for KOA.[68,104] On one hand, excess body fat is associated with chronic low-grade inflammation (i.e. increased levels of IL1-β, IL6, TNF-α and CRP),[68,105] but being overweight or obese also imposes more load on knee joints which exacerbates the inflammatory response of KOA.[106]

Metabolic syndrome

Metabolic syndrome (MetS) has a modulatory effect as a risk factor for KOA.[104,107] It is a non-specific cluster of conditions which can exacerbate oxidative stress and increase the risk(s) of heart disease, stroke, or type two diabetes.[108-111] Hyperglycemia in itself is associated with increased production of AGEs, and they contribute to the stiffening of collagen fibers in the ECM which predisposes them to breaking.[46,112,113] There are several definitions of MetS (Appendix 2: Supplementary Table 9-1); of which the criteria can include hyperglycemia, hyperlipidemia, hypertension, and BMI above normal according to the WHO. However, a BMI above normal does not necessarily reflect excess body fat,[114,115] and central (android) fat distribution is considered superior to BMI in risk assessment of MetS.[116,117] Irrespectively, Noubiap et al.[117] found in their meta-analysis of data pooled from 28,193,768 participants worldwide, that the prevalence of MetS across all definitions increased with age and was higher in females compared to males. Android fat accumulation often occurs during menopause,[118] so postmenopausal women can be considered at increased risk of KOA through their raised risk of developing MetS.[107]

Trauma / injury

Damage to cartilage through physical activity (i.e. sports injury) is a major risk for post-traumatic (secondary) KOA.[93,104,119,120] A meta-analysis of 1 million participants across 53 studies by Poulsen et al.[121] found the risk of developing secondary KOA increased 4-6-fold after a knee injury. It differs to idiopathic (primary) KOA because the etiology is known, albeit quiescent chondrocytes lack the ability for optimal self-renewal after damage from disease or trauma (i.e. primary and secondary KOA).[30,122-124] The newly repaired ECM is fibrous with less flexibility than the undamaged areas,[122-125] and the inflammatory response exacerbates a cascade of catabolic conditions in the knee joint.[126]

2.3.4 The inflammatory response of knee osteoarthritis

2.3.4.1 Acute inflammation

Acute inflammation in general is an immediate response to injury or disease.[127] In cases of KOA, the surrounding microvascular system dilates and increases in permeability to allow acute phase proteins (APPs) such as C-reactive protein (CRP) into the synovium.[70,75,128] The ongoing presence of APPs produced locally leads to chronic (systemic) inflammation.

2.3.4.2 Chronic inflammation

Chronic inflammation is a highly organized cellular response. It is beyond the scope of this review to explore in full detail, but in brief, it involves pro-inflammatory cytokines such as interleukin-1-beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) being secreted by chondrocytes, among other cells such as adipocytes and macrophages. These cytokines exacerbate a catabolic milieu within the ECM and synovial fluid which causes swelling (i.e. clinical sign), and promotes pain which is the predominant symptom of KOA.[126,129-132] However, the origins of knee pain in KOA are complex with nociceptive and neuropathic components,[70,133,134] and inflammation is only one root cause of pain,[135] especially among women during menopause.[101,103,136]

2.3.5 Pain management

There is no cure for KOA yet, nor are there any effective disease modifying OA drugs (DMOADs). Symptom control is the priority,[104] with contemporary therapies ranging from oral analgesics such as paracetamol and nonsteroidal anti-inflammatory drugs (NSAIDs) through to intra-

articular (IA) injections of corticosteroids, or surgery (i.e. lavage or total joint replacement) as a last resort.[73,79,137] All of these options are limited in effect, if not highly invasive according to a meta-analysis by Thorlund et al.[138] Consequently, nutraceuticals have emerged as a concomitant consideration for improving the control of KOA symptoms.[139]

2.4 Nutraceuticals and knee osteoarthritis

Nutraceuticals are nutrition supplements with therapeutic effects for health conditions.[140-142] There are several micronutrients which have anti-inflammatory and/or antioxidant properties with the potential to attenuate inflammation and oxidative stress associated with KOA, but evidence of their effect in humans is generally inconclusive.[143-146] Meanwhile, the bioactive peptide (BAP) potential of collagen hydrolysate (CH) in promoting joint comfort (i.e. alleviating pain) is an emerging area of interest to researchers and consumers.[139,147,148]

2.4.1 Bioactivity of collagen hydrolysate

A pioneering *in vitro* study by Oesser and Seiffert[149] found CH could upregulate Type II collagen synthesis. The authors compared Type II collagen secretion from chondrocytes incubated in CH or a control medium at concentrations of 0.5 mg/mL for 11 days. They found Type II collagen more than doubled when chondrocytes were cultured in CH compared to the control.[149] The authors also compared two molecular weights (MWs) of CH sourced from bovine hide (MW = 1.9 kDa and 3.3 kDa), against CH sourced from chicken cartilage (MW = 3.1 kDa), versus comparators of wheat protein hydrolysate (mean MW = 1.5 kDa), undenatured Type I collagen (UC-I), undenatured Type II collagen (UC-II) or a basal medium.[149] The chondrocytes incubated in CH secreted more than twice the amount of Type II collagen compared to the controls at concentrations of 0.5 mg/mL, 1 mg/mL and 5 mg/mL ($p<0.01$), but not the higher concentration of 10 mg/mL.[149]

Nakatani et al.[150] expanded on that finding with their *in vivo* and *in vitro* studies. The authors fed mice one of four standardized diets for three weeks; a control diet was compared with three treatment diets containing phosphorus to simulate a catabolic environment (i.e. for promoting ECM degradation). The treatment diets replaced 50 g protein with either 5 g/100 g CH (sourced from

porcine, average MW of 5 kDa), 0.3 g/100 g Pro-Hyp or a negative control of 5 g/100 g gluten. Compared to mice fed the normal diet without phosphorus, a loss of chondrocytes from the STZ and mid layers of the ECM occurred among mice fed the treatment diets, but the greatest loss was among mice on the negative control diet of gluten (*p values* not shown). Cartilage thickness was approximately 1.3 times greater in the mice fed CH and Pro-Hyp diets than the negative control diet, so the authors concluded that treating mature quiescent chondrocytes with CH, or specifically with Pro-Hyp, lessened (but did not arrest) cartilage degradation.[150]

Nakatani et al.[150] further explored their findings with *in vitro* analysis. They compared chondrocytes cultured in a medium with phosphorus to simulate an OA model for 35 days against mediums with phosphorus and either CH or Pro-Hyp. They found the chondrocytes with CH or Pro-Hyp remained active and produced significantly more aggrecan than the chondrocytes in the control culture. Aggrecan increased ≈1.6-fold in CH compared to control (*p=0.03*) and ≈2.3-fold in Pro-Hyp compared to control (*p=0.002*). The authors also found significantly less mineralization deposits (i.e. ossification from Type X collagen) in contrast to the control (*both p<0.0001*). Therefore, treatment with CH, or specifically with Pro-Hyp, protected the chondrocytes from premature terminal differentiation on exposure to the catabolic condition synonymous with KOA.[150] However, the authors examined the effect of CH on chondrocytes extracted from healthy murine cartilage.[150] Similarly, Oesser and Seiffert[149] conducted their research on chondrocytes extracted from healthy bovine cartilage rather than diseased cartilage. Schadow et al.[151] have since extracted cartilage from humans who were having knee-joint replacement surgery for KOA. Chondrocytes from the diseased tissues were treated with three preparations of CH, all sourced from bovine hide (i.e. UC-I) but with differing MW ranges (RDH = 3.1-12.3 kDa; RDH-n = 0.4-3.3 kDa; Alpha = 2.9-8.1 kDa). Ultimately, the *in vitro* study by Schadow et al.[151] aimed to assess the effect of CH treatment on collagen turnover (i.e. synthesis and degradation) over six days versus a control culture without CH.

Collagen synthesis was affected by the three formulations of CH differently.[151] There were no effects observed for any of the three treatments compared to the control at a concentration of 0.1

mg/mL, but RDH (MW = 3.1-12.3 kDa) significantly reduced collagen synthesis at higher concentrations of 0.5 mg/mL ($p \leq 0.001$), 1.0 mg/mL ($0.001 < p \leq 0.01$), 2.0 mg/mL ($p \leq 0.001$) and 10.0 mg/mL ($p \leq 0.001$). Alpha (MW = 2.9-8.1 kDa) caused a significant decrease in collagen synthesis compared to control at concentrations of 2.0 mg/mL ($0.001 < p \leq 0.01$) and 10 mg/mL ($p \leq 0.001$), but not the lower concentrations of 0.5 mg/mL and 1.0 mg/mL. Meanwhile, RDH-n (MW = 0.4-3.3 kDa) did not impact on collagen synthesis compared to the control.[151] The authors' general conclusion was that CH treatment either attenuated or had no effect on collagen synthesis which contrasted with the earlier findings from Nakatani et al.[150] and Oesser and Seiffert.[149]

Similar effects were observed for cartilage breakdown.[151] RDH (MW = 3.1-12.3 kDa) caused the concentration of collagenase-1 (MMP-1) to significantly increase ≈40-fold compared to untreated controls at concentrations of 0.5 mg/mL ($0.001 < p \leq 0.01$), 1.0 mg/mL ($0.01 < p \leq 0.05$), 2.0 mg/mL ($p \leq 0.001$) and 10 mg/mL ($0.001 < p \leq 0.01$). The lower MWs of RDH-n (MW = 0.4-3.3 kDa) and Alpha (MW = 2.9-8.1 kDa) did not induce a statistically significant effect on MMP-1 compared to controls, except RDH-n at 10.0 mg/mL. In that instance, it caused a 6-fold increase in MMP-1 compared to the control ($0.01 < p \leq 0.05$). Meanwhile, RDH (MW = 3.1-12.3 kDa) significantly increased MMP-13 at concentrations of 0.5 mg/mL ($0.01 < p \leq 0.05$) up to and including 10.0 mg/mL ($p \leq 0.001$), but RDH-n (MW = 0.4-3.3 kDa) and Alpha (MW = 2.9-8.1 kDa) did not affect MMP-13 at any concentration compared to control.

The effect of CH on TIMP-1 secretion, which deactivates proteases,[61,152] was also evaluated by Schadow et al.[151] Its secretion by chondrocytes was reportedly unaffected by RDH (MW = 3.1-12.3 kDa) or RDH-n (MW = 0.4-3.3 kDa) but it decreased when chondrocytes were cultured in Alpha (MW = 2.9-8.1 kDa) at a concentration of 10 mg/mL.[151] The data and p value for that observation were not shown, but the study found treatments of CH from bovine hide at varying doses and MW ranges were generally ineffective for correcting an imbalance in collagen turnover.[151] In some instances, the authors found CH could exacerbate the catabolic ECM environment and promote breakdown, especially at a higher MW range of 3.1-12.3 kDa.[151]

Schadow et al.[153] produced more research which challenges the early evidence that CH is a chondroprotective agent. With their *in vitro* study (which also used chondrocytes extracted from humans with KOA), the authors compared CH sourced from porcine skin (MW = 3.5-7.4 kDa) with CH sourced from fish of two different MW ranges (Peptan F2000, MW = 6.7-17.9 kDa and Peptan F5000, MW = 31-654 kDa). None of the three treatments induced a significant increase in collagen synthesis at any concentration from 0.1 mg/mL up to 10 mg/mL over six days.[153] The CH sourced from porcine skin caused an increase of ≈125% compared to the control at culture concentrations of 0.1 mg/mL, 0.5 mg/mL and 1 mg/mL, but the CH preparations sourced from fish were less than 100% at the same concentrations. The observations were not statistically significant,[153] but introduced a notion that the animal source of CH may influence its potential bioactivity (as explored in the upcoming section 2.4.2).

Schadow et al.[153] also examined the effect of CH treatments on protease activity. They found that CH treatments could downregulate aggrecanase secretion in a dose-response manner, but it could increase its activity too.[153] ADAMTS-4 production was significantly less at concentrations of 0.1 mg/mL for all three treatments compared to control cultures without any CH (*0.001<p≤0.01*). For the higher MW of CH sourced from fish (Peptan F5000, MW = 31-654 kDa), ADAMTS-4 remained significantly less than the control up to and including the highest concentration of 10 mg/mL (*0.01<p≤0.05*). This held true for the lower MW of CH from fish (Peptan F2000, MW = 6.7-17.9 kDa), but only at a concentration of 0.5 mg/mL (*0.001<p≤0.01*). It (Peptan F2000) increased ADAMTS-4 approximately 10-fold at the higher culture concentration of 10 mg/mL compared to the control (*0.001<p≤0.01*) and it upregulated ADAMTS-5 secretion at concentrations of 5 mg/mL and 10 mg/mL (*0.001<p≤0.01*). In contrast, Peptan F5000 (MW = 31-654 kDa) and CH sourced from porcine (MW = 3.5-7.4 kDa) had no effect on ADAMTS-5.[153]

The effect of CH on collagenase activity was generally in keeping with the aggrecanase findings. CH sourced from porcine (MW = 3.5-7.4 kDa) increased the level of MMP-1 at concentrations of 2 mg/mL and 10 mg/mL (*0.01<p≤0.05*), but not the lower concentrations of 0.1, 0.5 or 1 mg/mL.[153] Peptan F5000 of fish origin (MW = 31-654 kDa) increased levels of MMP-1 at 10 mg/mL (*p≤0.001*) but

not any of the lower concentrations. It (Peptan F5000) significantly increased levels of MMP-13 at 2 mg/mL and 10 mg/mL (*0.01<p≤0.05*) but not at concentrations of 0.1, 0.5 or 1 mg/mL. Peptan F5000 was the only CH treatment to increase MMP-13,[153] whereas Peptan F2000 (also sourced from fish but with the lower MW of 6.7-17.9 kDa) had no effect on collagenase levels.[153]

Overall, the preclinical evidence for CH as a 'chondroprotective' ingredient is inconclusive. Schadow et al.[151,153] provided valid data for questioning the earlier conclusions of Oesser and Seifert[149] and Nakatani et al.[150] The origins of articular cartilage explants used in their respective studies were noticeably different, which might explain the contrasting results considering the morphology of chondrocytes within diseased cartilage differs to that of healthy cartilage.[15,154] Nonetheless, these preclinical trials have formed the foundation for human trials on the potential bioactivity of CH, but their results are generally inconclusive due to heterogeneity in study designs.[1,76,155] For example, most of the study samples have included male and female participants, and some of those studies recruited people with self-reported knee pain,[156,157] while others only included participants with a radiographically confirmed diagnosis of KOA.[158,159] Furthermore, the MWs of CH formulations have varied, as have the animal origins of those CH interventions.[155] Therefore, the influence of bioavailability is worthwhile to consider as a contributing factor to the heterogeneity in results of clinical trials to date.[155]

2.4.2 Bioavailability of collagen hydrolysate

The bioavailability of CH is a complicated concept. The molecular structure of UC-I extracted from a host animal can affect its potential BAP profile (i.e. concentration of Pro-Hyp). Type I fibrils are predominantly found in the skin and bone of vertebrates,[2,4,5] and they are produced by fibroblasts in the same way chondrocytes make Type II fibrils in articular cartilage.[31,160] However, Type I fibrils are heterotrimers rather than homotrimers (Figure 2-13); they have two identical Type I alpha-one polypeptide chains (α1), encoded by the COL1A1 gene, and one Type I alpha two (α2) chain, encoded by the COL1A2 gene (as opposed to three Type II α1 chains in cartilage encoded by the COL2A1 gene).

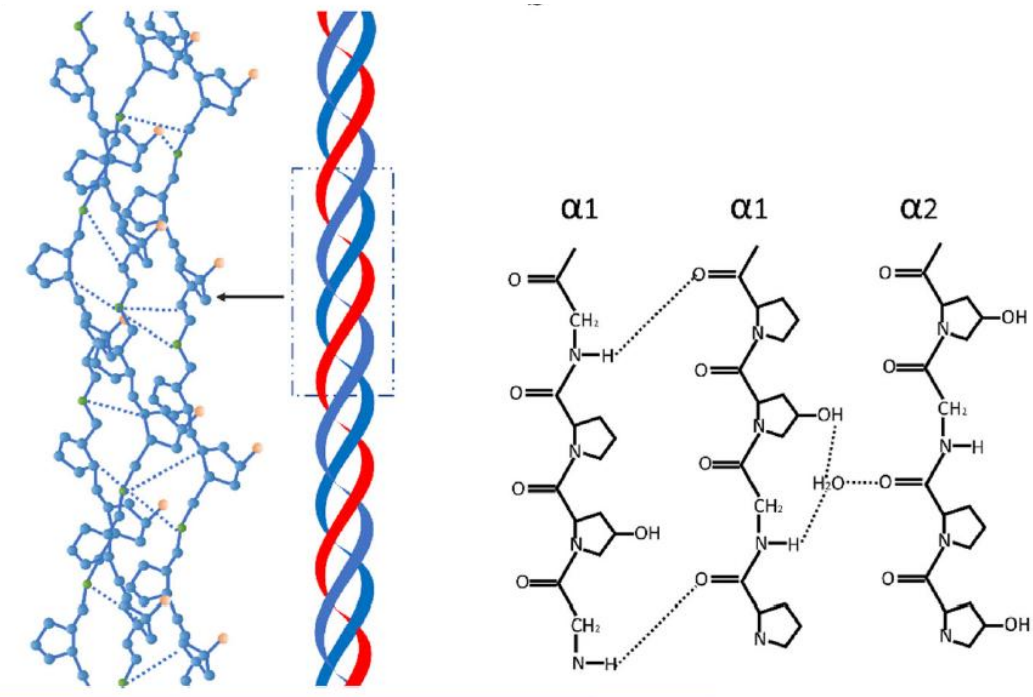

Figure 2-13. Image adopted with permission from Zhang et al.[41] showing the structural and molecular arrangement of Type I collagen fibrils.

Mature Type I and II fibrils are virtually mutual in structure and composition.[42,161] Type II fibers (i.e. supramolecular structures of fibrils) have more crosslinking between their telopeptides than Type I fibers,[31] but the uninterrupted helices of both types predominantly consist of Gly-Pro-Hyp.[31,36] However, Type I α2 chains are more variable in their X-Y residues than their two accompanying Type I α1 chains,[162,163] thus native Type I fibrils in the skin (i.e. UC-I) may have more variability in Gly-Pro-Hyp content compared to Type II fibrils in cartilage which impacts on the MW of CH after it is extracted from a host and hydrolyzed.[44,164]

2.4.2.1 Molecular weight

Undenatured Type I collagen (UC-I) has a length of 280-300 nm and MW circa 300 kDa.[3,165] In this native form, it is resistant to intestinal digestion,[1] but denaturation (partial hydrolysis) breaks the intramolecular bonds between its α chains to form gelatin with a MW of ≈100 kDa (Figure 2-14). Gelatin is generally used in the food industry as a thickening agent,[49] or to encase medicines and nutrition supplements in capsules.[166] Unlike UC-I, it can be absorbed, but only after proteolytic digestion in the gastrointestinal tract.[167,168] Meanwhile, extrinsic hydrolysis of gelatin produces

peptides with a MW generally between 2-6 kDa.[148,165] The MW of dipeptides, tripeptides and free AAs is <0.5 kDa,[169] thus a higher MW reflects the presence of longer oligopeptides which impacts on the transport efficiency of gelatin and CH after oral ingestion.[168-171]

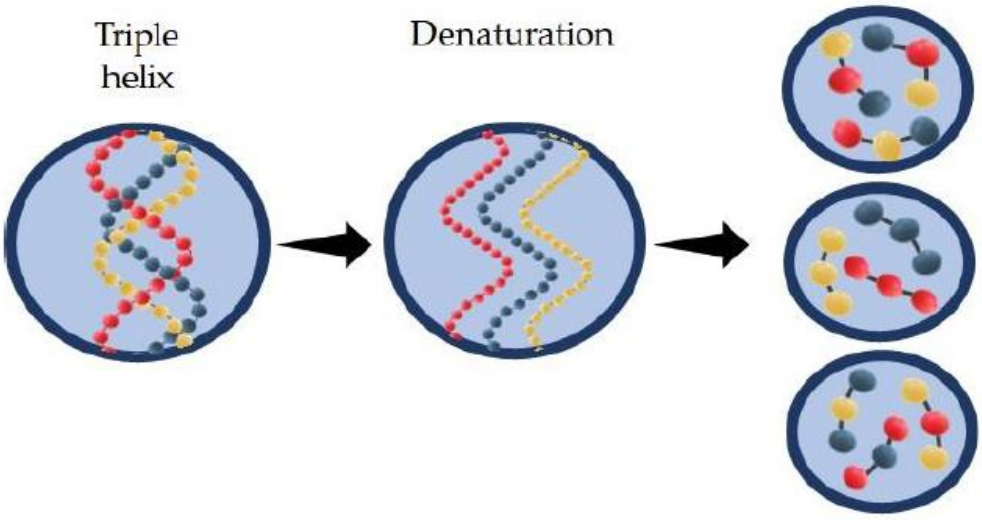

Figure 2-14. Denaturation (partial hydrolysis) of undenatured collagen (UC), and release of smaller peptides after hydrolysis (adopted with permission from León-López et al.[165]).

2.4.2.2 Absorption

Di- and tripeptides, if not free amino acids (AAs) are immediately available for absorption.[167] Figure 2-15 shows how they can pass through the Peptide Transporter 1 (PEPT1) on the brush border of the small intestine at a greater rate than AAs which use alternative systems.[37,38,167,172] Within the enterocyte, most of the small peptides are digested into AAs before passing through the basolateral membrane and into the portal vein,[167] but Pro-Hyp are resistant to peptidases in the cytoplasm so they pass into the blood stream through PEPT1 (or alternative active transporters).[167,170] The Gly-Pro-Hyp triplets can be absorbed this way,[173] but to a lesser extent considering they can be partially hydrolyzed by aminopeptidase N on the apical membrane of the intestinal tract (i.e. creating free Gly and Pro-Hyp dipeptides).[174]

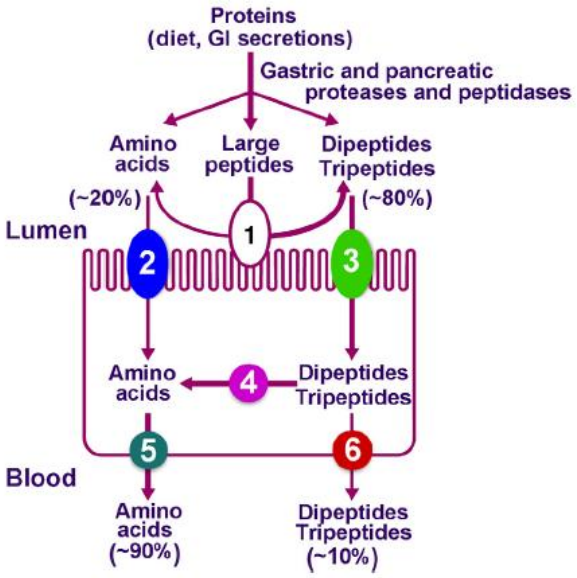

Figure 2-15. An overview of intestinal absorption adopted with permission from Bhutia and Ganapathy.[167] Undenatured proteins are resistant to absorption and undergo endogenous digestion by proteases and peptidases (1). Free amino acids use amino acid transport systems located on the brush border membrane for passing into enterocytes (2). Di- and tripeptides pass through the Peptide Transporter 1 (PEPT1) (3). Within the enterocyte, peptides are generally digested into AAs (4) for passing through the basolateral membrane and into the portal vein (5). The Pro-Hyp dipeptides are resistant to peptidases in the cytoplasm so can cross into the blood stream through PEPT1 if not other active transporters (6).[167,170]

The bioavailability of CH is superior to gelatin and UC-I. In a study of nine healthy adult females, Iwasaki et al.[175] compared the absorption of gelatin sourced from fish scales (Tilapia species) with two forms of CH differentiated by MW and derived from fish skin (Basa species) or scales (Tilapia species). The authors observed significant increases of plasma Hyp in its free form ($p<0.01$) and Pro-Hyp ($p<0.01$) across all three groups after ingestion, but peak plasma levels were achieved by 60 minutes in both CH groups and after 120 minutes for gelatin.[175] The levels of Hyp and Pro-Hyp were comparable for both CH formats, and they were higher than after ingesting gelatin ($p<0.01$). Therefore, the authors showed preferential absorption of CH peptides in contrast to the larger oligopeptides in gelatin. Moreover, they demonstrated foods containing gelatin (i.e. processed meats, bakery products, medicines encased in capsules) are dietary sources of Pro-Hyp which corroborated an earlier finding by Yamamoto. et al.[176]

Yamamoto. et al.[176] conducted their study on four healthy men fasted for 12 hours. The participants ingested solutions comprising various tripeptides in concentrations of ≈90% (CTP-100,

MW ≈0.3 kDa) and ≈50% (CTP-50, MW ≈0.6 kDa) at doses of 80 mg/kg. Specifically, Gly-Pro-Hyp tripeptides constituted 34% of the CTP-100 solution but only 7% of the CTP-50 solution for comparing with a control without any Gly-Pro-Hyp (MW ≈5 kDa). Blood samples were drawn pre-ingestion, and at five, 15, 30-, 60-, 120- and 240-minute intervals afterwards.[176] Plasma Gly-Pro-Hyp levels peaked within 30-60 minutes among those who consumed CTP-100 (≈20 nmol/mL) and CTP-50 (≈10 nmol/mL) whilst remaining negligible for the control ($p<0.05$). Plasma Pro-Hyp levels also increased, but to a greater extent than Gly-Pro-Hyp after ingestion of CTP-100 (≈60 nmol/L) and CTP-50 (≈30 nmol/mL) compared to control ($p<0.05$). By 240 minutes, plasma Gly-Pro-Hyp had returned to negligible levels whereas Pro-Hyp was still present and significantly higher ($p<0.05$) in the CTP-100 group (≈15 nmol/mL) versus CTP-50 group (≈5 nmol/mL), and the CTP-50 group was significantly higher than the control ($p<0.05$).

Yamamoto. et al.[176] showed endogenous digestion of Gly-Pro-Hyp occurs in a dose-response manner. They confirmed earlier results from Ichikawa et al.[177] who conducted their study on five healthy males who ingested CH sourced from fish, with a mean MW 5 kDa and dose of 0.385 g/kg body weight. However, Yamamoto. et al.[176] also did urinalysis of samples collected over 12 hours after ingestion. They found Pro-Hyp were the most abundantly excreted metabolite across all groups, followed by Gly-Pro-Hyp tripeptides which showed they remained intact in the circulatory system for eventual excretion in urine from the kidneys.[176] However, the authors did not assess the uptake of these peptides to other cells within the body (i.e. distribution), which is clearly difficult to do in humans.[164]

2.4.2.3 Distribution

Watanabe et al.[178] found in their murine study that Gly-Pro-Hyp tripeptides rapidly accumulated in the kidneys after ingestion more than other tissues including cartilage. Previously, Oesser et al.[179] had found in their murine study that CH peptides accumulated in cartilage within 12 hours of ingestion and peaked by 48 hours before starting to decline. They also found the CH peptides reached other organs including the kidneys and skin, although the uptake in skin peaked by 12 hours

and at a higher concentration than cartilage.[179] In other words, Oesser et al.[179] showed several cells are potential targets for CH peptides, and their finding has been corroborated by Kawaguchi et al.[172] in a similar study on rats. Therefore, identifying a source of UC-I with a high concentration of Gly-Pro-Hyp may optimize its bioactivity potential,[180] especially after gastrointestinal losses and distribution to competing target cells such as fibroblasts within the skin.[172,179]

2.4.3 Origins of collagen hydrolysate

2.4.3.1 Undenatured collagen

The animal source of UC-I is a key determinant of its Pro-Hyp yield. In a crossover single-blinded trial by Ohara et al.,[181] five healthy men ingested CH sourced from fish scale (CH_{scale}), fish skin (CH_{skin}), or porcine skin ($CH_{porcine}$). The mean MW of these supplements were comparable (≈5 kDa), and they were ingested at a dose of 0.385 g/kg body weight (approximately 25 g for a body weight of 65Kg) with a seven-day washout in between. Blood monitoring immediately before, and at 0.5, one, two, four, seven and 24-hour intervals after ingestion found plasma concentration of free form Hyp significantly increased and peaked between 1 and 2 hours for all three treatments.[181] The highest peaks were achieved after consuming CH_{scale} (≈120 nmol/L) and $CH_{porcine}$ (≈120 nmol/L), and they were both reported as significantly higher than CH_{skin} (≈80 nmol/L) without *p values* or confidence intervals shown. A similar result was observed for Hyp-containing peptides which peaked at two hours after ingestion for all three groups. There was a statistically significant greater increase in Hyp-containing peptides reported for CH_{scale} (≈140 nmol/L) compared to $CH_{porcine}$ (≈80 nmol/L), and that was reported as significantly higher than CH_{skin} (≈60 nmol/L), without estimates of error or *p values* shown.[181]

The study by Ohara et al.[181] is important in the context of this thesis. It provides insight into the heterogeneity in potential BAP profiles of CH extracted from UC-I of various animal sources. For example, Gly-Pro-Hyp tripeptides were absent in the study participants' plasma after consuming $CH_{porcine,}$ and they were negligible after ingesting CH_{skin} (3%) and CH_{scale} (5%) which can be explained by endogenous partial hydrolyzation of Gly-Pro-Hyp.[174,181] However, almost all the absorbed peptides recovered from participants who consumed $CH_{porcine}$ were Pro-Hyp (95%) which more than doubled

the amounts recovered from participants who consumed CH_{skin} (42%) or CH_{scale} (39%).[181] Therefore, $CH_{porcine}$ was a richer source of Pro-Hyp which validated earlier research from Iwai et al.[182]

Over two phases, Iwai et al.[182] examined the bioavailability of CH derived from UC-I derived from porcine skin ($CH_{porcine,}$ MW = 5 kDa), UC-I from chicken feet ($CH_{ChickFeet}$, MW = 12 kDa), and undenatured Type II collagen (UC-II) extracted from chicken cartilage ($CH_{ChickCart}$, MW = 12 kDa). First, they assessed the absorption of 9.4 g $CH_{porcine}$ consumed orally by five healthy adults fasted for 12 hours. The group's mean plasma level of free Hyp and Pro-Hyp were not presented, but the participants' peaks in free Hyp and Pro-Hyp were shown to range between 40-130 nmol/L and 10-40 nmol/L respectively by 60 minutes, if not before.[182] The free form of Hyp was absorbed at a ratio of ≈3:1 relative to Pro-Hyp which contrasts with more recent findings from Osawa et al.[183] In their *in vivo* study on rats, Osawa et al.[183] found intestinal absorption of peptide-bound Hyp was significantly higher ($p<0.05$) than free Hyp (16.6 µmol versus 6.6 µmol) during 60 minutes of vascular perfusion.[183] In other words, free Hyp was absorbed at a ratio of ≈1:3 relative to Pro-Hyp which is the direct opposite of what Iwai et al.[182] observed in their human trial. The discrepancy may be explained by the fact that absorption studies on animals do not necessarily transfer to humans,[184] or it may relate to the methods of extraction and hydrolysis which can influence the final MW (i.e. composition of AAs, and di or tripeptides as explained in the upcoming section 2.4.3.3).[44,166,169,171]

Iwai et al.[182] went on to assess the absorption of CH sourced from UC-I of chicken feet ($CH_{ChickFeet}$) and UC-II from chicken cartilage ($CH_{ChickCart}$). With seven healthy adults, they found $CH_{ChickFeet}$ (i.e. UC-I origin) ingested at a dose of 11 g per 60 kg body weight and $CH_{ChickCart}$ (i.e. UC-II origin) at a higher dose of 23 g per 60 kg body weight resulted in peak plasma levels of Hyp in its free form by 120 minutes among both groups. Peptide-bound Hyp levels peaked by 60 minutes among those consuming $CH_{ChickFeet}$ (UC-I) and by 120 minutes for the $CH_{ChickCart}$ (UC-II) group. The ratio of free Hyp to peptide-bound Hyp absorption was approximately 3:1 in both groups which is consistent with their finding from $CH_{porcine}$. However, $CH_{ChickFeet}$ (i.e. UC-I origin) evoked a greater rise in plasma Hyp and peptide-bound Hyp compared to $CH_{ChickCart}$ (≈150 nmol/L and 60 nmol/L versus ≈70 nmol/L and 20

nmol/L). The difference was not tested for significance,[182] but the study showed how the origins of UC (i.e. UC-I or UC-II, extracted from terrestrial mammals or avians) can influence the concentration of potential BAPs it may contain.[182] For example, CH sourced from porcine skin had the highest composition of Pro-Hyp (95%) compared to CH sourced from chicken feet (92%) and chicken cartilage (>70%).[182]

Bovine and porcine hides are traditionally the main sources of UC-I for extraction and hydrolysis.[166,185] Religious restrictions and concerns of contracting bovine spongiform encephalopathy (BSE) or swine flu have led to interest in poultry and sea-dwelling animals (i.e. fish) as alternative sources.[1,165,166] However, avian flu or contamination with heavy metals are concerns associated with those sources,[186] and they are different classes in the animal kingdom which means they have inherent differences in their thermoregulatory mechanisms.[187-190]

With some exceptions (i.e. tuna and dolphins), water-dwelling animals are cold-blooded (ectothermic). They rely on external (i.e. environmental) conditions for maintaining their core body temperature,[189] in contrast to terrestrial mammals and avians which are warm-blooded (endothermic) and maintain higher core temperatures through internal means.[190,191] Birds may have a higher internal temperature than terrestrial mammals,[190,191] but they do not have sweat glands, so they trap and release air between their skin and feathers.[189,190] Conversely, terrestrial mammals dissipate heat from their skin (covered with hair or fur depending on the genus and species) by conduction, convection, radiation and/or evaporation of sweat.[189,190] Porcines only have sweat glands on their snouts, so they rely on the other forms of heat loss from their skin.[189,192] Overall, these differences in thermoregulatory mechanisms impact on the thermostability of the host animal's UC-I.[44,187]

2.4.3.2 Thermostability

Thermostability of UC-I relates to its strength of coiling during synthesis.[5,36,40] Type I collagen fibers in the skin or hide of animals are created by fibroblasts in the same way as Type II fibers are formed within articular cartilage.[41,187,193] Tighter coiling is achieved by post-translational hydroxylation

of proline,[41,53,187] and the ensuing fibrils are strengthened by covalent crosslinks between Lys-Hyl in the telopeptide regions of tropocollagen monomers which self-assemble into collagen fibers.[35,39,41]

The thermostability of UC-I is expressed as a denaturation temperature (T_d). It is the critical point for initiating partial hydrolysis (i.e. denaturation) after extraction from the animal source, and it increases in proportion to the amount of Gly-Pro-Hyp within that host animal's UC-I fibers.[41,187,194] Porcine and bovine sources (i.e. terrestrial mammals) are comparable in composition of their UC-I,[163] and they contain more Gly-Pro-Hyp than sea-dwelling animals or avians.[163,187-190] However, animals living in warmer climates have a greater need for thermostability than animals in cooler conditions,[5,187,195] and the breed of animal can cause a difference too.[44] For example, sheep with black wool have higher skin temperatures than sheep with white wool, and the same may be seen in cattle with black hair (i.e. Holstein Friesian breed) rather than brown (i.e. Jersey breed).[190] Ultimately, terrestrial mammals are a richer reservoir of Gly-Pro-Hyp, but older animals have more non-enzymatic crosslinks between telopeptides within their native UC-I fibers due to the accumulation of AGEs.[44,45] Therefore, the extraction and hydrolysis of UC-I from older animals or those from warmer climates can be more challenging which impacts on the MW.[44,169,171,196]

2.4.3.3 Extraction and hydrolysis

The MW of CH formulations are highly variable. They differ between animal sources and even between batches of the same animal.[151,153,197,198] There are several methods available for hydrolysis, as there are for the pretreatment (removal of non-collagenous substances) and extraction methods preceding that step.[44,164,169,171] For instance, UC-I can be extracted by salt, acid, alkali, enzymes or a combination of those agents, if not by ultrasound or high-pressure means.[44,187,199] The hydrolysis techniques which follow include enzymatic, acidic or heat treatment, although subcritical water hydrolysis (maintaining water at a temperature of 100-374^{o}C and pressure 0.1-22 MPa) is also an option.[1,44,171,187] Overall, the chosen method for processing UC-I (i.e. extraction and hydrolysis) influences the MW and bioavailability of CH, irrespective of its animal source.[44,173]

Enzymatic hydrolysis is the preferred method for preparing CH after extraction of UC-I.[44] In their review, Hong et al.[44] outline how enzymes cleave specific peptides and allow for greater standardization in producing a low MW compared to acid and alkali treatments. Skov et al.[200] demonstrated the superiority of enzymatic hydrolysis in a human trial of 10 healthy young men. They compared the bioavailability of 35 g doses of enzymatically hydrolyzed collagen (ECH) derived from bovine bone with a placebo of water or a non-enzymatically hydrolyzed alternative (NCH) which had been prepared through heat treatment (H. Christine, email communication, October 23, 2024). Plasma levels of Gly-Pro-Hyp and the free forms of Gly, Pro and Hyp all increased significantly in the ECH and NCH groups compared to placebo ($p<0.0001$), but the effect was greater for ECH compared to NCH.[200] The mean plasma concentrations of Gly-Pro-Hyp were 849.1 µM versus 715.4 µM ($p=0.003$), while the free forms of Gly, Pro, and Hyp for the ECH and NCH groups were 448.7 µM versus 380.7 µM ($p=0.0059$), 335.4 µM versus 285.8 µM ($p=0.0074$), and 64.9 µM versus 48.9 µM ($p=0.0087$). The differences appeared after 20 minutes and were sustained for more than 240 minutes, but the peak of Gly-Pro-Hyp tripeptides in the ECH group was reached at 60 minutes, whereas peaks in the free forms of Gly, Pro and Hyp were achieved by 60, 90, and 120 minutes after ingestion.[200] The MWs of ECH and NCH were not reported, but the AA profiles were comparable which means the study showed how hydrolysis methods alone affect the bioavailability of CH peptides.[200] However, the choice of enzyme also has an effect.[169]

There are several commercially available enzymes for hydrolysis of UC-I.[44] Using UC-I extracted from bovine hide, Feng and Betti[169] examined alcalase, flavourzyme, and trypsin alone or in combination (e.g. alcalase, flavourzyme, alcalase-flavourzyme), and they found alcalase was the most effective individual treatment. Flavourzyme was the slowest acting and produced the greatest variability in MW whereas alcalase and trypsin were more effective at producing peptides with a MW less than 2 kDa, especially alcalase.[169] However, flavourzyme was generally more effective at releasing free AAs than the other enzymes, as evidenced by the free form of Hyp only being detected after hydrolysis by flavourzyme.[169] The free form of Gly was more variable between treatment with alcalase,

flavourzyme, trypsin or a combination of those enzymes,[169] but the free form of Pro was not detected after any of the treatments which is surprising considering some Hyp was released after treatment from flavourzyme. The authors attributed the anomaly to a likelihood that most Pro-Hyp dipeptides were intact after hydrolysis which is consistent with its ability to reach the kidneys, cartilage and skin after ingestion and absorption as previously explained. Overall, Feng and Betti[169] re-illustrated how hydrolysis of UC-I can influence its MW, thus bioavailability as CH.

2.4.4 Safety of collagen hydrolysate

Oral intake of CH is generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA).[159,201] The European Food Safety Authority (EFSA) also consider CH derived from bovine hide as safe for human consumption, thereby allaying historical concerns of contracting BSE.[187,202-205]

CH supplements are well tolerated. Their water solubility increases after hydrolysis of gelatin as the gelling properties are removed.[175,206] They can be mixed with many foods or fluids without issue, especially as a relatively high concentration of Gly results in a neutral taste compared to hydrolysates of other proteins which can be bitter.[207] However, the MW (i.e. extent of hydrolysis) will impact on the osmolarity of the solution,[167] and a lower MW leads to hyperosmolarity whereas more peptides and less free AAs will reduce its hyperosmolarity and lessen the likelihood of gastrointestinal upsets.[167] A systematic review by Choi et al.[208] examined 11 studies with a total of 805 participants, and they found no adverse effects of CH supplements were reported for doses of 2.5-10 g each day in interventions of four to 24 weeks. Abrahams et al.[209] also found in their mixed methods study of 14 females without any medical conditions and aged 46±6.2 years, that 20 g CH (bovine source) consumed twice daily as 10 g doses for six weeks resulted in reduced symptoms of bloating, constipation, stomach pain and acid reflux compared to 14 days of their usual diet without CH. The study had a high dropout rate and inconsistent reporting of symptoms by participants,[209] but it generally supports a point that CH supplements are well tolerated.

Oral supplements of CH are low in immunogenicity.[1,163] The chances of eliciting an immune response are low because the antigenic sites (epitopes) on native UC-I helices are removed during partial hydrolysis.[1,163,187] Epitopes can stimulate the oral response in the gut-associated lymphoid tissue (GALT) of the intestinal tract,[210-212] and UC-II epitopes may be transported into dendritic cells which promote T cells travelling to articular cartilage to inhibit the proinflammatory environment of cartilage breakdown (Figure 2-16). Only UC-II epitopes can stimulate this response in cartilage (which is primarily comprised of Type II rather than Type I collagen),[1,213,214] so UC-I from animal hide must undergo hydrolysis for any potential therapeutic effect as a nutraceutical for KOA.[187] In other words, UC-II (i.e. from chicken cartilage) has an entirely different proposed mechanism of action as a nutraceutical for KOA relief which explains why oral doses of CH are generally higher than doses of UC-II.[1,215,216]

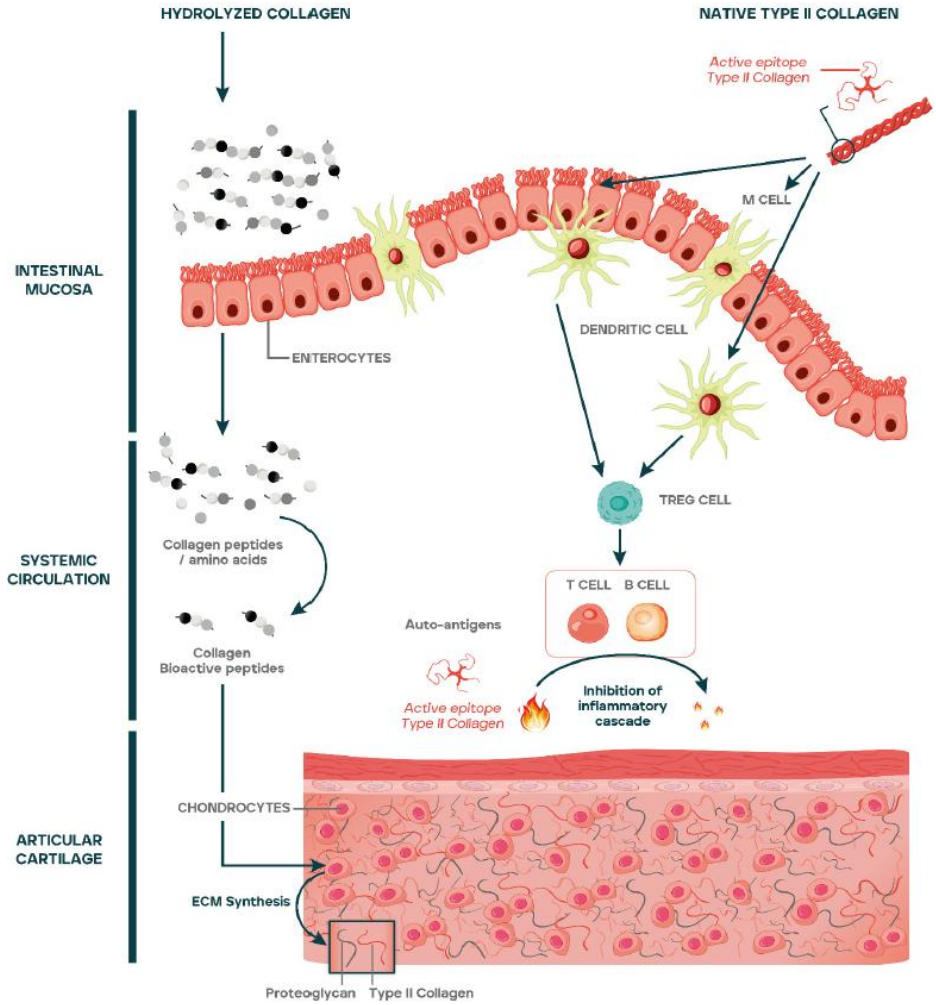

Figure 2-16. Differentiating between the proposed mechanisms of action of collagen hydrolysate (CH) and undenatured Type II collagen (UC-II) as a nutraceutical. UC-II is postulated to induce the oral response within the gastrointestinal tract whereas CH may exert a direct effect on the ECM after first-pass metabolism (adopted with permission from Martínez-Puig et al.[1]).

Oral intake of CH must allow for gastrointestinal losses in first-pass metabolism.[215,217] A daily dose anywhere up to 10 g/day is typical in human studies on CH, but this arbitrary dose is based on a study from more than 25 years ago by Moskowitz.[218] Jiang et al.[158] and Kumar et al.[159] found daily doses of 8-10 g CH (porcine and bovine sources) for 12 weeks to six months were safe in reference to liver and kidney function (serum and urine biomarkers), and considerably more research has been conducted since the study by Moskowitz[218] which suggest higher daily doses are safe for assessing the efficacy of CH with intervention trials on human subjects.[219,220]

Wu et al.[219] conducted their study on rats for eight weeks. With doses of CH (porcine source) that were approximately equivalent to 10 g/day, 100 g/d and 1,000 g/day in humans with a body

weight of 60 kg, they found nil adverse effects on liver function over the intervention. After fasting for 12 hours, Shigemura et al.[220] gave four healthy human subjects (two male, two female) CH sourced from fish (MW 1-5 kDa) in single doses of 30.8 mg/kg, 153.8 mg/kg, and 384.6 mg/kg which equated to doses of 2 g, 10 g, and 25 g for a human body weight of 65 kg. Plasma levels of free Hyp and peptides containing Hyp began to increase after 30 minutes postprandially and peaked for all doses by two hours. The effect of 2 g was negligible, but higher doses had an incremental dose-dependent effect ($p<0.05$). Maximum absorption was not reached with 25 g, so a higher dose could be expected to have an even greater rise in plasma levels of Hyp and peptide-bound Hyp.[220] Therefore, these studies show that daily doses above 10 g are safe for human trials to assess the BAP potential of CH.[219,220] In fact, daily doses between 20 g and 60 g have been ingested in similar studies examining the efficacy of CH in promoting Type I collagen biosynthesis within muscles without any adverse effects reported.[221-223]

2.4.5 Intervention efficacy

2.4.5.1 Subjective assessment

Relevant outcomes are vital for evaluating interventions for symptom relief of KOA.[224,225] Patient reported outcome measures (PROMs) provide data from study participants' perspectives,[224,226,227] but they are highly variable between individuals as they depend on demographics, genetics and psycho-social factors such as individuals' pain tolerance levels.[228,229] Furthermore, PROMs are subject to recall/response bias (i.e. a respondent's inability to remember their baseline score at endpoint), especially in the orthopedics field of study on KOA.[230,231] Therefore, the OARSI encourages capturing PROMs in research with valid and reliable instruments.[224]

Assessment instruments

The visual analogue scale (VAS) is a single-item measure of pain intensity.[232,233] It was designed in paper format, although it has been validated for self-administration on computers and smart phones.[234,235] It can be used on a numerical scale or with a horizontal line measuring 100 mm for greater sensitivity,[232,236] as respondents are restricted to scores on the ordinal scale (i.e. 0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe).[237-239] Alternatively, they can place a mark anywhere on the horizontal line to express their unique feeling of being pain free (i.e. far left) to having pain as

bad as can be on the far right.[224,236,239] Both formats provide comparable data, but the ordinal scale is preferred by respondents and researchers for reasons of simplicity in completing it or interpreting the results.[232,239,240] Statistically significant improvements are generally accepted with differences ≥30 mm or score changes ≥1,[224,241,242] thus participants must have enough perceived pain at baseline for detecting statistically significant effects at endpoint.[241]

The VAS may serve best as a screening tool for clinical trials. It is a unidimensional measure of pain intensity which does not give insight into the activity causing the pain, or whether the pain occurs during or after the activity.[232,236] In other words, the VAS is limited in validity as a construct compared to alternatives such as the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), the Knee Injury and Osteoarthritis Outcome Score (KOOS), or the Intermittent and Constant Osteoarthritis Pain (ICOAP) tool.[243-246]

The WOMAC was recommended for use in clinical trials by Altman et al.[224] It is self-administered, and more patient-focused than the instruments which preceded it such as the Le Quesne Index of Severity for Osteoarthritis of the Knee (LISOK) and the Lysholm scoring scale.[247,248] The LISOK and Lysholm scale are clinician-administered, and neither of them assess respondents' functional capability. The LISOK was validated for triaging patients awaiting joint replacement surgery and to assess the efficacy of drug therapy for pain relief.[247,249] The Lysholm scale was developed to assess participants' post-operative response to knee ligament surgery.[248] It has been used in studies of OA symptom management,[156,158] and it measures respondents' beliefs about how much activity they think they can perform before noticing instability or pain.[248] However, the Lysholm scale is criticized for assessing symptoms and functions in one score rather than differentiating between the two constructs as WOMAC, and subsequently KOOS have been tailored to do.[246]

The WOMAC was designed by Bellamy et al.[243] for elderly individuals with radiographic evidence of hip or KOA (i.e. KL Grade ≥II). It is typically delivered on a 5-point numerical scale of 24 questions within three domains of pain (five questions), stiffness (two questions), and physical function (17 questions). The summed scores of each domain aggregate to a total out of 96 which

reflects each respondent's perceived affliction from KOA on a best-worst scale.[243] However, combining the three domain scores impacts on the interpretability of data according to Roos and Lohmander.[250] For example, the primary outcome of a study may be to evaluate pain specifically (i.e. maximum score of 20), but that domain score is diluted within the total out of 96.[226] Another limitation is that WOMAC does not assess respondents' perceived ability in sport and recreation activities, nor does it assess the impact of KOA on their quality of life (QoL).[246] Therefore, Roos et al.[246] developed the KOOS for younger cohorts with or without radiographic evidence of KOA.

The KOOS has arguably superseded WOMAC for usefulness in clinical trials on KOA.[250,251] Both WOMAC and KOOS can be administered reliably in hard copy or electronic format,[252] and they are validated for use in several languages.[250,253] Furthermore, WOMAC and KOOS are more specific than other available self-administered tools like the ICOAP which evaluates pain across 11 items without exploring other constructs (i.e. functional capability and impact of symptoms).[245] However, the KOOS elicits a deeper understanding of a respondent's perceived burden from KOA than WOMAC.

The KOOS adopts all 24 questions from WOMAC. It has a total of 42 questions across five domains of pain, symptoms, activities of daily living (ADLs) which is equivalent to the function domain from WOMAC, sport and recreation function (sport) and quality of life (QoL). The QoL domain is an essential metric to assess according to the Outcome Measures in Rheumatology (OMERACT) – OARSI recommendations,[225] but the sport domain may be irrelevant for respondents with KOA who may not feel able to engage in the activities of running, jumping or squatting within that domain.[251,254] For example, Collins et al.[244] reported 74% of the sport domain questions in KOOS were considered "not applicable" ("n/a") by older adults undergoing knee replacement surgery. Roos and Toksvig-Larsen[251] tried adding a sixth response of "n/a" to that domain in their study of patients with KOA awaiting knee replacement surgery, but the authors advised against doing so in future to avoid issues with missing data. For instance, "n/a" responses are considered missed rather than scored according to the KOOS user guide instructions,[251] and the raw score is invalid If three out of the five sport domain questions are missed or marked as "n/a."[255] Nonetheless, KOOS is suitable for active cohorts at increased risk of

primary and secondary KOA, if not already at an early stage but still able to engage in sport and recreation activities.[246] Ultimately, it offers more constructs for evaluating as secondary outcome measures than WOMAC.[226,246]

The scoring and interpretation of KOOS is distinctly different to WOMAC. Raw scores of each KOOS domain are on the best-worst scale (i.e. same as WOMAC), but they remain separate for transforming onto a worst-best scale out of 100.[246] For example, a baseline raw score of 36/36 for the pain domain (i.e. indicating extreme pain) converts to 0/100,[255] and a reduction in pain after an intervention would correspond to a lower raw score thus increased score out of 100 (i.e. the raw score may improve from 36/36 to 30/36 which corresponds to 0/100 improving to 16.7/100). This is the exact opposite of how improvements in WOMAC scores are detected on the aggregated best-worst scale which can be confusing when critiquing studies with PROMs derived from WOMAC or KOOS.[226,256]

An algorithm was introduced by Roos et al.,[246] to facilitate the conversion of domain raw scores from WOMAC (pain, symptoms, function) onto separate scales to compare with KOOS.[250,255] Authors using WOMAC must report each of the three domain scores for that to be possible, but that is not standard practice. For example, a study by Benito-Ruiz et al.[257] showed a change in the WOMAC pain domain score for their intervention and comparator groups without reporting the groups' mean pain domain scores at baseline and endpoint. Similarly, Jiang et al.[158] appear to have reported their participants' mean response score for each of the five questions in the pain domain (i.e. 0 to 4) rather than a mean value for that domain which has a maximum score of 20. In both instances, the pain domain scores cannot be compared with data derived from KOOS, even though WOMAC and KOOS are comparable in reliability and responsiveness.[244,250,258]

Reliability and responsiveness

Reliability relates to the reproducibility of results from an instrument. It is a measure of accuracy in the tool used within and between testers over time.[259,260] Responsiveness is the instrument's ability to detect a change considered important by a participant or practitioner.[240,260,261]

It is a psychometric referred to as the minimal clinically important difference (MCID) or minimally important change (MIC).[230,262,263]

There is more than one way to estimate an MCID.[262,264] The anchor-based method involves participants indicating if they believe their symptoms have improved, worsened or deteriorated after an intervention.[261,265] Thereafter, an estimate is made through several options including predictive modelling (i.e. logistic regression), the mean change score of responders reporting an improvement, or using the receiver operating characteristic (ROC) curve which focusses on the area under a curve (AUC) data to discriminate between improved and unimproved responders.[264,266] Alternatively, the consensus method disregards participant perceptions (i.e. there is no anchor question) and uses a threshold agreed upon by experts in the discipline as important.[261,265] Lastly, the distribution method is grounded in statistical theory.[264,265] It can be calculated with the standard deviation of an entire sample, or by multiplying the mean standard error of measurement (SEM) in changes of non-responders to treatment by the upper 90% or 95% confidence interval.[259,264,265,267]

The anchor-based method is generally preferred for estimating the MCID. It is respondent-focused, with predictive modelling offering more accuracy than the mean change or ROC methods.[261,266,268] Conversely, an integrative approach of combining the anchor and distribution-methods allows for greater precision according to Molino et al.[269] Irrespective of how the MCID is calculated, it allows for thoughtful interpretation of statistics because it can be compared with the minimal detectable change (MDC) which is the point beyond random variability.[260,261,265,270] Otherwise known as the smallest amount of change attributable to an intervention rather than chance,[230,259] an MDC above the MCID value indicates participants' perceived improvements are probably explained by error or random variance in sampling,[226] while an MDC lower than the MCID suggests the perceived improvements are likely explained by an effect from the intervention.[226,265] However, achieving an MCID value higher than the MDC does not guarantee a patient acceptable symptom score (PASS) has been reached.[226]

The PASS is an estimated absolute value of participants' perceptions about success or satisfaction after an intervention.[226,265] It is focused on participants reaching a particular score at the end of the intervention rather than a change in score from baseline to endpoint as per the MCID.[226,271,272] A participant may report a noticeable improvement in their pain (i.e. their change in pain score from baseline to endpoint surpassed the MDC and MCID values), but they may not feel well enough or notice enough pain relief to consider the treatment successful as shown by Roos et al.[271] They analyzed postoperative data from participants who received early or delayed reconstructive knee surgery in combination with physiotherapy for an anterior cruciate ligament (ACL) injury. Almost all the participants in both groups reported improvements in pain scores which exceeded the MCID after two years,[271] but only 61.3% of those who received early surgery reported their pain to have improved enough to consider their treatment a success (i.e. surpassing the PASS score). In contrast, 54.2% of those who received delayed surgery considered it a success, thus they were less satisfied despite both treatments having provided noticeable relief from pain.[271] Overall, the PASS and MCID values are complementary to each other in the interpretation of PROMs, but the heterogeneity in methods for estimating MCID values can compromise the usefulness of that metric according to Franceschini et al.[264]

Interpreting PASS, MCID and MDC data derived from KOOS is complicated.[226,230,272] Roos[226] identified a PASS score of 89/100 for the pain domain based on a study by Ingelsrud et al.[273] The data were from young adults who sustained an ACL injury and underwent reconstructive surgery for follow up at 6 months, 12 months and 24 months.[273] The participants' mean baseline pain domain score was 72.5±19.3/100 which is considered mild,[226] and the PASS score of 89 is within the range considered completely free from pain.[226] In contrast, Boffa et al.[267] reported PASS values of 73.6/100 and 76.4/100 for their pain domain mean at six and 12 months after intra-articular (IA) injection of platelet-rich plasma in a more recent study of older participants with medically diagnosed KOA. The baseline pain score for their participants was 67.6±17.1/100 which is within the range considered mild,[226] as are the PASS values of 73.6 and 76.4 at six and 12 months.[226] Therefore, the participants' characteristics at

baseline (i.e. age, baseline pain levels and presenting clinical condition) can influence their PASS value or expectations from treatment at endpoint.[226]

There is a shortage of PASS data for KOA patients receiving non-surgical therapy for pain assessed with KOOS at present. The MCID for pain was originally estimated as a change of eight to 10 points based on WOMAC.[250,256] The MDC value for KOOS was estimated as six for patients with a knee injury or 13 for patients with KOA,[256,274] but more studies have emerged since the inception of KOOS, all of which have varying MCID and MDC values for the pain domain.[264,275] Along with the numerous methods available for estimating the MCID (i.e. intrinsic factors), extrinsic factors such as study characteristics (i.e. participant group and treatment type) are just as influential.[230,262,264,266] For example, Molino et al.[269] showed in their retrospective study on patients who received knee replacement surgery for KOA that the MCID for pain was 16 or 31 depending on the anchor question used. It was 31 or six depending on the cutoff defining intervention efficacy, and 25 or 31 depending on the sample size of 50 or 100 participants. Boffa et al.[267] reported a lower MCID of nine in their study of more men than women with KOA who received IA injection of platelet rich plasma, whereas Mostafaee et al.[268] estimated an MCID of 15 for a cohort consisting of more women than men with radiographic evidence of KOA who received physiotherapy for four weeks. Meanwhile, Ingelsrud et al.[266] reported a smaller MCID of 2.5, albeit outlining their participants were not suffering KOA and were post-operative of ACL repair. In other words, their participants' low pain domain scores at baseline left little room for improvement at endpoint.[266] Ultimately, MCID values are estimates based on subjective data,[230] and Molino et al.[269] recommend calculating an MCID value for each individual study rather than comparing its data with published values which are highly variable and depend on the estimation method used.[264]

2.4.5.2 Objective assessment

Objective outcomes evaluate effects of interventions with more certainty than PROMs.[226,227,231,276] Perceptions of pain can affect participants' performances in functional tests,[277] but a correlation between perceived ability and physical performance in walking or sitting tests is relatively

weak.[276,278] Therefore, collecting objective data alongside PROMs improves the rigor of a study.[224,225,278,279] Examples of objective data include functional tests and/or monitoring biochemical markers (biomarkers) of cartilage turnover and inflammation.[280,281]

Functional tests

The progression of KOA from an early, pre-radiographic stage (i.e. KL Grade <II) to advanced stages (i.e. KL Grade ≥II) correlates with a decline in muscle strength, especially for women.[282] Assessing walking speed offers prognostic value for clinicians, as the risk of radiographic KOA (KL ≥II) can increase 8% for every 0.1 m/s decline over 12 months.[260] Dobson et al.[280] recommended a battery of activities which were endorsed by OARSI to assess functional impairment from symptoms of KOA (Table 2-3). They are intended for researchers and clinicians,[280] and were designed for cohorts aged ≥40 years with diagnosed KOA (i.e. KL Grade ≥II), including those either awaiting or having had joint replacement surgery.[280] Therefore, the activities are safe among populations at an earlier stage of KOA before a diagnosis is possible. Three activities are considered essential (core), while the remaining two were offered as optional for including in certain situations such as clinical trials.[280] All of the activities were designed with guidelines for ensuring optimal testing conditions are held constant (i.e. ideal heights of chairs or lengths of courses are used and reported), and they are freely accessible on the OARSI website with video demonstrations on how to administer each test.[280,283,284] Even so, Chen et al.[285] found in their meta-analysis that very few studies have incorporated the functional tests into their respective studies of KOA interventions.

Table 2-3. The recommended activities for testing functional capability among those with symptoms of knee osteoarthritis (adapted with permission from Dobson et al.[280]).

Functional Activity[A]	**Recommended Test**
Sit-to-stand	*30 second chair stand*
Walking short distances	*4x10m fast-paced walk*
Stair negotiation	*Nothing recommended*
Ambulatory transitions	*Timed up and go*
Aerobic capacity / walking long distances	*Six-minute walk*

[A]Grey shading denotes core activities to include in testing.

30-second Chair Stand (30s-CST)

The 30-second Chair Stand Test (30s-CST) was ranked as the best sit-to-stand activity of all options explored by Dobson et al.[280] Participants start in a seated position with their back on the rear

support of a chair and arms crossed at the chest. A chair apron height of 43 cm was recommended,[280] although alternative heights are acceptable, assuming the same chair is used for repeated measures. After a count down, the participants stand up straight then immediately return to the seated position to complete one repetition. They continue doing so for 30 seconds, if not reaching exhaustion beforehand. The number of completed repetitions over 30 seconds is counted, and an adapted score is used if the participant was unable to begin in the crossed arms position.[280] If they begin as per protocol but break that position during the test, then only the number of completions with crossed arms are counted.

The 30s-CST has high test-retest reliability in clinical research.[286] An improvement from baseline is more repetitions completed within 30 seconds, but there are very few studies using this test so MDC and MCID values are difficult to find. Dobson et al.[259] found among a sample of 27 men and 24 women aged 51-81 (mean = 64.5±6.2) years with medically diagnosed KOA, that the MDC was 2.0 (calculated at 90% confidence level). Similarly, Holm et al.[287] reported the MDC of 2.4 repetitions among 40 participants (n=22 women) aged 68.8±8.3 years with radiographically confirmed KOA (i.e. KL Grade ≥II). Wright et al.[288] reported an MCID (i.e. perceived improvement) as 2.0-2.6 repetitions after their study on 65 patients with hip rather than KOA. Therefore, at least 2.0 repetitions would reflect a change in performance based on this existing evidence.

40m Fast-Paced Walk Test (FPWT)

The 40-metre Fast-Paced Walk Test (FPWT) requires participants to walk at their fastest speed, without running, as safely and comfortably as they can over 40 m.[280] The course can be set in various configurations (i.e. 2x20 m, 4x10 m or 8x5 m lengths), although 4x10 m is ideal.[280] Study participants complete four lengths of 10 m by rounding a cone placed 1 m beyond the testing zone and returning to the start for rounding another cone, also 1 m beyond the testing zone. Each participant continues doing so until they have covered the 40 m distance walking in a straight line. The time taken to complete the task within the measured area is recorded and a pace calculated thereafter.[280] A rehearsal of at least 1-2 turns (i.e. 10-20 m) is recommended to check participants' understanding and

improve the reliability of test results.[260,280] For instance, a practice can mitigate the impact of any potential learning effect (i.e. improvement through familiarization) which could confound results.[289]

Improvement in the FPWT is an increased (faster) walking pace.[260] Dobson et al.[259] found among their sample of men and women with medically diagnosed KOA, an MDC (calculated at 90% confidence level) of 0.19 m/s for the test while Holm et al.[287] reported an MDC of 0.20 m/s among their participants with radiographically confirmed KOA (i.e. KL Grade ≥II). Holm et al.[287] used a 2x20 m configuration rather than the 4x10 m course used by Dobson et al.,[259] and the MDC values are essentially the same. In contrast, Master et al.[260] reported an MDC of 0.32 m/s for the test performed by participants walking at their usual pace rather than their fastest which outlines the importance of reporting testing conditions to facilitate fair comparisons between studies.[260]

Stair Climb Test (SCT)

Stair negotiation is a difficult task for anyone with KOA.[290] Improving performance is a rehabilitation goal of clinicians and an outcome of interest to researchers,[291] but a specific test was not recommended for the core activity by Dobson et al.[280] due to a lack of data available at the time. Even so, the authors did encourage a protocol of nine steps, with each step measuring between 16-20 cm in height.[280]

The Stair Climb Test (SCT) is apt for testing stair negotiation. Participants walk at their fastest pace as safely and comfortably as possible up and down a flight of stairs. A start line is placed at the base of the stairs and participants can use the handrail for support going up or coming down after rounding a cone on the stair landing. They perform the timed activity twice, once as a rehearsal and then after resting from that practice. Time to complete the second attempt is recorded by at least two testers so an inter-rater average can be calculated.[280]

The SCT has high test-retest reliability.[292] Iijima et al.[292] found an MDC (95% confidence level) of 0.102 s for an 11-step SCT in their study of 59 individuals (73% female) aged 50-69 years in the pre-radiographic phase of KOA (i.e. KL Grade <II). Dobson et al.[259] reported a higher MDC of 2.33 s after their 11-step SCT on participants who were medically diagnosed with hip or KOA, while Holm et al.[287]

reported a slightly lower MDC of 2 s (calculated at 95% limits of agreement – LOA) in their sample of participants with radiographically confirmed KOA on a 9-step protocol. Iijima et al.[292] and Holm et al.[287] instructed their participants to begin on the landing and descend the flight of stairs before ascending, although Iijima et al.[292] had previously found in their pilot study that starting at the top or bottom of the stairs bore no influence on results.

Most recently, Sharma et al.[291] pooled evidence from four controlled trials of 12 weeks which used the SCT. In total, 182 women and 215 men were included, all with radiographically confirmed hip or KOA and a mean age of 63.8±8.0 years. Participants ascended/descended six steps at a daily pace rather than their fastest.[291] The authors reported an MDC of 1.21 s which is lower than the estimates of Dobson et al.[259] and Holm et al.,[287] but the SCT was on fewer steps and with participants walking at their normal daily pace rather than their fastest. Therefore, it is imperative to consider the instructions given to study participants before interpreting SCT outcomes for comparison with other studies.[260]

The baseline characteristics of study participants are also important to consider. Lange-Maia et al.[293] employed a 4 step, 3-repetions per attempt protocol in their study of women aged 44-66 years within the Women's Health Across the Nation Study in the USA. The authors did not report MDC or MCID values for their longitudinal study, but they found performance generally declined with age (i.e. time to complete increased). They also observed a deterioration in performance among women with general health conditions, including OA in any joint, and faster performances were associated with greater levels of physical activity.[293] Therefore, the study showed how the SCT can be confounded by variables including participants' physical fitness and stage or level of affliction from knee pain.

Timed Up and Go (TUG) Test

The Timed Up and Go (TUG) combines several activities endorsed by OARSI.[280] It simulates ADLs with movement from sitting to standing, walking short distances, and changing direction when walking.[294] Participants begin in a seated position with their back on the rear support of a chair and their arms resting on its armrests. After a count down, they walk at their usual pace to a cone placed

3 m from the chair. After rounding the cone, they return to the seated position with their back firmly against the rear support.[280]

There is a growing pool of data for MDC values but not MCID values for the TUG. Yuksel, et al.[295] estimated 1.62 s as the MDC calculated at a 95% confidence interval. They conducted a study on men and women (mean age of 58.5±12.9 years) who had total hip replacement at least six months before the test, and they were instructed to walk at their fastest rather than usual daily pace.[295] Previously, Yuksel et al.[296] had estimated a higher MDC of 2.27 s (calculated at the 95% confidence level) for men and women aged 65.6±9.7 years having undergone knee replacement surgery, but the participants were also instructed to walk at their fastest rather than usual daily pace. Alghadir et al.[297] estimated an MDC of 1.10-1.14 s (calculated at the 95% confidence interval) in a sample of sixty-five men and women aged 45-70 years with early-stage KOA (KL Grade I-III), but they did not report if participants walked at a usual pace or their fastest. In contrast, Dobson et al.[259] estimated an MDC of 1.21 s (calculated at the 90% confidence interval) among a sample of 51 men and women (n=24 women) aged 51-81 years (mean = 64.5±6.2) with medically diagnosed KOA and walking at their usual pace. In their earlier review, Dobson et al.[298] identified an MIC (i.e. MCID) of 0.8-1.4 s for the TUG test. In that case, the perceived improvements may have been due to random variability or measurement error considering the more recent MDC values.

Two-Minute Walk Test (2MWT)

The two-minute walk test (2MWT) is an evidence-informed proxy to the six-minute walk test (6MWT). Both timeframes require dynamic balance when changing directions whilst walking long distances.[280,299] However, the 6MWT was designed to evaluate medical interventions among cardiorespiratory cohorts rather than individuals with musculoskeletal conditions.[300] The distance to cover in six minutes may overburden participants with symptoms of KOA,[301,302] so the 2MWT was validated among populations of older adults and those with a medical diagnosis of KOA.[299,302-304]

For the 2MWT/6MWT, participants walk at their fastest pace, without running, as safely and comfortably as they can over a measured distance for two/six minutes.[280] They complete as many

lengths as possible over that time by repeatedly walking up to a cone placed ≥20 m from the start line and returning to the start line where another cone is placed for rounding.[283] Seats are provided along the way, and participants are welcomed to rest if needed while the two/six-minute timer continues.[283] After two/six minutes, participants are instructed to freeze so a mark can be placed at the toe of whichever foot is on the ground. A tester measures the distance from the toe to the cone most recently rounded for adding to a tally of completed 20 m distances.

An improvement in the 2MWT equates to a greater distance covered over two minutes. A meta-analysis of four studies by Bohannon et al.[305] reported reference distances (mean ±SE) covered in the 2MWT by various cohorts. The mean distance for healthy women aged 50-59 years was 169.1±10.0 m, 163.7±6.9 m for healthy women aged 60-69, and 150.3±1.3 m for those aged 70-79. Bohannon et al.[306] had previously reported an MDC value of 42.5 m for the test among their population-based sample of free-living adults (n = 1137).

Unnanutuana et al.[302] reported the mean distance covered at baseline by participants in their study as 46.2±19.6 m. It is three to four times less than the distance reported by Bohannon et al.,[305,306] and only marginally above their estimated MDC value of 42.5 m. However, Unnanutuana et al.[302] studied 162 participants (87.2% female, mean age 69.6±8.1 years) directly before total knee joint replacement surgery. Their participants were severely limited in physical capability which explains the discrepancy compared to the normative data.[302,305,306] The authors observed progressive improvements compared to baseline in their repeated measures at three, six and 12 months post-operatively (all being *p<0.001*), and the mean distance covered at 12 months was 66.1 m. Subsequently, they estimated (with a distribution-method) an MCID of 12.7 m for the 2MWT.[302]

Yuksel et al.[295] had previously reported a larger MDC of 17.56 m (calculated with the 95% confidence interval). Their study was on post-operative hip replacement patients rather than the knee,[295] but Yuksel et al.[296] had already estimated 14.96 m as the MDC (calculated with the 95% confidence interval) for their cohort of knee replacement patients who performed the 2MWT twice in one day. Chaudhary and Suhail[289] have since reported an MDC of 5.52 m (calculated at a 95%

confidence level) for their sample of 82 men and women (n = 55 women) aged 60.4±6.7 years with radiographically confirmed bilateral KOA. Their participants completed the 2MWT twice within 48 hours to determine an excellent test-retest reliability (intraclass correlation = 0.98) of the 2MWT.[289]

Interestingly, Johnston et al.[307] estimated MCID values of 4 m and 5.5 m for the 2MWT depending on the estimation method used (i.e. anchor or distribution-based). There were 59 participants (n = 31 women) with a mean age of 68.4±9.8 years, but they were patients with stable chronic obstructive pulmonary disease who performed the 2MWT before and after an 8-week rehabilitation program.[307] The two MCID values are either comparable to, or less than the MDC values derived from studies with participants suffering from KOA which again demonstrates the need for caution before comparing MDC and MCID data from other studies with potentially different participant characteristics and interventions.[264,269]

Biomarkers of cartilage turnover and inflammation

Articular cartilage is avascular (Figure 2-17). Unlike other tissues with blood supply, it relies on compressive loading and diffusion for nutrition and removal of waste products.[33,308,309] This helps explain why repair from cartilage injury is slower than muscle,[4,310] as nutrients for regeneration of the damaged ECM must pass from the circulatory system and move through the synovial fluid (SF) to reach chondrocytes in the ECM.[20,125] Conversely, byproducts of cartilage synthesis (e.g. procollagen peptides) and breakdown (e.g. CTX-II and COMP) must diffuse out of the ECM and SF to reach the blood stream for elimination in urine. Consequently, the byproducts of cartilage turnover (synthesis and breakdown) are measurable as soluble biomarkers in the urine, blood and SF.[74,308]

Figure 2-17. The avascular knee joint which relies on diffusion for supply of nutrition and removal of byproducts in cartilage turnover (adopted with permission from Kahraman et al.[311]).

The field of study on KOA biomarkers is diverse.[73] Clinicians may be able to use soluble biomarkers for detecting the disease sooner than current diagnostic standards,[312] but a consensus on one set for identifying risk or monitoring the progression of disease is yet to be reached (despite long standing attempts at doing so according to recent status updates from OARSI).[73,313] Meanwhile, researchers can evaluate the effect of drugs or nutraceuticals on cartilage turnover and inflammation over a series of repeated measures in longitudinal studies,[73,74] and in that sense the soluble biomarkers are drug development tools (DDTs).[314] For instance, they can detect molecular changes in the ECM before structural changes or performance improvements may have had enough time to materialize and become measurable.[74,312,315]

Collagen and cartilage synthesis

Collagen propeptides reflect the rate of collagen synthesis in the ECM.[316] They are cleaved from tropocollagen monomers during fibril formation (Figure 2-18).

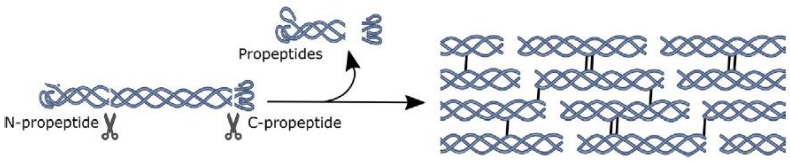

Figure 2-18 The formation of a tropocollagen monomer for fibril formation after propeptide cleavage (adopted with permission from He et al.[63]).

The carboxyl terminus propeptide of Type II collagen (PIICP or CPII) is cleaved by bone morphogenic protein-1 (BMP-1), while the amino terminus (PIINP) can be cleaved by ADAMTS-2,-3, or-14.[2,49] The propeptides reach the circulatory system,[317,318] but PIINP and PIICP cannot be interpreted interchangeably as PIINP exists in two isoforms.[318,319] Alpha PIINP (PIIANP) contains a 69-amino acid, cysteine-rich, globular domain which is encoded by exon 2, but the beta amino terminus propeptide of Type II collagen (PIIBNP) does not (Figure 2-19).

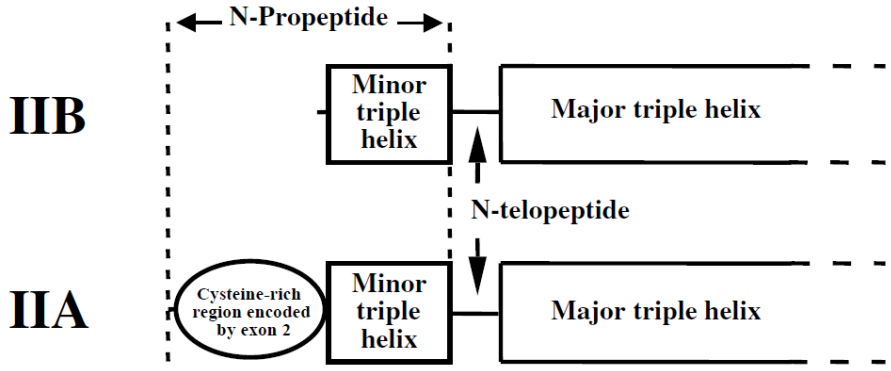

Figure 2-19. Differentiation between PIIANP and PIIBNP with PIIANP containing a 69-amino acid, cysteine-rich, globular domain encoded by exon 2 (adopted with permission from Rousseau et al.[320]).

Chondroprogenitor cells (i.e. chondroblasts) produce PIIANP during embryogenesis. By early adulthood, the ECM is mature, and the rate of collagen synthesis is negligible,[22,321,322] so PIIANP would not be expected when monitoring collagen synthesis of non-diseased cartilage among healthy adults.[5,323,324] Mature chondrocytes can express PIIBNP,[324,325] but it is not an accurate biomarker of total collagen synthesis among adults undergoing ECM repair from injury or disease considering PIIANP is also expressed.[323,324,326]

Early degradative damage of KOA occurs at the outer layer of the ECM.[22,321] It extends into the middle then deep zone of the ECM as the disease progresses,[327] and previously quiescent chondrocytes in the mid layer revert to progenitor cells (chondroblasts) in response to the upregulation of cytokines or growth factors trying to promote ECM repair.[323] For instance, chondroblasts produce PIIANP rather than PIIBNP, so PIIANP has potential as a diagnostic biomarker for the "silent" pre-radiographic phase of KOA.[63,315,323] It also offers prognostic value to identify patients at greater risk of disease progression after a diagnosis is made.[74,315] For example, Sharif et

al.[328] found serum PIIANP levels were significantly higher (*p<0.05*) among sufferers of medically diagnosed KOA who experienced worsening of symptoms over 5 years (progressors) compared to those diagnosed with KOA showing relative stability in their symptoms over the same timeframe (non-progressors). As such, the study showed how progression of KOA is non-linear which complicates monitoring PIIANP in longitudinal trials.[315,317] For instance, repeated measures from non-progressors (i.e. with relatively stable PIIANP levels) can confound data from progressors with increasing PIIANP levels.[317]

The complexities in monitoring PIIANP longitudinally prompted Nemirovsky et al.[326] to examine PIIBNP as an alternative biomarker of collagen synthesis. They found plasma levels of PIIBNP were approximately five times higher among a reference group of males and females aged 55-70 years without radiographic evidence of KOA compared to participants with confirmed diagnoses.[326] The observation makes sense considering radiographically confirmed cases of KOA can be expected to produce PIIANP rather than PIIBNP,[323] but Nemirovsky et al.[326] reported a coefficient of variation (CV) for inter-subject variability above 50% which indicates a large degree of variance between participants.[314] Luo et al.[324] corroborated the finding in their study which assessed collagen synthesis by measuring PIIANP and PIIBNP from participants with confirmed KOA (KL Grade II-IV) versus controls (KL Grade 0-I). In addition to increased serum PIIBNP among their 'healthy' control group versus the KOA cases (*p=0.0118*), Luo et al.[324] found the 'controls' had significantly higher levels of PIIANP than the KOA cases (*p=0.0485*). Therefore, their finding suggests the 'healthy' controls were at an earlier stage of KOA, albeit asymptomatic, as evidenced by the inclusion criteria of healthy participants with possible signs of disease in reference to radiographic data (i.e. KL Grade I). In other words, the study showed both groups of participants were simultaneously producing the two isoforms (i.e. PIIANP and PIIBNP).

Garnero et al.[329] and Rousseau et al.[320] had previously found serum PIIANP levels were significantly lower among their participants with KOA than healthy controls (*p<0.001*). The participants in both studies included postmenopausal women, and they are the group at greatest risk of developing

"menopausal OA" in multiple joints.[101,136,330] As such, the 'healthy' controls in both studies were plausibly at an earlier, asymptomatic stage of undiagnosed KOA considering PIIANP is only produced during embryogenesis and in diseased adult cartilage.[74,323]

In summary, the existing research on PIIANP and PIIBNP demonstrates a difficulty in taking repeated measures of PIIANP from participants who have not been defined as progressors or non-progressors within a sample population (i.e. different KOA phenotypes).[317,320,324,326,329] The studies also support a suggestion by Garvican et al.[61] that monitoring both isoforms of PIINP (i.e. PIIANP and PIIBNP) is necessary to assess total collagen synthesis among individuals with KOA in longitudinal studies, irrespective of their KOA phenotype.[77] However, the ratio of collagen synthesis to breakdown is a better indicator of cartilage turnover and KOA progression than one biomarker of either outcome measure alone (i.e. synthesis or breakdown).[331]

Collagen and cartilage breakdown

The primary cleavage site of mature collagen is at Gly_{775}-Leucine$(Leu)_{776}$ which creates collagen fragments of ¾ and ¼ length.[313] The proteases involved with cartilage breakdown (i.e. MMP-13, ADAMTS-4 and ADAMTS-5) are widely distributed within the body and lack specificity to the knee joint, but ECM fragments (i.e. collagen, PGs and COMP) are reflective of their degradative action.[74] For example, aggrecan depletion precedes collagen breakdown which occurs when the disease is more established and the damage is irreversible.[332]

Carboxyl-terminal telopeptides of Type II collagen (CTX-II) are a well-known biomarker of collagen breakdown.[317,328,329,332-335] They appear on the ¼ fragment of cleaved Type II collagen, with a peptide sequence Glu_{1049}-Lys-Gly-Pro-Asp-$Pro_{1054.}$[336] There is a shortage of data on serum CTX-II (sCTX-II), but urinary CTX-II (uCTX-II) has been heavily investigated as the fragments are stable in urine held at room temperature for up to 24 hours, and they can resist several freeze-thaw cycles which facilitates logistics between the time of collection through to analysis of the samples.[336] Currently, uCTX-II is the gold standard for measuring OA progression or discriminating between cases with or

without the disease.[333,337] In addition to its value as a diagnostic and prognostic biomarker for clinicians, uCTX-II also has use as a biomarker of collagen breakdown in clinical trials.[73]

Cheng et al.[333] found in their meta-analysis (13 studies, n=2,856 participants) that uCTX-II levels were higher (*p<0.0001*) in the group with radiographically confirmed KOA (KL Grade ≥II) compared to 'healthy' controls. Furthermore, the uCTX-II levels were higher (*p<0.0001*) among participants with later (advanced) stages of KOA (KL Grade III-IV) compared to an earlier stage (KL Grade II), and Sharif et al.[328] found higher levels of uCTX-II among progressors versus non-progressors of KOA symptoms over five years (*p<0.05*). However, Luo et al.[335] found uCTX-II levels did not correlate with serum CTX-II (sCTX-II) in a cross-sectional study of 188 participants diagnosed with KOA versus 57 controls without KOA (*p=0.6487*). The authors speculated a reason may be that several CTX-II fragments in the blood undergo further degradation and only one appears in the urine.[335] Therefore, uCTX-II is superior to sCTX-II as a biomarker for clinical trials.

Cartilage oligomeric matrix protein (COMP) is abundant in the ECM of healthy cartilage.[338] It anchors chondrocytes to the ECM and modifies cell signaling (e.g. down regulating Runx2 expression) to maintain homeostasis in cartilage turnover.[339-343] Its fragments have been measured at higher concentrations in the SF and serum of study participants with radiographically confirmed KOA compared to healthy controls without knee pain,[337,344,345] and Verma and Dalal[345] found serum COMP levels were positively correlated with well-known risk factors for KOA such as age (*p<0.001*) and BMI (*p<0.001*). The authors also found COMP was correlated with pain intensity, as assessed with the VAS (*p<0.001*), and negatively correlated with the duration of disease (p<0.001).[345] In other words, intact COMP starts to decline from an early stage of disease before collagen degradation, so it also has potential as a diagnostics biomarker for earlier detection of cartilage breakdown.[315,337] Furthermore, it fits the criteria for biomarkers evaluating efficacy of treatment in clinical trials,[346] and a meta-analysis by Hao et al.[337] concluded serum COMP (sCOMP) is appropriate for monitoring in lieu of SF samples. In fact, Henrotin[77] recommends measuring sCOMP alongside uCTX-II as exploratory endpoints in clinical trials.

Inflammation

The concentrations of ECM fragments increase during cartilage breakdown.[154,347] They are detected by chondrocytes which respond by upregulating their release of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α which activate CRP and potentiate the inflammatory response and ECM degradation as already discussed (Section 2.3.4).[348,349] Consequently, these soluble biomarkers of inflammation can complement monitoring biomarkers of cartilage turnover in repeated measures.[73,74,308] However, systemic biomarkers of inflammation (i.e. serum samples) are diluted and more difficult to interpret than SF samples drawn from a specific knee joint.[317,322,350]

Furthermore, C-reactive protein (CRP) is a generic biomarker of inflammation. It is released from the liver in response to injury and infection.[351] The high-sensitivity CRP (hs-CRP) assay can detect CRP at lower levels than the traditional measure,[352] and elevations above normal are seen among subjects with OA compared to healthy controls.[352] However, particularly high levels (e.g. >50 mg/dl) are usually caused by bacterial infections, and elevations of a lesser extent are not exclusively due to OA.[351] For example, excess body fat, diabetes and autoimmune conditions including RA can all cause CRP to increase, especially among women as they age.[351,353,354] Conversely, alcohol and medications including NSAIDS or statins can cause false decreases.[355-357] Similarly, essential fatty acids and micronutrients with anti-inflammatory properties (i.e. omega 3- or 6- oils, magnesium, zinc and selenium) may also attenuate the inflammatory response of KOA.[358-361] Overall, CRP alone can give misleading or inconclusive data about inflammation in KOA,[74] so the proinflammatory cytokines associated with KOA (i.e. IL-1β, IL-6 and TNF-α) are worthwhile to include as outcome measures. They can correlate with clinical signs (i.e. radiographic findings) and symptoms (i.e. pain) of disease progression.[362,363]

Data collection protocol

Monitoring soluble biomarkers in KOA research is not straight forward. The body fluid(s) chosen for evaluation can distort results as seen by comparisons between serum and SF. Nelson et al.[322] have shown PIICP reflects the total rate of collagen synthesis, and it has been found significantly higher within the SF of subjects with mild to moderate KOA confirmed by radiography compared to

healthy controls without signs of the disease.[322,364,365] The consistent finding may be explained by an increased rate of synthesis attempting to repair an even greater rate of breakdown associated with the progression of KOA,[365] but Nelson et al.[322] found PIICP levels in the SF were not correlated with serum. The mean PIICP concentration in SF was approximately double (*p<0.001*), and the authors speculated this being due to reduced cartilage synthesis in other body joints unaffected by OA.[322] In other words, systemic (whole body) measures of serum are diluted in contrast to direct measures of SF from the affected joint.

The heterogeneity in KOA phenotypes also means biomarkers will perform differently over time within and between individuals.[73,313-315] Some participants may be non-progressors whereas others are progressors with divergent rates of cartilage synthesis and breakdown at baseline and endpoint (Figure 2-20). Furthermore, some participants may have OA in multiple joints (i.e. the hip and knee), but the rate of cartilage turnover in each joint can differ.[322,366-368] Similarly, serum and urine (i.e. systemic) biomarkers of inflammation are confounded by autoimmune conditions including RA or inflammatory bowel disease which also cause elevations.[315,336,369,370] Therefore, systemic samples lack specificity in contrast to SF samples, but they are less invasive to collect and accepted in the research setting for monitoring soluble biomarkers.[308,317,371] Hepatic and renal disease can impair their clearance which explains why participants with those comorbidities are generally excluded as a standard operating procedure for intervention studies.[315]

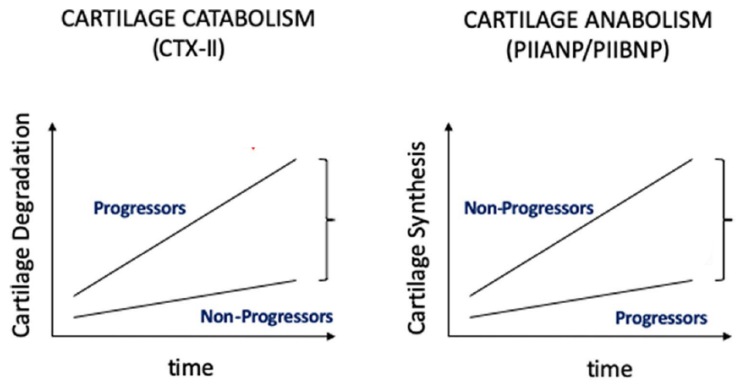

Figure 2-20. Demonstration of the disparity between progressors and non-progressors in their rates of cartilage turnover across time (adopted with permission from Kraus et al.[317]).

A standard operating procedure for collecting and analyzing data on biomarkers gives clinical trials validity.[224,314] Holding constant the time of day when samples are collected controls for diurnal variation,[336,372,373] and the OARSI Biomarkers Working Group recommends collecting second morning voids of urine and collecting blood samples at least two hours after rising.[314,368] The Group also suggests storing blood separately as serum (following gentle inversion immediately after collection, then centrifugation at ≈3500rpm and allowing 30-60 minutes for clotting), then keeping the samples below -70°C until they can be analyzed altogether rather than individually, immediately after collection.[224,368] Overnight fasting may not be necessary,[314,368] but ADLs and exercise can affect the concentration of biomarkers in the SF, bloodstream and urine.[372] Therefore, a study protocol should ensure biomarkers are collected before any functional performance tests, and guidance on whether overnight fasting is required should be communicated to participants for consistency. Overall, the OARSI Biomarkers Working Group recommendations are a quality control resource to facilitate comparability of data across trials.[314,368]

Pain relief

Monitoring participants' use of pain medication during an intervention provides objective data as a secondary outcome.[224] However, there are multiple approaches to collecting the information from participants which can affect its reliability.[374] Kiadaliri et al.[375] used self-reporting at baseline and endpoint to categorize their participants' use of analgesics over a 12-week intervention with an online self-management program for pain, while Bruyère et al.[376] compared the proportion of participants in their CH treatment and control groups who reported taking at least one pain medication during an intervention of six months. In both instances, the accuracy of data depended on self-reporting,[374] whereas Kivitz et al.[377] provided their participants with a supply of pain rescue medication for use *ad libitum* over 24 weeks after an intra-articular (IA) corticosteroid injection versus control. At follow up, self-reported use of pain medication on a diary was validated by counting the number of returned pills.[377] While this method relies on participants returning all their unused medication, it offers a more objective measure than self-reported use alone.

2.5 Summary

Type II collagen is the main organic component of articular cartilage. Its degradation is associated with the onset and progression of KOA which is characterized by signs of inflammation and symptoms of pain. The global prevalence of KOA is growing rapidly as the population ages, and postmenopausal women are at an increased risk. There is no cure for KOA yet, but there is a market for CH as a nutraceutical to manage its symptoms. However, CH supplements are highly variable in composition and MW which may impact on its bioactive peptide (BAP) potential in human trials. The MW of CH depends on the animal source of UC-I, along with the methods used for its extraction and hydrolysis. Terrestrial mammals have a higher concentration of Gly-Pro-Hyp in their UC-I than sea-dwelling animals or avians, so a review of the existing literature on the effect of CH from terrestrial mammals on knee pain in humans is indicated as a next step in evaluating its efficacy as a nutraceutical.

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Conception and design of the systematic review: DG and JC;
Data extraction and interpretation for narrative synthesis: DG
Risk of bias assessment: DG and Janet Weber (JW);
Drafting of the chapter and preparation of its revisions for a final version: DG;
Critical review of drafts for intellectual content and approval of the final version: JC, Marlena Kruger (MK), JW.

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Chapter 3: The effect of oral supplementation with collagen hydrolysate (CH) from terrestrial mammals on knee pain: a systematic review

This chapter critiques existing research on CH from a terrestrial mammal and its effect on knee pain. It served to identify strengths and limitations of that evidence to inform the CHAMP study design.

3.1 Abstract

Pain is a hallmark symptom of knee osteoarthritis (KOA). The disease is underdiagnosed, especially among groups with multiple risk factors. Collagen hydrolysate (CH) is marketed as a nutraceutical, but evidence of its effect on pain is inconclusive due to heterogeneity in study designs. Animal sources of CH have varied, but terrestrial mammals have a higher concentration of postulated bioactive peptides (BAPs) than avians and fish. Therefore, this review was registered with Prospero (CRD4202345642) to appraise human trials investigating the effect of CH from terrestrial mammals on knee pain. The Scopus, Medline and SciFinder N databases were searched from their dates of inception until March 2024. Data extraction included each study's design, participants' characteristics, intervention regimen and reported impact of CH on knee pain. Of 236 records identified, 11 were included and 5 reported a statistically significant effect of CH on knee pain compared to its comparator. However, all 11 studies were assessed as having some concerns or high risk of bias with the Cochrane RoB 2.0 tool, limiting the reliability of results for extrapolating to specific cohorts at risk of KOA such as postmenopausal women. Overall, the existing evidence of effect of CH from terrestrial mammals on knee pain is inconclusive, and more robust research is needed.

Keywords:
Knee osteoarthritis (KOA), bioactive peptides (BAPs), collagen, bovine, porcine, pain

3.2 Introduction

Knee osteoarthritis (KOA) is characterized by pain and an imbalance in articular cartilage turnover.[1] It has a complicated pathogenesis,[1,2] and medical diagnoses only occur after structural changes are evident.[3-5] Underdiagnosis is common,[6] especially among individuals with multiple risk factors.[3,7] Postmenopausal women are expected to be over-represented in the burgeoning prevalence of KOA worldwide,[8] indicating an effect from estrogen depletion on the cascade of events causing cartilage degradation, if not through other sex-related differences.[9-13]

Articular cartilage is an extracellular matrix (ECM) on the surface of subchondral bone.[14,15] It has three distinct layers, of which Type II collagen fibrils coexist with collagen Types IX and XI to form fibers of varying thickness.[16] In harmony with water and proteoglycan aggregates, collagen fibers enable the ECM to provide smooth joint articulation at the outer layer and absorption of force in the middle and deep zones.[16,17] However, the ECM relies on chondrocytes dispersed within its three layers for structural and functional integrity.[18,19] Usually, a low rate of cartilage breakdown is matched with synthesis in mature cartilage, but the equilibrium is disturbed in states of disease such as KOA.[20,21] For example, chondrocytes can upregulate their release of proinflammatory cytokines and a catabolic milieu arises, so they may be targets for stalling the onset or progression of KOA.[1,21,22]

Collagen hydrolysate (CH) has attracted interest as a 'chondroprotective' agent.[23-25] Also known as collagen peptides, these fragments are derived from undenatured collagen in its native form, which is the main structural protein of vertebrates (and a byproduct of the livestock industry).[26-30] Type I fibrils predominate in the skin or hide, and Type II fibrils are the main component of cartilage. Both types consist of Gly-X-Y repeating motifs,[30,31] with prolyl-hydroxyproline (Pro-Hyp) often occurring in the X-Y positions.[31-34] The virtually mutual structure of Types I and II is important for recognizing Type I collagen derived from animals as a potential nutraceutical ingredient to promote Type II synthesis in the articular cartilage of humans.

Bioactivity of a food or nutraceutical describes its therapeutic effects on a health condition.[23,35,36] Undenatured collagen is resistant to intestinal digestion, so it must undergo external denaturation and hydrolysis to release its postulated bioactive peptide (BAP) potential as CH.[37] Some preclinical studies have shown that Gly-Pro-Hyp tripeptides and Pro-Hyp dipeptides can increase the rate of Type II collagen synthesis by chondrocytes *in vitro,*[38,39] but Schadow et al.[40,41] found with their *in vitro* research that CH treatment had no effect on Type II collagen synthesis compared to placebo, and in some instances it upregulated the release of proteases involved in cartilage breakdown.[40,41] Overall, the preclinical evidence is inconsistent, and human trials are equally inconclusive due to heterogeneity in study designs.[23,37,42] For example, the molecular weights of CH have varied, as have the animal sources of the CH interventions among other variables including study participants' characteristics.[42]

There are numerous methods of extraction and hydrolysis which affect the molecular weight of CH.[29] Identifying an animal source of Type I undenatured collagen with a rich reservoir of Pro-Hyp may optimize its potential BAP yield,[43] and terrestrial mammals have a higher concentration of Gly-Pro-Hyp tripeptides in their hides than avian or fish sources.[44,45] Therefore, the aim of this systematic review was to appraise the studies which used CH sourced from terrestrial mammals (i.e. porcine and bovine hide) to investigate its effect on knee pain in humans.

3.3 Methodology

The systematic review with a narrative synthesis was designed in reference to best practice guidelines described by Siddaway et al.[46] It was registered on PROSPERO (CRD4202345642) and it aligned with the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA).[47]

3.3.1 Search strategy

3.3.1.1 Primary search

The Scopus, Medline via Pubmed and SciFinder N databases were searched by one author (DG) from their dates of inception up until January 04, 2024 (Table 3-1). The searches were saved as ongoing alerts on each database for any articles published thereafter until March 31, 2024.

Table 3-1. Primary search strategy for the systematic review.

Database	Terms
Scopus, Medline via Pubmed, SciFinder N	(Collagen AND hydro* OR peptide*) AND (supplement* OR capsule* OR nutraceutical*) AND (osteoarthritis OR “joint pain” OR “joint discomfort”)

3.3.1.2 Secondary search

A secondary search was performed after compiling a list of studies to include from the primary search. Their reference lists were reviewed by one author (DG), and relevant titles, if not already found in the primary search, were retrieved for subjecting to the selection process which the primary search results had undergone.

3.3.2 Selection process

3.3.2.1 Screening

The titles and abstracts of all records found in the primary search were screened on the PICO Portal without using its AI capabilities.[48] Between 17 January and 04 April 2024, two reviewers (DG and JW) independently judged if the studies met the eligibility criteria (Table 3-2). In cases of insufficient detail in the title or abstract (i.e. unspecified animal source of CH), the full text was retrieved. If the full text also lacked detail, the corresponding author was contacted by email. If a response was not received within two weeks, a follow up email was sent. Web searches were also performed for the CH interventions reported as brand names to try and identify the animal source, but the studies were excluded if an email response was not received after an additional two weeks and the source could not be identified from the web search.

3.3.2.2 Inclusion / exclusion criteria

The eligibility criteria for screening are shown in Table 3-2. Several studies were ineligible for multiple reasons, so a hierarchy was applied by the reviewers in retrospect to quantify the primary reason for exclusion (Table 3-2). Pilot studies were considered to broaden the search scope, as were observational or non-blinded intervention trials but only full articles available in English were included. A meeting was held between DG, JW and a third reviewer (JC) on April 30, 2024, to confirm all studies

identified as eligible during screening. Split decisions were discussed with JC as the judicator to include or exclude any studies in question.

Table 3-2. Inclusion / exclusion criteria of the systematic review.

Inclusion:
• Collagen Hydrolysate (CH) from a terrestrial mammal source
• Randomized Double-Blind Controlled Trial (RDBCT)
• Pilot study, observational or non-blinded intervention trials
• Primary or secondary outcome measure on the effect of CH on knee joint pain
Hierarchy of Exclusion:
1 Systematic reviews or meta-analyses
2 Animal or *in vitro* / preclinical studies
3 Undenatured collagen or non-oral CH study (i.e. intra-articular injection)
4 Primary or secondary outcome measure not evaluating the efficacy of CH on knee joint pain
5 Collagen Hydrolysate (CH) from a non-terrestrial mammal source (i.e. avian or marine)

3.3.3 Data collection

A template was designed to extract data for subsequent narrative syntheses (Table 3-3). The main outcome of interest for the review was a patient reported outcome measure of pain.

Table 3-3. Information sought for data extraction in the systematic review.

1. Study Details
- authors' names
- year of publication
- trial registration number
- design and duration
2. Population
- total recruited and completed
- sex and mean age
3. CH source and dose
4. Comparator (composition/type and dose)
5. Key findings (e.g. mean difference with 95% CIs)

3.3.4 Risk of Bias

Risk of bias (RoB) assessments were independently performed by DG and JW with the Cochrane RoB 2.0 tool.[49] Both authors were guided in learning to use the tool by Moore et al.[50] The Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) tool was reserved for any studies without randomization,[51] and the RoB 2.0 for crossover trials was used to assess studies of crossover designs.[49] The full article of each study was reviewed alongside its trial registration document (if a registration number was reported and it could be accessed from a public database). Any conflicting judgements between DG and JW were resolved by discussion between the two authors for consensus.

3.4 Results

3.4.1 Study selection

3.4.1.1 Primary search

A total of 236 records was identified in the Medline (76 records), Scopus (112 records) and SciFinder N databases (48 records). Once uploaded to the PICO portal, 71 records (68 duplicates and 3 supplemental records) were automatically removed and 165 records from the primary search were screened (Figure 3-1).

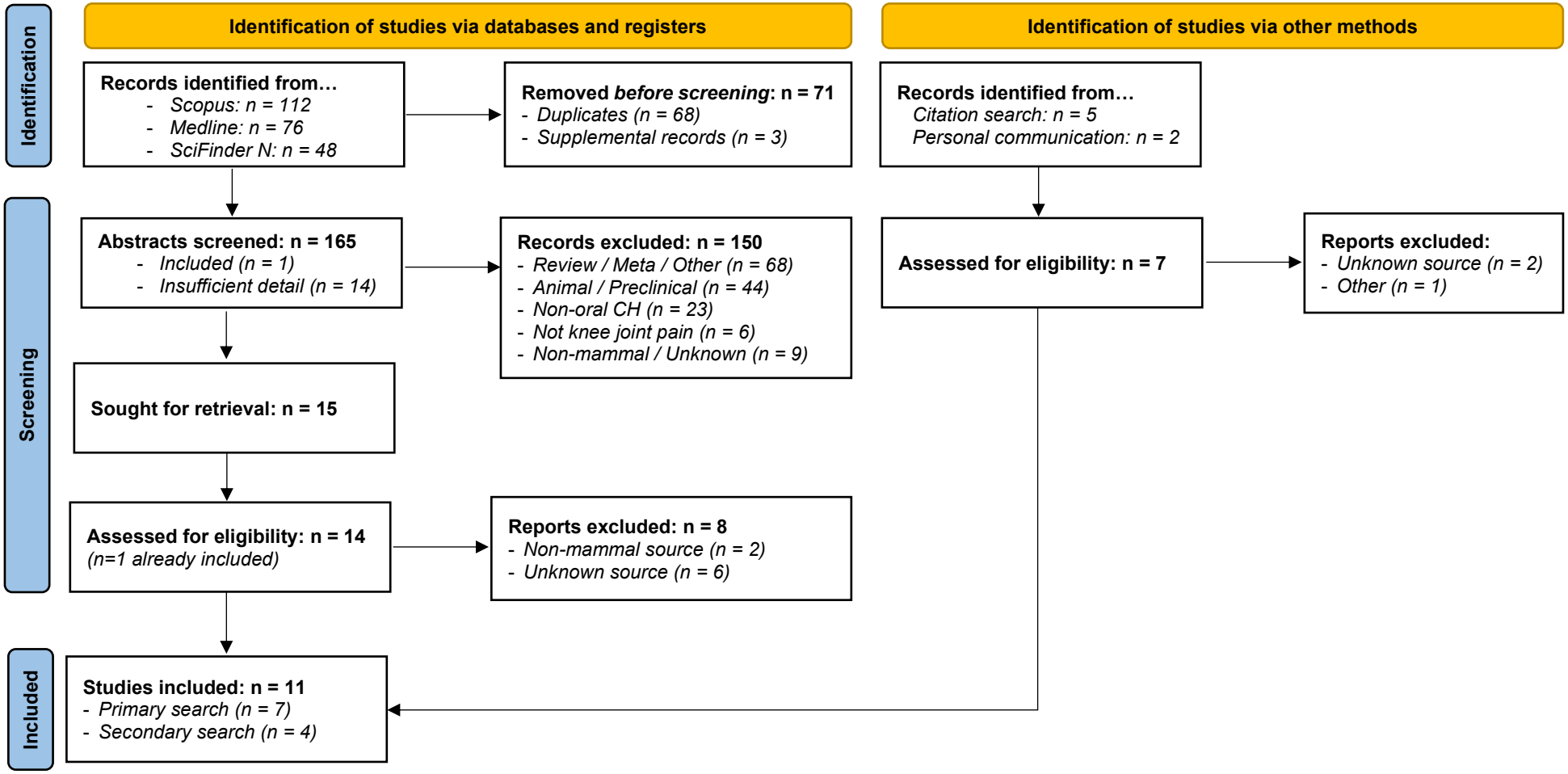

Figure 3-1. PRISMA diagram of the systematic review screening process (adapted with permission by creative commons license from Page et al.[47]).

3.4.1.2 Screening phase

Titles and abstracts

After screening the 165 primary search result titles and abstracts, one study was included as the CH source was reported as a terrestrial mammal.[52] Meanwhile, 150 records were excluded, and 14 were sought for retrieval in full text to assess their eligibility considering the CH source was unspecified.

Full text

Four studies were immediately included after retrieving and reading the full articles. The authors clearly stated the CH source was bovine or porcine,[53-56] but one study reported a synthetic preparation of CH so it was excluded.[57] Nine emails were sent to corresponding authors for information about the origins of the CH used in their intervention trial.[58-66] In two instances, the CH source was stated as porcine, but it was unclear if the intervention was CH or its native (undenatured) format,[60] or if post-extraction methods (i.e. filtration, centrifugation and 'salting out') were used to recover specific Pro-Hyp peptides which would essentially make it a synthetic formulation.[29,66]

Email enquiries

Four email replies were received in response to three of the sent enquiries (Prof. Olivier Bruyère, email reply, 18 January 2024; Dr. Christina Larder, email reply in follow up to Prof. Bruyère, 19 January 2024; Dr. Francessca Oliviero, email reply, 17 January 2024; Prof. Michael Ormsbee, email reply, 16 January 2024). The CH source was named as a terrestrial mammal for studies by Bruyère et al.[58] and Kviatkovsky et al.,[61] but it was avian in the study by Oliviero et al.[63] which was excluded. There were no responses to six of the nine emails sent.[59,60,62,64-66] The requested information could not be discerned from a web search for brand names of any of those six enquiries, so the studies were excluded and a preliminary list of seven was formed for inclusion at the end of the primary search.

3.4.1.3 Secondary search

Three articles were initially identified from the reference lists of primary search results.[67-69] A study by Moskowitz[68] was excluded because the CH source was unspecified and the sole author passed away in 2018. Benito-Ruiz et al.[67] reported using Colnatur® from the bones of a non-ruminant, but the

corresponding author could not be contacted to clarify if that animal was porcine (i.e. for inclusion) or avian (i.e. for exclusion). The website of Colnatur®[70] reports its source as bovine, which is a ruminant, so the study was excluded on the grounds of inconsistencies in reporting of the source.

A further four articles were retrieved in the secondary search. A study by Trč and Bohmová[69] was identified in the reference list of a primary search result by Kumar et al.[52] The authors reported their CH intervention as Colatech® without stating its source.[69] Attempts at contacting the authors failed, but their study made reference to other studies having used the same CH product.[71,72] Adam[71] did not report the source but Ribas-Fernandez and Perez[72] reported it was porcine, albeit under a different brand name (Artivit Biosol). Consequently, the studies by Trč and Bohmová[69] and Adam[71] were included, although the study by Ribas-Fernandez and Perez[72] was excluded because it aimed to assess the effect of CH on cartilage thickness rather than pain among healthy athletes which is outside the scope of this review. Lastly, two studies[73,74] were provided by a third party whilst advising on the source of CH in the study by Bruyère et al.[58] Those two studies by Bernardo and Azarcon[73] and Feliciano et al.[74] were confirmed as having used CH from a bovine source so they were included (Dr. Christina Larder, email reply, 19 January 2024). Ultimately, 11 studies were included for data extraction at the end of the selection process (Figure 3-1).

3.4.2 Risk of Bias

Five registration documents were retrieved for the risk of bias (RoB) assessments. They were performed with the RoB 2.0 tool for 10 of the 11 included studies,[52-56,58,61,69,73,74] and its crossover variant was used for the study by Adam.[71] The ROBINS-I tool was not used to assess two single-blind studies as they were randomized so were assessed with the RoB 2.0 tool.[49,56,73] Three studies assessed with the ROB 2.0 were judged at an overall high risk of bias, with the remaining seven raising some concern (Figure 3-2). Two of the studies at high risk of bias reported a positive effect from CH versus the comparator,[52,69] and the third reported a positive effect on all joints studied, except the knee.[58]

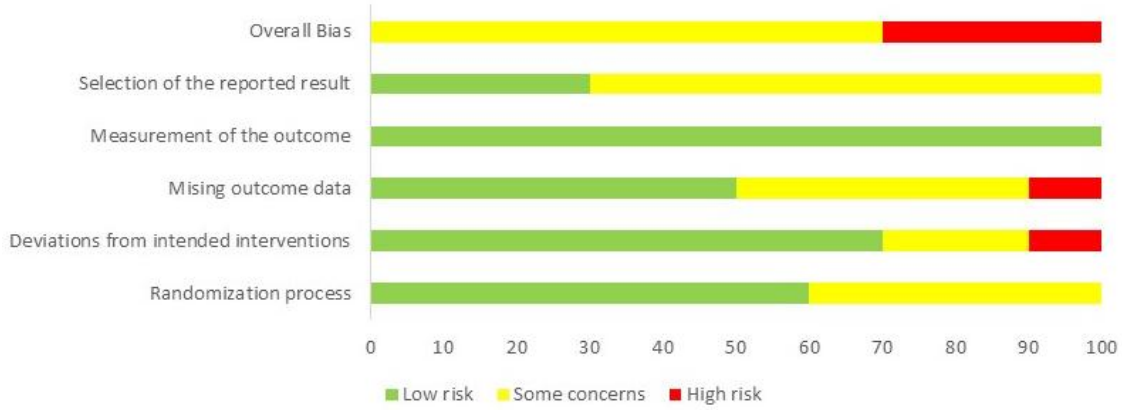

Figure 3-2. Risk of bias assessment summary.[49]

Figure 3-3 shows domain-level summaries for each study. Selective reporting was the most common reason for having at least some concern for bias in 70% of the studies assessed, while missing outcome data was reason for at least some concern in 50% of the studies. One study by Trč and Bohmová[69] was upgraded to a high overall risk during the consensus meeting due to concerns with domains one (randomization) and five (selective reporting) which were considered borderline high risk.

Study	Risk of Bias Domain					
	D1	D2	D3	D4	D5	Overall
Bernardo & Azarcon (2012)	+	+	!	+	!	!
Bongers et al. (2020)	+	+	!	+	+	!
Bruyere et al. (2012)	!	+	-	+	+	-
Feliciano et al. (2017)	+	+	+	+	!	!
Jiang et al. (2014)	!	+	+	+	!	!
Kimira et al. (2019)	+	!	+	+	!	!
Kumar et al. (2015)	+	-	+	+	+	-
Kviatkovsky et al. (2023)	+	+	!	+	!	!
Newman et al. (2023)	!	!	!	+	!	!
Trč & Bohmová (2011)	!	+	+	+	!	-

Key	
+	Low risk
!	Some concerns
-	High risk
D1	Randomisation process
D2	Deviations from the intended interventions
D3	Missing outcome data
D4	Measurement of the outcome
D5	Selection of the reported result

Figure 3-3. Domain-level summary for each study assessed with the RoB 2.0 tool.[49]

The crossover study by Adam,[71] which also reported a positive effect from CH versus its comparator, was identified as high risk in two domains and some concern in three for an overall high risk of bias (Figure 3-4).

Study	Risk of Bias Domain						
	D1	DS	D2	D3	D4	D5	Overall
Adam (1991)	!	!	-	+	-	!	-

Key			
		D1	Randomization process
+	Low risk	DS	Bias arising from period and carryover effects
!	Some concerns	D2	Deviations from the intended interventions
-	High risk	D3	Missing outcome data
		D4	Measurement of the outcome
		D5	Selection of the reported result

Figure 3-4. Domain-level summary for each study assessed with the RoB 2.0 crossover variant.[49]

3.4.3 Study characteristics

Table 3-4 summarizes the data extracted for the ensuing narrative synthesis.[46] Compared to its comparator, a statistically significant effect from CH on knee pain was reported in five out of 11 studies.[52,54,56,69,71] Of the six studies which did not report a difference between groups at endpoint,[53,55,58,61,73,74] Bongers et al.[53] reported statistically significant improvements within their CH and placebo groups compared to baseline, while Bernardo and Azarcon[73] reported a statistically significant improvement within the CH group versus baseline, without a statistically significant difference in pain scores between the treatment and comparator groups at endpoint. Furthermore, Bruyère et al.[58] reported a statistically significant improvement for the CH group compared to placebo for all joints except the knee and Feliciano et al.[74] reported a statistically significant effect for CH versus placebo only after post-hoc analysis on a sub-group of non-adherent exercisers. Overall, there were inconsistencies between studies with how their results were reported and in many cases effect size data were missing (Table 3-4).

Table 3-4. Clinical trials assessing the effect of collagen hydrolysate from terrestrial mammal sources on knee joint pain.

<table>
<tr><th>STUDY</th><th>POPULATION</th><th>COLLAGEN HYDROLYSATE (CH)</th><th>COMPARATOR(S)</th><th>KEY FINDINGS</th></tr>
<tr><td><u>Author(s)</u>:
Adam (1991)[71]
<u>Trial registration</u>:
Unreported
<u>Design</u>:
Randomized, non-blinded crossover with randomization and allocation methods unspecified
<u>Duration</u>:
60d / treatment (2mo in between)</td><td><u>Total</u>: n=52
M: n=28, F: n=24
Age: 56 years (SD not given)
<u>Ailment</u>:
- KOA: n=21 confirmed in x-ray as Kellgren-Lawrence (KL) Grade I-III
- Hip OA: n=41 (unilateral: n=10, bilateral n=31)</td><td><u>Source</u>:
Porcine [A]
(Treatment 1 = T1)
<u>Dose</u>:
10 g/d via 20x 0.5 g tablets</td><td><u>Treatment 2 (T2)</u>:
Gelatin
<u>Treatment 3 (T3)</u>:
Gelatin + CaPo4H20 (hydroxyapatite)
<u>Treatment 4 (T4)</u>:
Egg albumin
<u>Dose</u>:
10 g/d via 20x 0.5 g tablets</td><td><table><tr><th></th><th>Pain[B] Score Reduction of:</th><th></th><th></th></tr><tr><th></th><th><26%</th><th>26-50%</th><th>>50%</th></tr><tr><td><u>T1:</u></td><td>n=10/52</td><td>n=17/52</td><td>n=25/52</td></tr><tr><td><u>T2:</u></td><td>n=7/52</td><td>n=15/52</td><td>n=29/52</td></tr><tr><td><u>T3:</u></td><td>n=10/52</td><td>n=18/52</td><td>n=24/52</td></tr><tr><td><u>T4:</u></td><td>n=19/52</td><td>n=7/52</td><td>n=5/52</td></tr></table>NB: T4 reported as insubstantial & insignificant influence. All other treatments reported as a substantial reduction of symptoms and were statistically different compared to T4 except for Sensitivity to weather. Additionally, T1 was significantly different to T2, T3, T4 for Tiredness Pain, T2 was significantly different to T1, T3, T4 for Pain at Night. Statistical analysis via Lehmacher & chi quadrat tests (without p values shown)</td></tr>
<tr><td colspan="5">[A] Not explicitly stated as porcine but cited by Trč and Bohmová [69] as being the same used in that study and that of Ribas-Fernandez and Perez[72];
[B] Pain: On starting to walk; stiffness on starting to walk; Pain at night; Sensitivity to weather; Exhaustion; Sensitivity to cold; Stress pain; Tiredness pain; Muscle pain; Pressure pain over joint; Pain in hip; Pain on movement; End phase pain.</td></tr>
<tr><td><u>Author(s)</u>:
Bernardo & Azarcon (2012)[73]
<u>Trial registration</u>:
Unreported
<u>Design</u>:
Randomized, single-blind, open-label with 1:1 group allocation using an unspecified auto-generation method
<u>Duration</u>:
6 months</td><td><u>Total</u>:
Recruited: n=150 M & F
Completed: n=113
<u>Group A</u> (CH): n=55
M: n=16, F:n=39
Age: 68±8.4y
BMI: 24.0±2.0 kg/m^2
<u>Group B</u> (NSAID): n=58
M: n=19, F: n=39
Age:69s±7.8y
BMI: 24.0±1.5 kg/m^2
<u>Ailment</u>: diagnosed with KOA (Kellgren-Lawarence Grade ≥II)</td><td><u>Source</u>:
Bovine
<u>Dose</u>:
1,200 mg/d at night (3x400 mg capsules)</td><td><u>Type</u>: active NSAID
<u>Dose</u>:
100 mg 2x/d for the first five days then as needed thereafter</td><td>Western Ontario and McMaster University Osteoarthritis Index (WOMAC): Mean±SD<table><tr><th></th><th>Group A CH (n=55)</th><th>Group B NSAID (n=58)</th><th>A vs B</th></tr><tr><td>Month 0</td><td>2.83±0.7</td><td>2.91±0.7</td><td>p=0.47</td></tr><tr><td>Month 1
vs m0</td><td>2.38±0.6
p=0.33</td><td>2.59±0.7
p=0.37</td><td>p=0.42</td></tr><tr><td>Month 2
vs m0</td><td>2.04±0.8
p=0.23</td><td>2.42±0.8
p=0.32</td><td>p=0.35</td></tr><tr><td>Month 3
vs m0</td><td>1.65±0.7
p=0.13</td><td>2.29±0.8
p=0.29</td><td>p=0.25</td></tr><tr><td>Month 4
vs m0</td><td>1.30±0.8
p=0.08</td><td>2.26±0.9
p=0.29</td><td>p=0.16</td></tr><tr><td>Month 5
vs m0</td><td>1.05±0.9
p=0.07</td><td>2.17±0.9
p=0.26</td><td>p=0.14</td></tr><tr><td>Month 6
vs m0</td><td>0.76±0.9
p=0.04</td><td>1.87±1.2
p=0.21</td><td>p=0.11</td></tr></table>NB: Missing effect size data & unclear presentation of WOMAC score</td></tr>
</table>

<table>
<tr><th>STUDY</th><th>POPULATION</th><th>COLLAGEN HYDROLYSATE (CH)</th><th>COMPARATOR(S)</th><th>KEY FINDINGS</th></tr>
<tr>
<td><u>Author(s)</u>:
Bongers et al. (2020)[53]
<u>Trial registration</u>:
Dutch Trial Register No: NTR5825
<u>Design</u>:
Parallel RCDBT with 1:1 group allocation via computer-generated random numbers in blocks of 10
<u>Duration</u>:
12 wk (3 months)</td>
<td><u>Total Recruited</u>:
n=200 (50-70y)
M: n=115, F: n=85
<u>Total Analyzed:</u>
n=167[A]
M: n=99, F: n=68
Median age: 63y (IQR 56-68)
<u>CH Group</u>: n=77
M: n=49, F: n=28
Median age: 65y (IQR 58-68)
<u>Comparator Group</u>: n=90
M: n=50, F: n=40
Median age: 61y (IQR 55-67)
<u>Ailment</u>: Self-reported knee pain without a diagnosis of KOA recruited in preparation for a multi-day walking event (<u>https://www.4daagse.nl/</u>) and reporting a Visual Analogue Scale (VAS) score >1 at baseline</td>
<td><u>Source</u>:
Bovine (CH)
<u>Dose</u>:
10 g/day dissolved in 100 mL water & taken at breakfast</td>
<td><u>Type</u>: Placebo Maltodextrin (MDx)
<u>Dose</u>:
10g /day dissolved in 100 mL water & taken at breakfast</td>
<td>Knee Injury and Osteoarthritis Outcome Score (KOOS) (all domains)
0: worst, 500: best (increase = improvement)
<table>
<tr><th></th><th><u>CH</u></th><th><u>MDx</u></th><th></th></tr>
<tr><td>Week 0</td><td>302±68
95% CI: 287,317</td><td>308±64
95% CI: 294,322</td><td>p=0.55</td></tr>
<tr><td>Week 12 Δ</td><td>41±56
95% CI: 29, 54
p<0.001</td><td>31±68
95% CI: 16, 45
p<0.001</td><td>p=0.28</td></tr>
<tr><td>SMD</td><td>0.73</td><td>0.46</td><td></td></tr>
</table>
SMD: standardized mean difference

VAS score
(lower score at endpoint indicates improvement)
<table>
<tr><th></th><th><u>CH</u></th><th><u>MDx</u></th><th></th></tr>
<tr><td>Wk 0</td><td>4.7
IQR: 2.8-6.1
95% CI: 3.9, 4.9</td><td>4.7
IQR: 2.8-6.2
95% CI: 4.2, 5.0</td><td>p=0.50</td></tr>
<tr><td>Wk 12 Δ</td><td>-1.6±2.4
95% CI: -2.1, -1.1
p<0.001</td><td>-1.9±2.6
95% CI: -2.5, -1.4
p<0.001</td><td>p=0.42</td></tr>
<tr><td>SMD</td><td>-0.67</td><td>0.46</td><td></td></tr>
</table>
</td>
</tr>
<tr><td colspan="5">[A] n=16 excluded after allocation but before intervention with VAS score <1 at baseline visit, and n=17 dropouts during intervention.</td></tr>
<tr>
<td><u>Author(s)</u>: Bruyère et al. (2012)[58]
<u>Trial registration</u>:
ISRCTN76960238 controlled-trials.com
<u>Design</u>: Parallel RCDBT (1:1 via computer-generated random numbers in blocks of 4.
<u>Duration</u>:
26w (6mo)

(continued overleaf)</td>
<td><u>Total</u>: n=200 M & F ≥50y
<u>CH Group</u>[A]
M: n=27; F: n=73
Age: 65.7±7.8y
BMI: 27.6±4.7 kg/m^2
Knee: n = 34
Hip: n = 13
Back: n = 27
Shoulder/elbow/hand: n=26
<u>Comparator Group</u>[B]
M: n=35; F: n=65
Age 64.4±8.5y
BMI: 27.6±4.6 kg/m^2
<u>Knee</u>: n= 28, <u>Hip</u>: n= 11, <u>Back</u>: n= 39, Shoulder/elbow/hand: n=22</td>
<td><u>Source</u>: Bovine[C]
<u>Dose</u>: 1.2 g/day 3x400 mg capsules with 92.25 mg gelatin / shell</td>
<td><u>Type</u>: Placebo Maltodextrin (MDx)
<u>Dose</u>: 1.2g/day 3x400mg capsules with 92.25 mg gelatin / shell</td>
<td>Clinical responders (VAS > 20% change)
<table>
<tr><th colspan="4">ALL JOINTS</th></tr>
<tr><th></th><th>CH-bov (n=67)</th><th>Placebo (n=77)</th><th></th></tr>
<tr><td>3mo</td><td>44.1%</td><td>39.6%</td><td>p=0.53</td></tr>
<tr><td>6mo</td><td>51.6%</td><td>36.5%</td><td>p=0.036</td></tr>
<tr><th colspan="4">KNEE JOINT</th></tr>
<tr><th></th><th>CH-bov (n=32)</th><th>Placebo (n=27)</th><th></th></tr>
<tr><td>3mo</td><td colspan="3">Not reported</td></tr>
<tr><td>6mo</td><td>37.5%</td><td>51.9%</td><td>p=0.269</td></tr>
</table>
NB: Mean VAS scores at baseline and endpoint were not shown (unable to calculate and compare standardized mean difference between groups)</td>
</tr>
</table>

<table>
<tr><th>STUDY</th><th>POPULATION</th><th>COLLAGEN HYDROLYSATE (CH)</th><th>COMPARATOR(S)</th><th>KEY FINDINGS</th></tr>
<tr><td>Bruyère et al.[58]
continued</td><td><u>Ailment</u>: ambulatory with self-reported worst joint pain (VAS >30mm) in hip, knee, elbow, shoulder, hand, or spine</td><td></td><td></td><td></td></tr>
<tr><td colspan="5">[A] n=33 dropouts (n=15 reporting inefficacy); [B] n=23 dropouts (n=9 reporting inefficacy); [C] confirmed via email (C. Larder, personal communication, January 19, 2024).</td></tr>
<tr><td><u>Author(s)</u>:
Feliciano et al. (2017)[74]
<u>Trial registration</u>: Unreported
<u>Design</u>: Parallel randomized triple blind controlled with 1:1 allocation via computer-generated random numbers in unspecified blocks
<u>Duration</u>:
26w (6mo)</td><td><u>Total</u>:
Recruited: n=115 (M & F ≥50y)
Allocated: n= 109
<u>CH Group</u>:
Allocated: n=56
Completed: n=44[A]
M:13, F:43
Age: 64.1±8.6y
BMI: 26.4±3.9 kg/m^2
<u>Comparator Group:</u>
Allocated: n=53
Completed: n=43[B]
M:10, F:43
Age: 62.7±10.2y
BMI: 27.0±4.8 kg/m^2
<u>Ailment</u>: self-reported knee pain ≥1mo and a KOA defined by Altman's Criteria for Classification of Idiopathic OA</td><td><u>Source</u>: Bovine[C]
<u>Dose</u>: 1.2 g/d via 3x400 mg capsules with 92.25 mg gelatin / shell
AND
home exercise program ≥2x/wk</td><td><u>Type</u>: Placebo Maltodextrin (MDx)
<u>Dose</u>: 1.2 g/d via 3x400 mg capsules with 92.25 mg gelatin / shell
AND
home exercise program ≥2x/wk</td><td>
<table>
<tr><th></th><th>CH (n=44)</th><th>MDx (n=43)</th><th></th></tr>
<tr><td colspan="4">VAS (0: best, 10: worst)[D]: Mean±SD</td></tr>
<tr><td>0m</td><td>4.6±3.0</td><td>4.6±2.9</td><td>p=1.00</td></tr>
<tr><td>6m</td><td>2.2±2.6</td><td>2.2±2.9</td><td>p=0.66</td></tr>
<tr><td>SMD[E]</td><td>-0.82</td><td>-0.72</td><td>p=0.75</td></tr>
<tr><td colspan="4">WOMAC (0: best, 2400: worst) Mean±SD</td></tr>
<tr><td>0m</td><td>795.4 ± 545.2</td><td>752.2 ± 490.4</td><td>p=0.75</td></tr>
<tr><td>6m</td><td>596.4 ± 549.8</td><td>537.5 ± 400.0</td><td>p=0.99</td></tr>
<tr><td>SMD</td><td>-0.39</td><td>-0.36</td><td>p=0.46</td></tr>
</table>
Sub-analysis
(non-compliant exercisers)
<table>
<tr><th></th><th>CH (n=21)</th><th>MDx (n=14)</th><th></th></tr>
<tr><td colspan="4">VAS (0: best, 10: worst)[D:] Mean±SD</td></tr>
<tr><td>0m</td><td>3.7±2.7</td><td>5.6±3.0</td><td>p=0.09</td></tr>
<tr><td>6m</td><td>3.0±2.9</td><td>5.1±3.0</td><td>p=0.03</td></tr>
<tr><td>SMD</td><td>-0.42</td><td>-0.36</td><td>p=0.55</td></tr>
<tr><td colspan="4">WOMAC (0: best, 2400: worst)</td></tr>
<tr><td>0m</td><td>805.3± 569.4</td><td>686.2± 528.1</td><td>p=0.55</td></tr>
<tr><td>6m</td><td>646.5± 519.1</td><td>627.8± 403.5</td><td>p=0.74</td></tr>
<tr><td>SMD</td><td>-0.61</td><td>-0.30</td><td>p=0.23</td></tr>
</table>
</td></tr>
<tr><td colspan="5">[A] n=12 dropouts (n=10 lost in follow up); [B] n=10 dropouts (n=8 lost in follow up); [C] confirmed via email (C. Larder, personal communication, January 19, 2024);
[D] Scored on measured horizontal line with markings for corresponding to ordinal response (i.e. 100mm = 10/10 = extreme), [E] standardized mean difference.</td></tr>
<tr><td>(Table 3-4 continued overleaf)</td><td></td><td></td><td></td><td></td></tr>
</table>

<table>
<tr><th>STUDY</th><th>POPULATION</th><th>COLLAGEN HYDROLYSATE (CH)</th><th>COMPARATOR(S)</th><th>KEY FINDINGS</th></tr>
<tr>
<td><u>Author(s)</u>: Jiang et al. (2014)[54]
<u>Trial registration</u>: Clinical Trial Registration No: 2011-51[A]
<u>Design</u>: Parallel RDBCT with 1:1 group allocation and method of randomization unspecified
<u>Duration</u>: 26w (6mo)</td>
<td><u>Total</u>: Recruited: n=100 women (40-70y) Completed: n=94
<u>CH Group</u>: n=46
Age: 60.9±8.8y
BMI: 24.5±3.1 kg/m^2
<u>Comparator Group:</u> n=48
Age: 60.7±6.2y
BMI: 24.0±3.3 kg/m^2
<u>Ailment</u>: mild-moderate pain confirmed with KOA KL Grade I-III (KL Grade IV excluded)</td>
<td><u>Source</u>: Bovine: 8g/d
<u>Dose</u>: 8 g/day</td>
<td><u>Type</u>: Placebo Maltodextrin (MDx)
<u>Dose</u>: 5 g/day Consumed any time</td>
<td>
<table>
<tr><th></th><th>CH (n=46)</th><th>MDx (n=48)</th><th></th></tr>
<tr><td colspan="4">WOMAC – pain domain (0: best, 20: worst)
0: best, 4: worst (per question): Mean±SD</td></tr>
<tr><td>0m</td><td>3.41 ± 1.68</td><td>3.94 ± 1.42</td><td>p=0.105</td></tr>
<tr><td>6m</td><td>2.33 ± 1.55</td><td>3.67 ± 1.48</td><td>p<0.001</td></tr>
<tr><td colspan="4">WOMAC – stiffness domain (0: best, 8: worst)
0: best, 4: worst (per question): Mean±SD</td></tr>
<tr><td>0m</td><td>1.27 ± 1.36</td><td>1.40 ± 1.38</td><td>p=0.650</td></tr>
<tr><td>6m</td><td>0.71 ± 0.87</td><td>1.29 ± 1.27</td><td>p=0.012</td></tr>
<tr><td colspan="4">WOMAC – function domain (0: best, 68: worst)
0: best, 4: worst (per question: Mean±SD</td></tr>
<tr><td>0m</td><td>10.60 ± 3.50</td><td>10.5 ± 3.30</td><td>p=0.833</td></tr>
<tr><td>6m</td><td>8.24 ± 3.14</td><td>9.98 ± 3.30</td><td>p=0.010</td></tr>
</table>
NB: 95% CI and change in mean data not shown. An improved Total WOMAC score for CH vs MDx at 3m (p<0.001) & 6m (p<0.001) was presented in a truncated graph but not tabulated and differences in means were reported for MDx and CH at 3mo (MDx: 0.002 vs. CH: -1.07) and 6mo (MDx: -0.77 vs. CH: -3.93) without variance so SMD could not be calculated. Overall, unclear reporting of WOMAC domain scores impacting on the interpretation of results.
</td>
</tr>
<tr><td colspan="5">[A] The study protocol was reported as approved by the ethical committee of the 6th People's Hospital affiliated to Shanghai Jiaotong University and registered in the hospital's database. Genuine attempts were made for retrieving the documentation through email communications to no avail.</td></tr>
<tr>
<td><u>Author(s)</u>: Kimira et al. (2019)[55]
<u>Trial registration</u>: Clinical Trial Registration No: 000023033 (Josai University Hospital Medical Information Network)

(continued overleaf)</td>
<td><u>Total</u>: n=51 male students (18-21y)
<u>CH Group</u>:
Recruited: n=26
Completed: n=25 [A]
Age: 19.5±1.3y
BMI: 19.5±1.3 kg/m^2
<u>Comparator Group:</u>
Recruited: n=25
Completed: n=21 [B]
Age: 18.9±1.1y
BMI: 19.8±1.1 kg/m^2
<u>Ailment</u>: healthy/free living (without OA) recruited from a running club</td>
<td><u>Source</u>: Porcine (MW: 3.5-7KDa)
<u>Dose</u>: 5 g/day Consumed any time</td>
<td><u>Type</u>: Placebo Maltodextrin (MDx)
<u>Dose</u>: 5 g/day Consumed any time</td>
<td>
<table>
<tr><th></th><th>CH (n=25)</th><th>MDx (n=21)</th><th></th></tr>
<tr><td colspan="4">JKOM[C] II – pain and stiffness domain
(0: best, 32: worst): Mean±SD</td></tr>
<tr><td>0w</td><td>1.04 ± 2.68</td><td>1.43 ± 3.31</td><td>NS*</td></tr>
<tr><td>4w</td><td>0.16 ± 0.47</td><td>1.33 ± 2.39</td><td>p<0.05</td></tr>
<tr><td>8w</td><td>0.04 ± 0.20</td><td>0.48 ± 2.18</td><td>NS*</td></tr>
<tr><td colspan="4">(continued overleaf)</td></tr>
</table>
</td>
</tr>
</table>

<table>
<tr><th>STUDY</th><th>POPULATION</th><th>COLLAGEN HYDROLYSATE (CH)</th><th>COMPARATOR(S)</th><th>KEY FINDINGS</th></tr>
<tr><td>Kimira et al.[55]
continued

<u>Design</u>:
Parallel RDBCT with 1:1 group allocation and double-blind method of tabulated random numbers
<u>Duration</u>:
8 weeks</td><td></td><td></td><td></td><td>
<table>
<tr><th colspan="4">VAS
(0: best, 100: worst): Mean±SD</th></tr>
<tr><td>0w</td><td>18.5 ± 20.4</td><td>19.5 ± 20.7</td><td>NS*</td></tr>
<tr><td>4w</td><td>8.6 ± 15.7</td><td>19.0 ± 22.7</td><td>NS*</td></tr>
<tr><td>8w</td><td>18.4 ± 26.1</td><td>12.2 ± 19.1</td><td>NS*</td></tr>
</table>
*not significant (without p value shown)
NB: 95% CI and change in mean data not shown.
</td></tr>
<tr><td colspan="5">[A] n=1 dropout (personal reasons); [B] n=4 dropouts (personal reasons); [C] Japanese Knee Osteoarthritis Measure (JKOM) was developed from WOMAC with Japanese appropriate adaptions.</td></tr>
<tr><td><u>Author(s)</u>: Kumar et al.(2015)[52]
<u>Trial registration</u>: CTRI/ 2009/091/000993 (Clinical Trial Registry, India)
<u>Design</u>:
2x Parallel RDBCT[A] with treatment to control ratio of 2:1 assigned via computer-generated coding
<u>Duration</u>:
13 weeks

(continued overleaf)</td><td><u>Total</u>:
n=30 (study 1: CH porcine)
n=30 (study 2: CH bovine)
M & F (30-65y)
<u>Study 1</u>[B]: n=3 (M), n=27 (F)
n=19 CH-porcine
n=11 placebo
<u>Study 2</u>[C]: M: n=12, F: n=18
Completed: n=18 CH-bovine
Completed n=10 placebo
<u>Ailment</u>:
KOA with VAS≥40 and confirmed KL Grade II-IV</td><td><u>Source</u>:
Porcine (study 1)
Bovine (study 2)
<u>Dose</u>: 10 g/d via 2x 5g/d dissolved in 250 ml water or milk after food in the morning and night</td><td><u>Type</u>: Placebo Maltodextrin (MDx) for study 1 & 2
<u>Dose</u>: 10 g/d via 2x 5g/d dissolved in 250 ml water or milk after food in the morning and night</td><td><u>Study 1: CH porcine vs. MDx</u>
<table>
<tr><th></th><th>CH-por
n=19</th><th>MDx
(n=11)</th><th></th></tr>
<tr><td colspan="4">WOMAC-total (0: best, 96: worst): Mean±SD</td></tr>
<tr><td>Baseline</td><td>47.2±9.8</td><td>47.3±8.6</td><td></td></tr>
<tr><td>day 15</td><td>35.4±8.1</td><td>39.9±8.7</td><td></td></tr>
<tr><td>day 30</td><td>39.4±9.4</td><td>42.6±9.4</td><td></td></tr>
<tr><td>day 45</td><td>37.8±9.5</td><td>43.8±8.7</td><td></td></tr>
<tr><td>day 60</td><td>36.1±9.6</td><td>43.6±8.0</td><td>p<0.05</td></tr>
<tr><td>day 75</td><td>34.7±9.3</td><td>45.6±8.1</td><td>p<0.01</td></tr>
<tr><td>day 90</td><td>32.7±9.5</td><td>43.6±9.8</td><td>p<0.01</td></tr>
<tr><td>Endpoint</td><td>31.1±9.8
p<0.001</td><td>45.5±9.4*</td><td>p<0.01</td></tr>
<tr><td colspan="4">VAS (0 mm: best, 100 mm: worst): Mean±SD</td></tr>
<tr><td>Baseline</td><td>63.2±10.6</td><td>60.0±6.3</td><td></td></tr>
<tr><td>day 15</td><td>44.2±10.7</td><td>42.7±13.5</td><td></td></tr>
<tr><td>day 30</td><td>51.1±9.4</td><td>52.7±12.7</td><td></td></tr>
<tr><td>day 45</td><td>46.8±12.0</td><td>50.0±12.6</td><td></td></tr>
<tr><td>day 60</td><td>40.5±11.8</td><td>50.0±10.0</td><td>p<0.01</td></tr>
<tr><td>day 75</td><td>38.9±11.0</td><td>50.9±12.2</td><td>p<0.01</td></tr>
<tr><td>day 90</td><td>36.3±13.8</td><td>54.5±13.7</td><td>p<0.01</td></tr>
<tr><td>Endpoint</td><td>31.1±15.2
p<0.001</td><td>57.3±13.5*</td><td>p<0.01</td></tr>
</table>
*p value for endpoint vs baseline not shown
</td></tr>
</table>

<table>
<tr><th>STUDY</th><th>POPULATION</th><th>COLLAGEN HYDROLYSATE (CH)</th><th>COMPARATOR(S)</th><th>KEY FINDINGS</th></tr>
<tr><td>Kumar et al.[52]
continued</td><td></td><td></td><td></td><td><u>Study 2: CH bovine vs. MDx</u>
<table>
<tr><th></th><th>CH-bov (n=18)</th><th>MDx (n=10)</th><th></th></tr>
<tr><td colspan="4">WOMAC-total (0: best, 96: worst): Mean±SD</td></tr>
<tr><td>Baseline</td><td>50.3±9.6</td><td>50.1±14.7</td><td></td></tr>
<tr><td>day 15</td><td>38.1±7.7</td><td>35.3±7.4</td><td></td></tr>
<tr><td>day 30</td><td>38.5±8.3</td><td>45.6±13.9</td><td></td></tr>
<tr><td>day 45</td><td>34.8±8.6</td><td>47.4±17.9</td><td></td></tr>
<tr><td>day 60</td><td>32.9±9.2</td><td>46.9±18.3</td><td>p<0.01</td></tr>
<tr><td>day 75</td><td>31.8±9.6</td><td>45.2±17.5</td><td>p<0.01</td></tr>
<tr><td>day 90</td><td>29.1±10.0</td><td>45.9±18.6</td><td>p<0.01</td></tr>
<tr><td>Endpoint</td><td>25.8±11.3
p<0.001</td><td>47.3±19.4*</td><td>p<0.01</td></tr>
<tr><td colspan="4">VAS (0 mm: best, 100 mm: worst): Mean±SD</td></tr>
<tr><td>Baseline</td><td>66.0±12.3</td><td>62.0±14.0</td><td></td></tr>
<tr><td>day 15</td><td>40.0±13.4</td><td>50.0±12.5</td><td></td></tr>
<tr><td>day 30</td><td>44.5±11.5</td><td>52.0±12.3</td><td></td></tr>
<tr><td>day 45</td><td>38.5±11.8</td><td>51.0±17.3</td><td></td></tr>
<tr><td>day 60</td><td>39.5±10.5</td><td>53.0±14.9</td><td>p<0.01</td></tr>
<tr><td>day 75</td><td>36.5±11.4</td><td>52.0±13.2</td><td>p<0.01</td></tr>
<tr><td>day 90</td><td>32.0±10.6</td><td>54.0±19.6</td><td>p<0.01</td></tr>
<tr><td>Endpoint</td><td>28.0±10.9
p<0.001</td><td>55.0±20.1*</td><td>p<0.01</td></tr>
</table>
*p value for endpoint vs baseline not shown
NB: 95% CI and change in mean data not shown.</td></tr>
<tr><td colspan="5">[A] Separated into Study 1 (n=30) with CH-(porcine) vs Control and Study 2 (n=30) with CH-bovine vs Control; [B] n=1 dropout in CH group (adverse event) and n=1 dropout in placebo group, replaced by n=2; [C] detail not shown for dropouts in study 2.</td></tr>
<tr><td><u>Author(s)</u>:
Kviatkovsky et al. (2023)[61]
<u>Trial registration</u>:
NCT05751005[A]
ClinicalTrials.gov

(continued overleaf)</td><td><u>Total:</u> n=86[B] M & F (40-65y)
<u>CH-20 Group (n=19):</u>
M: n=11 (58.8±5.3y)
F: n=8(52.0±6.1y)
<u>CH-10 Group (n=19):</u>
M: n=7 (66.1±7.8y)
F: n=12 (56.0±6.5y)
<u>Comparator Group (n=21):</u>
M n=12 (52.6±7.5y)
F: n=9 (54.0±6.5y)</td><td><u>Source</u>: Bovine[E]
<u>Doses:</u>
CH-20: 20 g/d
(10 g, 2x/d)

OR

CH-10: 10 g/d
(5 g, 2x/d)</td><td><u>Type</u>: Placebo
Maltodextrin (MDx)
<u>Dose:</u> 10 g/d (5 g/d x2)</td><td>KOOS-pain (females) (0: worst, 100: best): Mean±SD
<table>
<tr><th></th><th>CH-20 n=8</th><th>CH-10 n=12</th><th>MDx n=9</th><th></th></tr>
<tr><td>0m</td><td>95.5±5.7</td><td>87.0±13.3</td><td>95.7±4.4</td><td>NS</td></tr>
<tr><td>3m</td><td>94.4±7.0</td><td>89.8±11.5</td><td>94.4±5.7</td><td>NS</td></tr>
<tr><td>6m</td><td>96.2±4.4</td><td>91.7±11.5</td><td>87.5±13.6</td><td>NS</td></tr>
<tr><td rowspan="2">9m</td><td>n=6</td><td>n=8</td><td>n=7</td><td></td></tr>
<tr><td>97.7±3.7</td><td>91.0±14.4</td><td>97.6±3.4</td><td>NS</td></tr>
</table>
NS: Nil between or within group difference</td></tr>
</table>

<table>
<tr><th>STUDY</th><th>POPULATION</th><th>COLLAGEN HYDROLYSATE (CH)</th><th>COMPARATOR(S)</th><th>KEY FINDINGS</th></tr>
<tr><td>Kviatkovsky et al.[61]
continued

<u>Design</u>: Parallel RDBCT, 1:1:1 allocation[B] stratified by sex using Excel random function
<u>Duration</u>:
Phase 1: 26 weeks (6 months)
Phase 2[C]:
26 to 39 weeks (9 months)</td><td><u>Ailment</u>: habitual exercisers[D] self-reporting non-specific joint pain (without osteoarthritis diagnosis). Specific joint pain reported by 19% of participants at baseline in their: back (14%), hip (13%), shoulder (11%), knee (9%), heel (2%),rib (2%)</td><td></td><td></td><td></td></tr>
<tr><td colspan="5">[A] Identified after email contact (M. Ormsbee, personal communication, May 26, 2024). The study was reported as approved by the Florida State University Institutional Review Board (STUDY00000044); [B] n=75 completed the 6-month phase (59 results analyzed due to the removal of data from those sustaining injury during intervention), n=51 from these 75 completed the optional extra 3 months (41 results analyzed due to the removal of data from those sustaining injury during intervention); [C] Participants willing to continue for an additional 3 months of intervention after the 6 month phase; [D] Habitual exercise: ≥4h/week for ≥15 years; [E] Identified via email reply (M. Ormsbee, personal communication, January 18, 2024).</td></tr>
<tr><td><u>Author(s)</u>: Newman et al. (2023)[56]
<u>Trial registration</u>: Unreported[A]
<u>Design</u>: Single blind real-world study with randomized treatment to control allocation ratio of 5:1 via the ‘Ingredients for Life’ phone app
<u>Duration</u>:
12 weeks then 4wk washout</td><td><u>Total</u>:
Recruited: n=213 (M & F, 18-72y)
Completed: n=201
<u>CH Group</u>:
n=177 (n=172 completed)
M: n=83, F: n=94 (F: n=2 dropouts)
Age:40.2 ± 11.0y
<u>Comparator</u>:
n=36 (n=29 completed)
M: n=18, F: n=18 (F: n=4 dropouts)
Age: 42.1 ± 11.0y
<u>Ailment</u>: self-reported activity-related joint pain[B] without a diagnosis of osteoarthritis</td><td><u>Source</u>: Porcine[H]
<u>Dose</u>: 1 g/d via 2x 0.5 g capsule also with chondroitin sulfate
AND
using the ‘Ingredients for Life’ phone app for logging pain score each week.</td><td><u>Type</u>: Placebo Maltodextrin (MDx)
<u>Dose</u>: 1 g/d via 2x 0.5 g capsule
AND
using the ‘Ingredients for Life’ phone app for logging pain score each week.</td><td><table><tr><th></th><th colspan="3">VAS score (approximate)
0: best, 10: worst</th></tr><tr><th></th><th>CH</th><th>MDx</th><th></th></tr><tr><td>0w</td><td>≈5/10</td><td>≈4.5/10</td><td>p=0.12</td></tr><tr><td>3w</td><td>≈4/10</td><td>≈4.5/10</td><td>p=0.009</td></tr><tr><td>12w</td><td>≈2/10</td><td>≈4.5/10</td><td>p=0.001</td></tr><tr><td>16w</td><td>≈3/10</td><td>≈4/10</td><td>p=0.001</td></tr></table>
NB: Mean scores and variance with estimates of error at baseline and endpoint were not shown (unable to calculate and compare standardized mean difference between groups)</td></tr>
<tr><td colspan="5">[A] The study was reported as approved by the Reading University Independent Ethics Committee (130819-4). Genuine attempts were made for retrieving the trial registration number and documentation through email communications to no avail; [B] Any site on body with aggravating activities ranging from low impact gardening to high impact marathon training.</td></tr>
<tr><td>(Table 3-4 continued overleaf)</td><td></td><td></td><td></td><td></td></tr>
</table>

<table>
<tr><th>STUDY</th><th>POPULATION</th><th>COLLAGEN HYDROLYSATE (CH)</th><th>COMPARATOR(S)</th><th>KEY FINDINGS</th></tr>
<tr>
<td><u>Author(s)</u>: Trč & Bohmová (2011)[69]
<u>Trial registration</u>: Unreported
<u>Design</u>: Parallel RDBCT with 1:1 group allocation (randomization method unspecified)
<u>Duration</u>:
12 weeks
(3 months) with a one-week washout beforehand</td>
<td><u>Total</u>:
Recruited: n=100 M & F (≥40y)
Completed: n=93[A]
<u>CH group (n=47)</u>:
Baseline demographics not shown
<u>Comparator group (n=46)</u>:
Baseline demographics not shown
<u>Ailment</u>: Free-living, diagnosed [B] with early-stage KOA and self-reported pain/discomfort ≥3mo</td>
<td><u>Source</u>: Porcine[C]
<u>Dose</u>: 10 g/d</td>
<td><u>Type</u>: Active Glucosamine sulphate (GS)
<u>Dose</u>: 1.5 g/d g</td>
<td>VAS (pain intensity improvement [D])
<table>
<tr><th>Score reduction:</th><th>CH</th><th>GS</th><th></th></tr>
<tr><td>Clear improvement (≥20 mm)</td><td>n=32 (68.1%)</td><td>n=17 (37%)</td><td>p<0.05</td></tr>
<tr><td>Marginal (10-20 mm)</td><td>n=8 (17.0%)</td><td>n=14 (30.4%)</td><td>p<0.05</td></tr>
<tr><td>No Improvement (<10 mm)</td><td>n=7 (14.9%)</td><td>n=15 (32.6%)</td><td>p<0.05</td></tr>
</table>
WOMAC Improvement
<table>
<tr><th>Score reduction:</th><th>CH</th><th>GS</th><th></th></tr>
<tr><td>≥15 pts</td><td>n=16 (34.8%)</td><td>n=6 (13.3%)</td><td>p<0.05</td></tr>
<tr><td><15 pts</td><td>n=30 (65.2%)</td><td>n=39 (86.7%)</td><td>p<0.05</td></tr>
</table>
NB: Mean scores for VAS and WOMAC at baseline and endpoint were not shown (unable to calculate and compare standardized mean difference between groups)</td>
</tr>
<tr><td colspan="5">[A] n=4 lost in follow up, n=2 transport issues; n=1 unrelated accident; [B] American College of Rheumatology (ACR) criteria score of I-III; [C] Not explicitly stated but cited as the same used in the studies by Ribas-Fernandez and Perez[72] and Adam[71]; [D] Pain intensity in four situations: now, at its best, the average/typical level, at its worst.</td></tr>
</table>

3.4.3.1 Study design

Most of the studies used a double-blinded, if not triple-blinded, randomized trial design.[52-55,58,61,69,74] Adam[71] conducted a randomized crossover trial, Bernardo and Azarcon[73] did a randomized single-blind open label-controlled trial, and Newman et al.[56] reported on their single-blind, 'real-world' study with a randomization ratio of 5:1. The method of randomization allocation was not reported in three studies.[54,69,71]

3.4.3.2 Study population

The number of recruited participants ranged from 30 to 200.[52,53,58] One study exclusively studied a cohort of peri- or postmenopausal females with medically diagnosed KOA confirmed by radiography,[54] and one study was on healthy young men aged 18-21 years without KOA to investigate the effect of CH on preserving joint condition among endurance runners with negligible pain at baseline.[55] The majority of participants were female in five studies,[52,56,58,73,74] but most of the participants in three studies were male.[53,61,71] The numbers of male and female participants were not reported in the study by Trč and Bohmová.[69]

3.4.3.3 Intervention regimen (comparator, dose and duration)

Comparator

Eight studies compared CH with a placebo of maltodextrin (MDx), matched by weight rather than energy.[52-56,58,61,74] Adam[71] used two comparator groups of protein (gelatin and egg albumin), also matched with CH by weight rather than protein content and without a placebo such as MDx. Meanwhile, Trč and Bohmová[69] compared a daily dose of 10 g CH with 1.5 g glucosamine rather than a placebo, while Bernardo and Azarcon[73] compared a daily dose of 1.2 g CH with pain medication taken *ad libitum* for the duration of their study.

Dose

Table 3-4 shows that the lowest daily dose of CH was 1 g as a capsule.[56] The highest daily dose was 20 g, split into two doses of 10 g, presumably (but not stated) as a powder mixed with an unspecified liquid.[61] Three studies tested daily doses of 1.2 g CH in capsules encased by 92.25 mg of gelatin (Prof. Olivier Bruyère, email reply, 18 January, 2024), thus providing an extra ≈275 mg partially hydrolyzed collagen each day for endogenous digestion.[58,73,74] In one study, participants consumed 5

g/day CH as a powder,[55] another at 8 g/day of powder,[54] while participants in the remaining four studies consumed doses of 10 g/day either as a powder[52,53] or capsules.[69,71]

Duration

The shortest intervention was approximately 60 days in two studies.[55,71] The longest was nine months in a sub-sample studied by Kviatkovsky et al.,[61] although most of the participants in that study ended their intervention as originally planned after six months. A further four interventions were six months,[54,58,73,74] and four were for 12-13 weeks.[52,53,56,69] Kumar et al.[52] reported their intervention as 13 weeks (91 days) in their methodology but presented and reported data for 14 weeks of intervention. Newman et al.[56] also included a comparison between their intervention and comparator groups after a washout of four weeks.

Dose-duration-response

Table 3-5 presents dose-duration-response data for the included studies. All 11 studies made between-group comparisons (i.e. CH vs comparator), and four of them also performed within-group analysis of their intervention and comparator groups (i.e. baseline vs endpoint).[52,53,61,73] There was no apparent pattern between the five studies which reported a positive effect from CH versus its comparator and the six which did not. The daily dose of 1 g/d for 12 weeks was reported as superior to MDx by Newman et al.,[56] but 20 g/d of CH for six months was no different to placebo in the study by Kviatkovsky et al.[61] Five studies captured their PROMs with VAS and either WOMAC,[52,69,74] KOOS[53] or the Japanese Knee Osteoarthritis Measure (JKOM).[55] Three studies used KOOS or WOMAC without VAS,[54,61,73] one study used VAS without KOOS or WOMAC[58] and two did not use VAS, KOOS or WOMAC.[56,71] Table 3-5 shows one study reported an effect from CH versus the MDx placebo with VAS but not WOMAC.[58] It also shows three out of the four studies which listed an author as an employee of the CH supplement company reported an effect from CH, but two of those studies did not declare their author(s) as a conflict of interest,[52,54] in contrast to Newman et al.[56] who did.

Table 3-5. Summary of a dose-duration-effect on pain for collagen hydrolysate (CH) in the 11 included studies.

	DURATION	2m (8w)		3m (12-13w)		4m (16w)		6m (26w)		9m (39w)	
DOSE	*Effect*[A]	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
1 g/d	YES			CH v MDx (VAS)[56]*		CH v MDx (VAS)[56]*					
	NO										
1.2 g/d	YES							BL v EP (WOMAC)[73] CH v MDx (VAS, all joints)[58] CH v MDx (VAS, sub-group)[74]			
	NO								CH v NSAID (WOMAC)[73] CH v MDx (VAS, knee)[58] CH v MDx (WOMAC, VAS)[74] CH v MDx (WOMAC, sub-group)[74]		
5 g/d	YES										
	NO		CH v MDx (JKOM, VAS)[55]*								
8 g/d	YES							CH v MDx (WOMAC)[54]**			
	NO										
10 g/d	YES	CH v Egg[71]		BL v EP (KOOS, VAS)[53] BL v EP & CH v MDx (WOMAC, VAS)[52]** CH v GS (WOMAC, VAS)[69]							
	NO				CH v MDx (KOOS, VAS)[53]				BL v EP & CH v MDx (KOOS)[61]		BL v EP & CH v MDx (KOOS)[61]
20 g/d	YES										
	NO								BL v EP & CH v MDx (KOOS)[61]		BL v EP & CH v MDx (KOOS)[61]

[A] Baseline (BL) vs endpoint (EP) or collagen hydrolysate (CH) versus comparator (maltodextrin: MDx, glucosamine sulphate: GS, Non-steroidal anti-inflammatory: NSAID)

* Author(s) listed as employees of supplement company and declared as a conflict of interest, ** Author(s) listed as employees of supplement company without declaring a conflict of interest

3.5 Discussion

The aim of this systematic review was to evaluate studies investigating the effect of CH from terrestrial mammals on knee pain among participants with or without a diagnosis of KOA confirmed by radiography. Postmenopausal women were not the sole focus, but they were of interest considering their increased risk of experiencing symptomatic KOA which is only diagnosed after the disease has progressed to a relatively advanced stage.[4,5] Only studies using CH sourced from terrestrial mammals were included because they are richer reservoirs of Gly-Pro-Hyp than avian and marine sources; a variable which may contribute to the heterogeneity in results on the efficacy of CH on knee pain to date.[37,42,44]

3.5.1 Risk of Bias

All 11 studies were found to have at least some concern for overall bias. Selective reporting was the predominant reason, despite being discouraged because it prevents dissemination of transparent research.[75] Even so, only three studies were low risk in that domain.[52,53,58]

Nine studies reported their CH intervention as a commercial brand name.[52,53,55,56,58,61,69,73,74] Two studies declared members of their research teams as employees of the supplement company used in their trial as a conflict of interest,[55,56] but two studies which reported an effect from CH on pain did not.[52,54] These facts suggest industry has a keen interest in the nutraceutical market, as health claims of joint pain relief are often made on promotional material for consumers and referenced to published research.[76-78] However, industry funded research may be more likely to find a positive effect than independently funded studies,[79,80] and a lay audience may not have the skills or insight for critical appraisal of that research to determine its generalizability to specific cohorts within the global population. For example, Feliciano et al.[74] reported a statistically significant effect of CH on pain only after post hoc analysis on a small subgroup of non-adherent exercisers with the Visual Analogue Score (VAS), but not the Western Ontario and McMaster Universities Arthritis Index (WOMAC). Therefore, outcome measurement tools are important to consider alongside the results they measure.

3.5.2 Outcome measures

The VAS is a validated single-item scale designed to measure pain intensity.[81] Statistically significant improvements are generally accepted between pre and post intervention measures ≥30mm on a measured line or with scores ≥1 on a numerical scale.[82-84] It has low sensitivity if used in isolation,[81,85] but it can complement other validated tools such as the WOMAC or the Knee Injury and Osteoarthritis Outcome Score (KOOS) which capture subjective measures, including pain, more comprehensively.[86] All except three of the 11 studies[56,58,71] used either WOMAC or KOOS to assess their participants' pain (Table 3-4), but perceptions (i.e. PROMs) are relative to a sufferer's perspective which can depend on demographics, genetics and psycho-social factors.[87,88]

Collecting objective data alongside self-reported measures improves the rigor of a study.[82,89,90] It allows for actual effects to be examined through testing for change in a participant's functional capability or their biomarkers of cartilage turnover and inflammation.[82,86,91] However, only two studies assessed biomarkers of inflammation (data not shown),[53,55] while none of the studies collected data using a set of functional tests endorsed by the Osteoarthritis Research Society International (OARSI).[92] Therefore, it is plausible that some of the reported effects from CH to date are partially or wholly attributable to the contextual effect.[91,93]

The contextual effect is also referred to as the placebo effect which is a common confounder in studies on pain relief.[93-95] A placebo is imperative to include in the design of studies in this field,[82] but three studies either compared CH with protein (i.e. egg albumin),[71] if not other potentially active ingredients of glucosamine or non-steroidal anti-inflammatory drugs (NSAIDs) rather than a placebo.[69,73] The CH treatments were reported as more effective than the positive controls of glucosamine and NSAIDs respectively (Tables 3-4 and 3-5), but the magnitude of their superiority over non-treatment is unclear without a placebo comparator. For example, Bernardo and Azarcon[73] reported a statistically significant improvement in WOMAC scores (i.e. pain, symptoms and function domains) compared to baseline for the CH but not NSAIDs group, but the difference between these two groups at endpoint was not statistically significant. Furthermore, the results were reported as a

mean response for each question (i.e. on a scale of 0-4) rather than each group's mean aggregated score out of 96 for the pain, symptoms and function domains as per the recommended scoring system of WOMAC.[96,97]

3.5.3 Study population

Postmenopausal women are at an increased risk of KOA,[10] but only one study exclusively studied peri- or postmenopausal females with KOA confirmed by radiography.[54] Kumar et al.[52] included males, although their participants were diagnosed with KOA confirmed by radiographic evidence and most of their participants were female of a similar age as the study by Jiang et al.[54] Likewise, Feliciano et al.[74] included males, but their participants were predominantly females within the same age-bracket and were suffering from clinically confirmed symptomatic KOA using the American College of Rheumatology (ACR) criteria.[98] Therefore, the results of these three studies are more applicable to postmenopausal women in the population than the remaining eight studies in this review. For example, Kimira et al.[55] exclusively studied young men and their included participants' baseline pain levels were below the minimum for detecting statistically significant changes in VAS scores at endpoint.[82]

Table 3-4 shows that participants were only included if a medical diagnosis of KOA was confirmed by radiography in six of the 11 studies.[52,54,69,71,73,74] A confirmed diagnosis of KOA was a reason for exclusion in four studies of participants with self-reported pain,[53,55,56,61] and of those studies, the knee was fourth most commonly reported for causing the pain after the back, hip and shoulder joints respectively in the study by Kviatkovsky et al.[61] Furthermore, participants in the study by Bongers et al.[53] were training for an endurance walking event which suggests their degree of pain at baseline was perhaps less prohibitive in contrast to medically confirmed cases of KOA. Therefore, the study participants' baseline characteristics, including extent of ailment from knee pain, are important conditions to consider when interpreting the end point data.

3.5.4 Dietary factors

3.5.4.1 Placebo comparator

Maltodextrin (MDx) was used as a placebo in eight of the 11 studies.[52-56,58,61,74] One of those studies reported statistically significant improvements in knee pain within but not between the groups consuming CH and MDx,[53] and three reported a statistically significant improvement in pain scores for the CH treatment group compared to MDx.[52,54,56] However, those three studies were based solely on subjective measures so contextual factors could explain the reported effect.[91,93] For example, the participants' daily diets may have had an influence within and between the intervention groups.

3.5.4.2 Dietary protein

Dietary protein upregulates intestinal transport of free amino acids and peptides.[99] The efficiency of transport declines with age,[99] but any additional protein consumed with CH can improve the bioavailability of peptides within that intervention.[100,101] For example, Walrand et al.[101] found when giving CH dissolved in a drinkable yoghurt versus water, that the group consuming drinkable yoghurt achieved significantly higher blood levels of collagen peptides compared to the water group. In the study by Bongers et al.,[53] participants were advised to mix their once-daily intervention in water (i.e. a protein-poor medium), ensuring a standard amount of protein was ingested with the supplement. In contrast, Kumar et al.[52] advised their participants to mix twice daily doses of CH or placebo in 250 mL water or milk (i.e. 500 mL of a protein-poor or milk protein-rich fluid each day). All other studies did not report what guidance, if any, was given to their participants.[54-56,58,61,69,71,73,74] Therefore. the participants' protein intakes whilst consuming the supplements each day may have affected the bioavailability of the CH interventions in those studies, irrespective of their total dietary protein intake.

Only Kviatkovsky et al.[61] monitored their participants' total dietary intake over their intervention. They collected data on macronutrient intake at baseline and at each monthly follow up, but the authors only presented the mean intake of macronutrients over their entire study rather than each month.[61] Participants in other studies were advised to follow their usual way of eating, but the

authors did not verify their advice had been followed.[52,53,55] Meanwhile, the remaining studies did not report what, if any, advice was given to their participants, nor did they assess dietary behavior.[54,56,58,69,71,73,74] Therefore, their participants' dietary intakes of protein were essentially unmonitored.

Protein foods of animal origin are sources of undenatured Type I collagen.[102] Rich dietary sources include chicken consumed with its skin or pork with crackling, and in both cases the cooking process causes denaturation (i.e. partial hydrolysis). It becomes gelatin which can be absorbed after further gastrointestinal digestion, albeit less efficiently than CH.[99,103,104] As such, the dietary sources of undenatured Type I collagen and products using gelatin for thickening (i.e. baked goods and processed meats) or to encapsulate medicines are potential sources of BAPs above the supplement dose of CH in the intervention trial.[104] Using data from the 2001-2002 and 2003-2004 National Health and Nutrition Examination Surveys (NHANES), Paul et al.[105] found that daily consumption of these foods can vary from roughly 3 g/d up to ≈23 g/d depending on the individual's dietary behavior, and Nulty et al.[106] found dietary collagen intake was lower among females and older adults than their younger counterparts and males. Therefore, dietary protein intake as a variable, especially foods of animal origin, is important for monitoring as a covariate considering the impracticality of controlling it over the duration of the studies in this review.

3.5.5 Limitations

This systematic review has its limitations considering some studies may have been excluded during screening. In the study by Benito-Ruiz et al.,[67] the CH source may have been porcine (i.e. for inclusion) or avian (i.e. for exclusion). The study was discussed in depth during the screening phase, but it was excluded in keeping with the eligibility criteria. However, those criteria allowed for the inclusion of studies on participants with relatively minor knee pain as already discussed. Furthermore, the search string inadvertently omitted "knee" as a term which risked off-target retrieval. On repeating the search over the same period with Scopus and including 'knee,' the number of hits was reduced from 112 to 60 which means more results went through manual screening and actually increased the

chance of detecting studies for inclusion. Even so, excluding studies on participants without KOA confirmed by radiography in future would focus on a more homogenous group of participants with knee joint pain attributable to the disease. Furthermore, a search on the Cochrane library was not done, but it may have served better than SciFinder N which tended to retrieve patent registration documents rather than peer reviewed studies. Lastly, only five trial registration documents could be retrieved, despite their value in executing a thorough risk of bias assessment.[50,107] For example, they allow the planned statistical analyses of studies to be compared with the actual analyses performed and reported on within that study.[50]

3.6 Conclusion and recommendations

In summary, this systematic review highlights a need for more specific research on the effect of CH on knee pain. It focused on studies using the richest reservoir of postulated BAPs from CH sourced from terrestrial mammals. The 11 included studies were all identified as raising some concern (n=7), if not having a high risk of overall bias (n=4). Given the variability in study designs to date, more targeted research is needed for a clearer understanding about the efficacy of CH as a nutraceutical for knee pain. Future studies may benefit from an exclusive focus on cohorts such as postmenopausal women experiencing knee pain with a diagnosis of KOA, and by capturing objective data alongside subjective patient reported outcomes for within group as well as between group comparisons. Ideally, dietary protein intake would be controlled, if not monitored as a covariate, and the instructions given to participants for consuming the intervention should be reported. Designing and implementing such research will allow future systematic reviews or meta-analyses to synthesise a body of unbiased evidence.

3.7 Chapter 3 references

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CHAMP study conception and planning: JC, Janet Weber (JW) and Katie Schraders (KS, Study Coordinator);
Data collection: DG, JC, KS;
Analysis and interpretation of results: DG;
Drafting of the chapter and preparation of its revisions for a final version: DG;
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Chapter 4: The effects of collagen hydrolysate and milk protein on knee pain and functional performance in a randomized double-blind controlled trial on women aged ≥50 years

This chapter achieved objectives 1 and 2 of the CHAMP research project. It reports on the effect of collagen hydrolysate versus milk protein and maltodextrin on knee pain among women aged ≥50 years. It also includes results for a secondary outcome of the study to examine the effect of CH and comparator groups on functional performance. An abstract was accepted for a presentation at the 2025 Osteoarthritis Research Society International (OARSI) World Congress on Osteoarthritis and publication in Osteoarthritis and Cartilage:

Gordon, D. Schraders, K. Weber, J. Kruger, M. Coad, J. The collagen hydrolysate and milk protein study on postmenopausal women with knee pain. Osteoarthritis Cartilage. 2025;33:S89. Doi: https://doi.org/10.1016/j.joca.2025.02.123

4.1 Abstract

The aim of this study was to assess the effect of 15 g protein as collagen hydrolysate (bovine hide) versus milk protein, or maltodextrin on knee pain and functional performance among peri- and postmenopausal women. The randomized, double-blind controlled trial of four months recruited females aged ≥50 years who self-reported knee pain not necessarily due to KOA. They completed a monthly online survey based on the Knee Injury and Osteoarthritis Outcome Score (KOOS), with mean change in pain score as the primary outcome. The four remaining KOOS domains and five functional tests were secondary measures. The data were examined with mixed design (split-plot) ANOVA, and presented as *F*-statistics, *p values* and partial eta (η_p^2). Pairwise comparisons were made with Bonferroni corrections, and per protocol (PP) analyses were reported as mean, standard error, and 95% confidence intervals after verification with intention-to-treat (ITT) analyses. Eighty-two participants were allocated to the three isoenergetic supplements. Eight participants dropped out and two were excluded during PP analysis (adherence to intervention <75%). There was a significant improvement in mean pain score ($F(2.879, 198.650) = 21.199$, $p<0.001$, $\eta_p^2 = 0.235$), but no interaction between the supplements and pain over time ($F(5.758, 198.650) = 0.303$, $p=0.930$, $\eta_p^2 = 0.009$). Compared to baseline, monthly improvements in pain were statistically significant within each group, and statistically significant within but not between-group improvements were found for the secondary KOOS domains and three functional activities. Ultimately, daily consumption of 15 g protein as CH was not any more effective than the two comparator groups for improving pain perceptions or functional performance.

Trial Registration: Australia and New Zealand clinical trials registry (https://www.anzctr.org.au/) - ACTRN 12622000779774 (31/05/2022).

4.2 Introduction

Knee osteoarthritis (KOA) is characterized by cartilage degradation causing pain and stiffness.[1] Women are disproportionately affected after menopause, indicating an effect from estrogen depletion on catabolic conditions, if not from other sex-related differences which are yet to be fully understood.[2-6] Cartilage is an extracellular matrix (ECM) covering subchondral bone, and its integrity is moderated by quiescent chondrocytes in the upper layers of a healthy mature matrix.[7] In diseased states (i.e. KOA), altered chondrocyte activity leads to irreversible erosion of the ECM,[8] so chondrocytes are considered targets for stalling the onset or progression of KOA.[3,9]

Collagen hydrolysates (CH) are marketed as a nutraceutical agent for KOA pain relief. They are derived from undenatured Type I collagen (UC-I), after being extracted from a host animal and hydrolyzed.[10,11] Undenatured collagen (UC) is the predominant structural protein of vertebrates, and UC-I is found in the skin while Type II (UC-II) is the main organic component of cartilage.[12] Both types are comprised of repeating Gly-X-Y sequences, with proline (Pro) and hydroxyproline (Hyp) often occurring in the X-Y positions.[12] The concentration of prolyl-hydroxyproline (Pro-Hyp) varies by source, and terrestrial mammals have a higher concentration of these potentially bioactive peptides (BAPs) in their UC-I than avian and marine alternatives.[13]

Pioneering *in vitro* research has shown Pro-Hyp can promote UC-II synthesis and prevent terminal differentiation of chondrocytes.[14,15] More recently, Schadow et al.[16] observed no such effect with their *in vitro* study on chondrocytes from diseased human cartilage. Overall, the evidence on CH efficacy is inconclusive considering human trials to date are also unconvincing. Several studies have reported a statistically significant effect of CH on joint pain compared to controls,[17-20] but they only employed subjective patient reported outcome measures (PROMs).[21,22]

Studies on KOA which measure PROMs and objective data are needed.[23-25] Of the few intervention studies on CH from terrestrial mammals which exist,[26-28] Bongers et al.[26] and Feliciano et al.[27] found statistically significant improvements within the treatment and control groups for PROMs

captured with the Knee Injury and Osteoarthritis Outcome Score (KOOS) and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) respectively. The authors did not find between-group differences in PROMs, nor any evidence of effect when measuring biomarkers of inflammation, cartilage turnover or thickness.[26,27] Furthermore, the researchers used maltodextrin (MDx) as a placebo matched by weight rather than energy, and they did not include an isoproteic comparator.[26,27] To our knowledge, nobody has compared CH sourced from terrestrial mammals with milk protein (MP), even though milk is a rich source of Proline, so it may have as much potential as CH in supporting collagen biosynthesis.[12,29,30] Therefore, the aim of this study was to assess the effect of CH on knee pain among peri- and postmenopausal women (i.e. aged ≥50 years) in comparison to energy, and protein matched placebos. In doing so, we assessed PROMs and objective outcomes of functional performance.

4.3 Methodology

The parallel randomized double-blind controlled trial (RDBCT) of four months (17 weeks) was registered with the Australia and New Zealand clinical trials registry on 31/05/2022 (ACTRN 12622000779774, https://www.anzctr.org.au/). It was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving humans were approved by the New Zealand Health and Disability Ethics Committee (2022 exp 12925). Written informed consent was obtained from all participants.

4.3.1 Participants

Females aged ≥50 years with self-reported knee pain not necessarily due to KOA were recruited between January and October 2023. Advertisements appeared on social media and at community groups within the Manawatū region of New Zealand. The study was also promoted on national radio, in print media and by word of mouth.

Participants completed an online survey (Qualtrics XM 2023), adapted from the pain domain of KOOS before it became licensed.[24,31] Nine screening questions were self-administered on a 6-point scale rather than the usual 5-points. “Not applicable” (“n/a”) was added so respondents could indicate

not having engaged in the activity in question (e.g. ascending/descending stairs). All "n/a" responses were considered missed rather than scored zero (i.e. indicating no pain during the activity). Raw scores ≥8/36 were included, but the criteria were refined midway through recruitment because several respondents with pain scores <8/36 reported changing their living routines for pain-related reasons (e.g. avoiding stairs which evoke pain). Thereafter, respondents with pain scores of 6-7/36 were provided with the quality of life (QoL) questions, and raw scores ≥7/16 for that domain were included.[31] All auto-immune conditions (except diet-controlled Coeliac Disease) were excluded, as were hepatic or renal diseases, and anyone who sustained knee trauma or underwent surgery in the previous six months. Similarly, any respondents who commenced hormone replacement therapy (HRT) or used oral nutrition supplements containing chondroitin, glucosamine, or collagen (undenatured or hydrolyzed) within the previous six months were excluded (Appendix 3: Supplementary Table 9-2).

4.3.2 Intervention

Participants were randomized with an equal representation ratio (1:1:1) for every nine admitted to the study using a computer-generated system (calculator.net). They were allocated on arrival at baseline to receive 15 g protein as CH (extracted from bovine hide), 15 g milk protein (MP), or the isoenergetic placebo of 19.2 g MDx (Table 4-1). To achieve an isoenergetic and isoproteic state, 4.2 g MDx was added to CH. The identically packaged supplements were presented to participants at the end of their baseline visit accompanied by verbal and written guidance to consume one sachet each day as a single dose or spread over the day in foods or fluids of each participant's choice. They were also given a supply of multivitamins for daily consumption to reduce the possibility that micronutrients required for collagen synthesis were rate-limiting (Appendix 3: Supplementary Table 9-3). Lastly, participants were asked to maintain their habitual diet and exercise patterns over the intervention. All researchers and participants were blind to the supplement groups until statistical analyses were complete.

Table 4-1. Nutritional information panel for the three isoenergetic supplement groups.

	Collagen Hydrolysate	Milk Protein	Maltodextrin
Energy (kJ)	325.8	325.8	325.8
Protein (g)	15	15	0
Fat (g)	0	1.2	0
Carbohydrate (g)	4.2	3.6	19.2
Total weight (g)	20.3	21.5	19.2

4.3.3 Data collection

Baseline and endpoint visits were hosted at the Human Nutrition Research Unit (HNRU) of Massey University in Palmerston North, New Zealand. They started between 0730-0930 and finished between 1130-1300 hours. The first baseline visit was in February 2023, and the last endpoint visit was in February 2024.

4.3.3.1 Patient-Reported Outcome Measures

After providing written informed consent at baseline, participants completed their first monthly online questionnaire (Qualtrics XM 2023 and 2024). It was slightly modified from KOOS,[31] and self-administered on a 5-point scale for the pain, symptoms and QoL domains, or 6-point scale for the activities of daily living (ADL) and sport and recreation function (sport) domains. The "n/a" response was added into the sport and the ADL domains, except for two ADL questions about ascending/descending stairs which remained on the 5-point scale. All "n/a" responses were considered missed rather than scored, and raw scores for each domain were converted onto a worst-best scale out of 100 with an algorithm which required a minimum number of scored responses for validation.[32] The participants were prompted by email to complete the survey each month at home until their endpoint visit at the HNRU.

4.3.3.2 Functional tests

Five functional activities were performed at baseline and endpoint (Table 4-2). A two-minute walk (2MWT) was used in lieu of the six-minute test given its suitability and validity for participants with knee ailments.[33,34] All five activities were described from a script offering minimal encouragement to avoid influencing performance,[35,36] and two testers kept time with a stopwatch to one hundredth

of a second on a laptop (Microsoft Clock App, 2023, HP Probook 635 Aero G8 Notebook) or personal cell phone.

Table 4-2. The five functional activities in order of execution and with their key reporting standards.[37]

40m Fast-Paced Walk Test (FPWT) • Course calibration = 4x 10 m • Walking pace = fast, without running
Two-Minute Walk Test (2MWT) • Course calibration = 20 m distance • Walking pace = fast, without running
Timed Up and Go (TUG) • Chair apron height = 47 cm, • Chair apron depth = 46 cm • Arm rest height = 70 cm (rear) and 67 cm (front) • Walking pace = usual daily (i.e. not fast)
30-second Chair Stand (30s-CST) • Chair apron height = 47 cm, • Chait apron depth = 46 cm
Stair Climb Test (SCT) • Number of stairs = 11 • Height = 17 cm • Depth = 30 cm • Walking pace = fast, without running

4.3.3.3 Dietary behavior

Participants were emailed four-day diet records (4DDRs) to complete at home in the preceding week of their baseline and endpoint visits. The data were intended to be analyzed with Foodworks (version 10.0.4266, premium edition, Xyris Software Pty Ltd, Australia) to verify intakes of protein were unchanged. Body weight was also measured in light clothes without shoes to the nearest 0.01 kg on digital scales (Tanita WB-110A, Japan) to examine for weight maintenance or change over the intervention. Standing height was taken to the nearest 0.1 cm with a stadiometer (Seca Medical Measuring Systems, Chino, CA, USA) and body mass index (BMI) was calculated.

4.3.3.4 Pain relief

A 'pain diary' was given to all participants at the end of their baseline visit. They were asked to record any oral analgesics used over the intervention, at what dosage and if they were taken for knee pain or for other reasons (e.g. headache). The participants were also asked informally to note other treatments (e.g. topical creams, intra-articular injections, acupuncture, physiotherapy or strength and conditioning exercise). The pain diaries were collected from participants at endpoint.

4.3.3.5 Adherence

All empty and unopened supplement sachets returned by participants were counted.[38] A conservative threshold of <75% was set for defining a protocol violation of non-adherence.

4.3.3.6 Adverse events

All participants were asked in their monthly surveys if they had any of the listed symptoms which have been experienced in similar studies on CH.[18,20,26] They could also report any ill effects with free text.

4.3.4 Statistical analyses

4.3.4.1 Primary outcome

Mean change in monthly pain domain score by supplement group was the primary outcome. Participants indicated at baseline if their pain was unilateral or bilateral, and those with bilateral pain were asked which knee was predominant for monitoring as the index.[38] If both were equally as painful, a hierarchy determined which knee would be the primary (Figure 4-1).

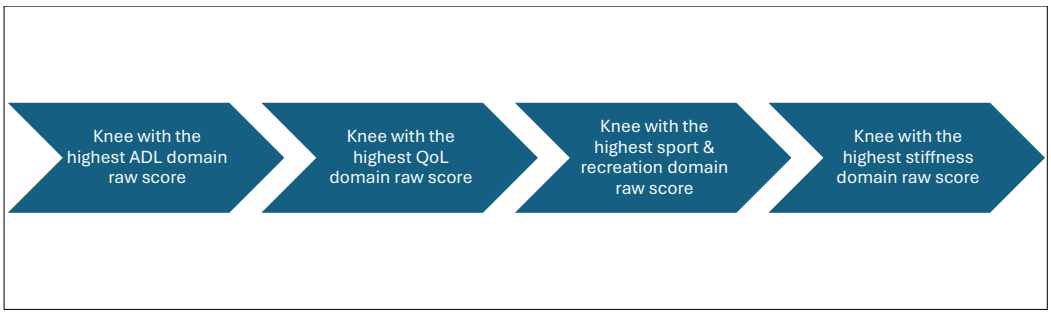

Figure 4-1. Scoring hierarchy for determining the worst knee at baseline.[24] If both knees were equally as painful, the knee with a higher ADL score was chosen, but if ADL scores were equal, then the knee with a higher QoL score was chosen. If ADL and QoL scores were equal, then the higher sport and recreation score was chosen, but if that score was equal, then the higher stiffness score was used to identify the worst knee.

4.3.4.2 Secondary outcomes

The mean change in scores of all four remaining KOOS domains for the worst knee between baseline and endpoint were secondary outcomes, as were performances in the five functional activities.[31,36] The mean change in scores in the five KOOS domains for the least painful knee were secondary outcomes for participants who reported bilateral pain at baseline.[24,38]

4.3.4.3 Data analyses

The statistical analyses were performed with SPSS (version 29, IBM SPSS statistics). A sample size of 27 per group was calculated with G*Power (version 3.0.10) at 90% power, and alpha error of 5% based on detecting change ≥6 in the KOOS pain domain score.[39] Baseline data were presented as means and standard deviations by supplement group and altogether. Distributions in variance within groups were evaluated by visual inspection of skewness (≤±1) and kurtosis (≤±2), Q-Q plots and the Shapiro-Wilk test. Homogeneity of variance between groups was assessed with Levene's test. Violations in the assumptions of normality for the primary outcome of pain were resolved by common (log10) transformation and removal of one extreme outlier from the CH group (Appendix 3: Supplementary Table 9-4). The data were examined with a two-way mixed-design (split-plot) ANOVA, and the Greenhouse-Geisser correction was applied to overcome unequal group sizes. All analyses of the index knee are presented as *F*-statistics, *p* values and partial eta (η_p^2) to examine for statistical significance and interpret effect sizes.[40,41] Any follow-up pairwise comparisons were made with Bonferroni corrections, presented as means, standard errors, and 95% confidence intervals with $p<0.05$ set for significance. All KOOS domain data on the second knee were assessed with non-parametric ANCOVA (Quade's test), adjusting for baseline values as covariates.

The primary and secondary outcomes of the index knee were checked with a sensitivity analysis model.[42,43] Intention-to-treat (ITT) data were analyzed before and after log10 transformation using the Expectation-Maximization (EM) method for imputation of missing values. The analyses were progressively repeated before and after log10 transformation per protocol (PP) without data from dropouts, without dropouts and participants with adherence rates <75%, and then without the one extreme outlier of pain in the CH group.

4.4 Results

82 participants were allocated to supplements and 74 completed the study (Figure 4-2). The most common reasons for discontinuation were disliking the taste (n=2) and private matters reported as unrelated to the study (n=2). One participant also withdrew after sustaining an injury, and one

started HRT soon after baseline so was excluded thereafter. One was lost in follow-up before the first monthly survey, and one had knee surgery after the third monthly survey but before endpoint. Unless otherwise stated, the results are reported for PP analysis of data without dropouts and adherence rates <75% (including the extreme outlier for pain), given the consistency of findings across all steps of sensitivity analysis examination.[43]

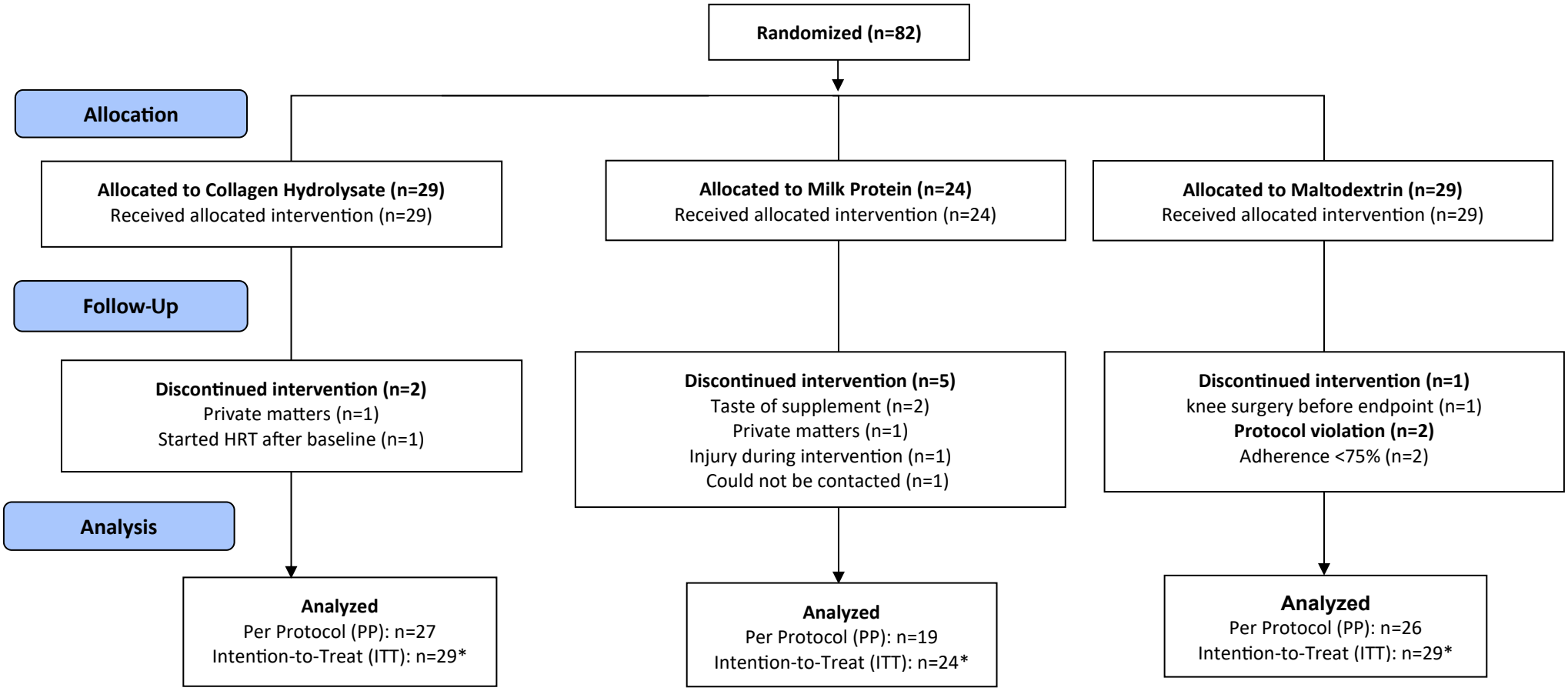

Figure 4-2. CONSORT diagram of participant flow from randomization to data analyses.

*Expectation-Maximation (EM) imputation for missing data for comparing ITT with PP data in the untransformed and log10 transformed formats.

Table 4-3. presents baseline data of the total sample and by supplement group. It includes comparisons of pain scores for the worst knee and the second knee among those with bilateral pain at baseline. As per KOOS scoring guidelines,[32] higher raw scores translate to lower scores out of 100 (i.e. maximum pain = 36/36 raw = 0/100 transformed).[24]

Table 4-3. Baseline characteristics of participants by supplement group and altogether.

	Supplement Group											
	Collagen Hydrolysate (CH)			Milk Protein (MP)			Maltodextrin (MDx)			Total		
	n=	Mean	SD	n=	Mean	SD	n=	Mean	SD	N=	Mean	SD
Age (years)	29	63.9	8.2	24	61.3	7.8	29	64.8	8.7	82	63.5	8.3
Baseline weight (kg)	29	74.1	14.3	24	83.4	14.6	29	78.4	15.8	82	78.3	15.2
Baseline BMI (kg/m^2)	29	27.8	5.0	24	30.6	5.5	29	29.5	6.0	82	29.2	5.6
Worst knee pain (0-100) *0= extreme, 100=absent*	29	62.4 *Mod* [A] *(37.5-62.5)*	16.3	24	58.3 *Mod* [A] *(37.5-62.5)*	13.4	28[B]	58.4 *Mod* [A] *(37.5-62.5)*	15.9	81	59.8 *Mod* [A] *(37.5-62.5)*	15.3
Second knee pain (0-100) *0= extreme, 100=absent*	12	74.8 *Mild* [A] *(62.5-87.5)*	16.6	11	72.2 *Mild* [A] *(62.5-87.5)*	13.4	16	72.7 *Mild* [A] *(62.5-87.5)*	15.3	39	73.2 *Mild* [A] *(62.5-87.5)*	14.9

[A] As categorized by Roos,[24]
[B] One participant missed 6 of 9 questions which invalidated their raw score for transforming onto the scale out of 100.[32]

4.4.1 Primary outcomes

4.4.1.1 Patient Reported Outcome Measure - Pain

Table 4-4 shows the change in pain scores by supplement group across time. Any missing data from partially completed monthly surveys were imputed with the Expectation-Maximization (EM) method (maximum 25 iterations).

There was a significant change in pain between baseline and endpoint ($F(2.879, 198.650) = 21.199$, $p<0.001$, $\eta_p^2 = 0.235$), but no interaction between the supplement groups and pain scores ($F(5.758, 198.650) = 0.303$, $p=0.930$, $\eta_p^2 = 0.009$). The mean pain scores were improved within each group at every month compared to baseline (Table 4-4), and they all reached statistical significance except for the MDx group at month 3 (mean change: 6.7±2.7, 95% CI: -1.2, 14.5. $p=0.160$). It was significant with ITT analysis (mean change: 7.8±2.6, 95% CI: 0.2, 15.4. $p=0.041$).

Table 4-4. Monthly mean KOOS pain domain scores by supplement group.

	Supplement Group													
	Collagen Hydrolysate (CH)					Milk Protein (MP)					Maltodextrin (MDx)			
	n=27	Mean	SE	95% CI		n=19	Mean	SE	95% CI		n=26	Mean	SE	95% CI	
Time	*F*(2.879, 198.650) = 21.199, ***p<0.001***, η_p^2 = 0.235														
Time*Group	*F*(5.758, 198.650) = 0.303, *p=0.930*, η_p^2 = 0.009														
Baseline *Classification*[24]		62.8 *Mild (62.5-87.5)*	3.0	56.8	68.7		58.5 *Mod. (37.5-62.5)*	3.5	51.4	65.5		60.7 *Mod. (37.5-62.5)*	3.0	54.6	66.7
Month 1 *Within-group p value* [A]		73.7 ***p<0.001***	3.0	67.7	79.7		68.8 ***p=0.003***	3.6	61.6	75.9		69.8 ***p=0.002***	3.1	63.7	76.0
Month 2 *Within-group p value* [A]		74.3 ***p<0.001***	2.8	68.7	79.9		70.4 ***p=0.004***	3.3	63.7	77.0		70.6 ***p=0.005***	2.9	64.9	76.3
Month 3 *Within-group p value* [A]		73.1 ***P=0.002***	3.1	66.9	79.3		69.4 ***p=0.009***	3.7	62.0	76.8		67.3 ***p=0.160*** [B]	3.2	61.0	73.6
Endpoint *Classification*[24] *Within-group p value* [A]		74.6 *Mild (62.5-87.5)* ***p<0.001***	3.1	68.4	80.9		70.7 *Mild (62.5-87.5)* ***p=0.005***	3.7	63.2	78.1		72.8 *Mild (62.5-87.5)* ***p<0.001***	3.2	66.5	79.2

[A] Timepoint versus baseline, [B] *p=0.041* (untransformed ITT data mean change: 7.8±2.6, 95% CI: 0.2, 15.4).

4.4.2 Secondary outcomes

Table 4-5 shows participants' secondary KOOS domain scores for their worst knee by supplement group at baseline and endpoint. Table 4-6 presents the domain scores of the second knee among those who reported bilateral pain at baseline and endpoint. Table 4-7 shows performances at baseline and endpoint by supplement group for each of the functional activities.

4.4.2.1 Patient Reported Outcome Measures

There were no statistically significant differences between groups (interactions) in the secondary KOOS domains across time (Table 4-5). Within-group improvements were statistically significant for all domains, except symptoms in the MDx group (mean change: 5.2±2.6, 95% CI: -0.1, 10.4 *p=0.052*). It was significant with ITT analysis (mean change: 5.6±2.3, 95% CI: 1.0, 10.2 *p=0.019*).

Table 4-5. Secondary patient reported outcome measures of participants worst knee at baseline (BL) and endpoint (EP) by supplement group.

	Supplement Group													
	Collagen Hydrolysate (CH)					Milk Protein (MP)					Maltodextrin (MDx)				
	n=	Mean	SE	95% CI		n=	Mean	SE	95% CI		n=	Mean	SE	95% CI	
Symptoms	27					19					26				
Time	$F(1.000, 69.000) = 31.042$, ***p<0.001***, $\eta_p^2 = 0.310$														
Time*Group	$F(2.000, 69.000) = 1.713$, *p=0.188*, $\eta_p^2 = 0.047$														
Baseline		62.0	3.5	55.0	69.0		58.5	4.2	50.2	66.9		67.1	3.6	60.0	74.3
Endpoint		73.8	4.0	65.9	81.8		68.0	4.8	58.6	77.5		72.3	4.1	64.2	80.4
Within-group p value		***p<0.001***					***p=0.003***					***p=0.052*** [A]			
Activities of Daily Living	27					19					26				
Time	$F(1.000, 69.000) = 37.569$, ***p<0.001***, $\eta_p^2 = 0.353$														
Time*Group	$F(2.000, 69.000) = 0.028$, *p=0.972*, $\eta_p^2 = 0.001$														
Baseline		73.4	3.3	66.9	80.0		70.5	3.9	62.7	78.3		71.3	3.4	64.6	78.0
Endpoint		83.9	2.9	78.0	89.7		80.0	3.5	73.0	87.0		81.1	3.0	75.2	87.1
Within-group p value		***p<0.001***					***p=0.003***					***p<0.001***			
Sport & Recreation	27					19					26				
Time	$F(1.000, 69.000) = 82.519$, ***p<0.001***, $\eta_p^2 = 0.545$														
Time*Group	$F(2.000, 69.000) = 0.247$, *p=0.782*, $\eta_p^2 = 0.007$														
Baseline		46.6	4.4	37.8	55.4		44.0	5.3	33.5	54.5		48.9	4.5	39.9	57.9
Endpoint		72.5	4.1	64.4	80.7		66.0	4.8	56.3	75.6		71.4	4.1	63.1	79.6
Within-group p value		***p<0.001***					***p<0.001***					***p<0.001***			
Quality of Life	27					19					26				
Time	$F(1.000, 69.000) = 45.875$, ***p<0.001***, $\eta_p^2 = 0.399$														
Time*Group	$F(2.000, 69.000) = 0.329$, *p=0.721*, $\eta_p^2 = 0.009$														
Baseline		32.6	2.8	27.1	38.2		29.2	3.3	22.6	35.8		36.4	2.8	30.7	42.0
Endpoint		45.4	3.8	37.9	52.9		43.7	4.5	34.8	52.6		47.1	3.8	39.5	54.7
Within-group p value		***p<0.001***					***p<0.001***					***p<0.001***			

[A] *p=0.019* (untransformed ITT data mean change: 5.6±2.3, 95% CI: 1.0, 10.2).

After controlling for baseline values of complete data (bilateral pain at baseline and endpoint), there were no significant differences between groups in the five KOOS domain scores for the second knee (Table 4-6). Visual inspection of the data found eight cases across all groups (CH: n= 2, MP: n= 1, MDx: n= 5) reported a worsening of pain in their second knee at endpoint, and nine participants with bilateral pain at baseline reported unilateral pain at endpoint (i.e. data for their second knee were unavailable for ANCOVA). Of those nine participants, six were from the CH group, one was from the MP group, and two consumed MDx.

Table 4-6. Baseline (BL) and endpoint (EP) mean KOOS domain scores (second knee) by supplement group.

	Supplement Group											
	Collagen Hydrolysate (CH)			Milk Protein (MP)			Maltodextrin (MDx)			Total		
KOOS Domain (Secondary Knee)	**n=**	**Mean**	**SD**	**n=**	**Mean**	**SD**	**n=**	**Mean**	**SD**	**n=**	**Mean**	**SD**
BL Pain (0-100)	12	74.8	16.6	8	68.4	12.6	15	74.4	14.2	35	73.1	14.5
EP Pain (0-100)	6	71.8	15.1	7	80.5	9.0	13	79.1	15.5	26	77.8	13.8
Quade ANCOVA [A]	*F*(2, 24) = 0.048, *p=0.953*											
BL Symptoms (0-100)	12	73.3	17.1	8	72.4	10.7	17	75.7	13.6	37	74.2	14.0
EP Symptoms (0-100)	6	69.0	21.4	7	79.1	15.2	14	77.6	13.3	27	76.1	15.7
Quade ANCOVA [A]	*F*(2, 25) = 0.528, *p=0.596*											
BL Activities of Daily Living (0-100)	12	83.5	12.7	7	73.1	12.6	16	78.8	14.8	35	79.2	13.8
EP Activities of Daily Living (0-100)	6	75.5	11.0	7	86.5	10.6	14	84.7	16.8	27	83.1	14.4
Quade ANCOVA [A]	*F*(2, 24) = 1.194, *p=0.320*											
BL Sport & Recreation (0-100)	10	67.7	23.1	5	51.7	14.0	12	62.4	18.9	27	62.4	20.0
EP Sport & Recreation (0-100)	2	51.9	11.5	5	64.7	11.7	9	68.7	22.9	16	65.3	18.9
Quade ANCOVA [A]	*F*(2, 12) = 0.190, *p=0.829*											
BL Quality of Life (0-100)	12	42.7	16.0	8	35.2	17.0	17	48.5	9.0	37	43.7	14.1
EP Quality of Life (0-100)	6	38.5	15.5	7	48.2	18.7	14	51.3	16.1	27	47.7	16.8
Quade ANCOVA [A]	*F*(2, 25) = 2.016, *p=0.154*											

[A] *Analysis performed with complete data only (i.e. baseline and endpoint values), adjusting for baseline.*

4.4.2.2 Functional tests

There were no statistically significant differences (interactions) between groups over time in the five functional tests. The three groups achieved statistically significant within-group improvements in the TUG (Table 4-7). They all improved in their chair stand (30s-CST) and stair climb (SCT) tests, but the changes were only statistically significant for the MDx and CH groups in the 30s-CST and for the CH group in the SCT. The MP group's improvement in the 30s-CST was statistically significant in ITT data analysis (mean change: 0.7±0.3, 95% CI: 0.1, 1.3 *p=0.033*), and the CH group's mean change in SCT performance was not significant in PP data analysis without the extreme outlier for pain (mean change: -0.8±0.4, 95% CI: -1.6, 0.02 *p=0.054)*. Inspection of the SCT data found four participants in the MP group, nine in the MDx group, and seven in the CH group were slower at endpoint compared to baseline. There were not any statistically significant within-group improvements for the Fast-Paced Walk (FPWT) or Two-Minute Walk Test (2MWT). Inspection of the 2MWT data found 10 participants within the CH group travelled less distance at the endpoint compared to baseline, as did seven within the MP group and nine within the MDx group.

Table 4-7. Functional test performances at baseline and endpoint by supplement group.

	Supplement Group													
	Collagen Hydrolysate (CH)					Milk Protein (MP)					Maltodextrin (MDx)				
	n=	Mean	SE	95% CI		n=	Mean	SE	95% CI		n=	Mean	SE	95% CI	
FPWT [A] (m/s)	27					19					26				
Time	$F(1.000, 69.000) = 1.031$, *p=0.313,* $\eta_p^2 = 0.015$														
*Time*Group*	$F(2.000, 69.000) = 0.601$, *p=0.551,* $\eta_p^2 = 0.017$														
Baseline		1.71	0.06	1.59	1.83		1.76	0.07	1.62	1.90		1.68	0.06	1.56	1.81
Endpoint		1.75	0.06	1.63	1.87		1.79	0.07	1.65	1.92		1.68	0.06	1.56	1.79
Within-group p value		*p=0.231*					*p=0.425*					*p=0.786*			
2MWT [B] (m)	27					19					26				
Time	$F(1.000, 69.000) = 4.258$, ***p=0.043*** [F], $\eta_p^2 = 0.058$														
*Time*Group*	$F(2.000, 69.000) = 0.064$, *p=0.938,* $\eta_p^2 = 0.002$														
Baseline		186.4	5.4	175.7	197.1		191.1	6.4	178.2	203.9		182.1	5.5	171.2	193.1
Endpoint		190.2	5.6	179.1	201.4		193.6	6.7	180.3	206.9		186.0	5.7	174.7	197.4
Within-group p value		*p=0.158*					*p=0.429*					*p=0.156*			
TUG [C] (s)	27					19					26				
Time	$F(1.000, 69.000) = 34.537$, *p<0.001,* $\eta_p^2 = 0.334$														
*Time*Group*	$F(2.000, 69.000) = 1.595$, *p=0.210,* $\eta_p^2 = 0.044$														
Baseline		9.52	0.31	8.90	10.13		8.70	0.37	7.96	9.43		9.49	0.31	8.86	10.12
Endpoint		8.67	0.31	8.06	9.29		8.08	0.37	7.35	8.81		9.08	0.31	8.46	9.71
Within-group p value		***p<0.001***					***p=0.003***					***p=0.022***			
30s-CST [D] (n=)	27					19					26				
Time	$F(1.000, 69.000) = 18.735$, *p<0.001,* $\eta_p^2 = 0.214$														
*Time*Group*	$F(2.000, 69.000) = 0.087$, *p=0.916,* $\eta_p^2 = 0.003$														
Baseline		9.11	0.52	8.08	10.15		9.55	0.62	8.32	10.79		9.00	0.53	7.94	10.06
Endpoint		9.98	0.51	8.96	11.00		10.26	0.61	9.05	11.48		9.90	0.52	8.86	10.94
Within-group p value		***p=0.006***					***p=0.058*** [G]					***p=0.005***			
SCT [E] (s)	27					19					26				
Time	$F(1.000, 69.000) = 6.912$, *p=0.011,* $\eta_p^2 = 0.091$														
*Time*Group*	$F(2.000, 69.000) = 0.295$, *p=0.745,* $\eta_p^2 = 0.008$														
Baseline		17.04	1.15	14.76	19.33		15.49	1.37	12.76	18.21		16.40	1.17	14.07	18.73
Endpoint		16.12	1.15	13.83	18.42		15.01	1.37	12.27	17.74		15.83	1.17	13.49	18.17
Within-group p value		***p=0.026*** [H]					*p=0.321*					*p=0.168*			

[A] 40m-Fast Paced Walk Test (FPWT), [B] 2-minute walk test (2MWT), [C] Timed Up and Go (TUG), [D] 30-second Chair Stand Test (30s-CST), [E] Stair Climb Test (SCT), [F] $F(1.000, 69.000) = 3.804$, *p=0.055,* $\eta_p^2 = 0.052$ (log10 PP data without 2x protocol violations), $F(1.000, 68.000) = 3.529$, *p=0.065,* $\eta_p^2 = 0.049$ (log10 PP data without 2x protocol violations and 1x extreme outlier for pain),

[G] *p=0.033* (ITT data: mean change: 0.7±0.3, 95% CI: 0.1, 1.3), [H] *p=0.054 (*PP data without 2x protocol violations and 1x extreme outlier for pain: mean change: -0.8±0.4, 95% CI: -1.6, 0.02*).*

4.4.2.3 Dietary behavior and pain relief

There was a significant change in body weight between baseline and endpoint (*F*(1.000, 69.000) = 7.632, *p=0.007*, η_p^2 = 0.100), but no interaction between the supplement groups and weight over time (*F*(2.000, 69.000) = 0.469, *p=0.628*, η_p^2 = 0.013). Each group's mean body weight increased from baseline (Table 4-8), but this was only statistically significant for the MDx group (mean change: 0.8±0.4, 95% CI: 0.1, 1.5 *p*=0.037).

Table 4-8. Baseline (BL) versus endpoint (EP) body weight comparisons within and between supplement groups.

	Supplement Group														
	Collagen Hydrolysate (CH)					**Milk Protein (MP)**					**Maltodextrin (MDx)**				
	n=	**Mean**	**SE**	**95% CI**		**n=**	**Mean**	**SE**	**95% CI**		**n=**	**Mean**	**SE**	**95% CI**	
Time	*F*(1.000, 69.000) = 7.632, ***p=0.007***, η_p^2 = 0.100														
Time*Group	*F*(2.000, 69.000) = 0.469, *p=0.628*, η_p^2 = 0.013														
BL weight (kg)	27	74.7	2.9	68.9	80.4	19	84.4	3.5	77.5	91.3	26	76.5	3.0	70.6	82.3
EP weight (kg)		75.0	2.9	69.2	80.8		85.2	3.5	78.2	92.1		77.3	3.0	71.3	83.2
Within-group p value		*0.369*					*0.088*					***0.037***			

The majority of returned 4DDRs lacked enough detail for nutritional analysis. Serving sizes were regularly missing and/or ingredients used in recipes were not reported. Furthermore, some of the 4DDRs were completed from memory at baseline or endpoint rather than in real time at home. Ultimately, the 4DDR data were unreliable and excluded from analyses, as were the pain diaries. Some of the diaries were returned with sufficient detail, but others were completely blank, which could mean nothing was taken or that days of using analgesics were not documented for whatever reason (e.g. time-burden). Due to competing tasks at endpoint, the research team were unable to consistently capture this metric from each participant so the pain diary data were incomplete.

4.4.2.4 Adverse events

Supplementary Table 9-5 (Appendix 3) shows general gastrointestinal discomfort was noted once by a participant consuming CH, twice by one participant in the MP group and seven times by the MDx group (two participants in the MDx group reported that complaint on two occasions between baseline and endpoint). Heartburn was specifically reported by two participants who consumed CH, and one participant in the MDx group reported heartburn twice (i.e. at months 1 and 2). Weight gain was reported by two participants in the MP group and two from the MDx group. Presumably unrelated

conditions including an itchy eyelid, Covid-19 cough, cholecystitis and gastrointestinal issues self-attributed to other foods or illnesses were reported by 13 participants (CH: n=6, MP: n=3, MDx: n=4).

4.5 Discussion

The CHAMP study evaluated the effect of 15 g CH protein (bovine hide) on the burden of self-reported knee pain not necessarily due to KOA among women aged ≥50 years. The attrition rate was comparable to previous studies,[18,20,26,27] and participants were only included if they reported enough pain at baseline for clinically and statistically significant differences to be detected at endpoint.[38,44] Functional performance was measured alongside PROMs,[23,25] and the two comparators were matched by protein content and energy which is a novelty in the study design. Previous studies have only included MDx or lactose matched by weight,[17,20,26,27] if not using glucosamine as an active comparator rather than a placebo.[45] As such, any observed effects in previous research are not necessarily attributable to CH due to study design limitations with the comparators.[46-48] The main finding of the CHAMP study was an improvement in the subjective measure of pain for all groups, but there were not any indisputable improvements in the objective data on functional performance tests within or between those three groups.

Each group improved from moderate pain at baseline to mild at endpoint which indicates minimal clinically important differences were achieved in the three supplement groups.[24] They all occurred within the first month and were sustained until endpoint, albeit without any group reaching a patient acceptable symptom state (PASS) reported by Roos.[24] Statistically significant improvements in the four secondary KOOS domains were also observed within each supplement group but not between them. Overall, these subjective findings of the CHAMP study align with previous research which collected PROMs and objective measures,[26,27] but they contrast with studies which only examined PROMs.[19,20]

Functional tests were included as objective measures to enhance the rigor of the study.[25] There were not any statistically significant between-group differences, although within-group

improvements were observed for three of the five activities (i.e. TUG, 30s-CST and SCT). The change in stair climb (SCT) performance was only significant for the CH group, although all the functional test improvements were below minimal detectable change (MDC) values reported for similar cohorts in those tests (Appendix 3: Supplementary Table 9-6). In other words, they may be due to random error rather than true change,[49] in which case they align with Genç et al.[28] who did not find any significant difference in their study on CH from a terrestrial mammal source which used the same functional tests.

Our findings may demonstrate the power of the contextual effect. It is inevitable in studies of this nature,[22] and it modulates clinical outcomes (i.e. pain perceptions) through non-specific variables encompassing the placebo effect, co-therapies, participants' hopes or expectations of treatments (affect heuristics) and their experience interacting with practitioners or research team members.[21,22,25] Co-therapies such as acupuncture, physiotherapy, exercise and weight loss may be therapeutic for KOA,[50-52] but their benefits are outweighed by the placebo effect.[21,53] Even so, our results are not definitive evidence of the placebo effect due to its limitations.

Concomitant therapies are important to monitor in clinical trials.[38] The participants were given a 'pain diary' for tracking their use of analgesics and other treatments, but the inconsistent quality of returned diaries prevented statistical analyses of that data. Similarly, adherence to the daily multivitamin was not monitored, even though nutritional adequacy is vital for collagen biosynthesis and ameliorating oxidative stress associated with cartilage degradation.[12] Consequently, the improved PROMs within all three supplement groups may relate to an effect from the multivitamin, if not from self-directed use of analgesics which warrants further investigation.

The participants were asked to maintain their usual dietary behavior and exercise routines. Body weights were measured at baseline and endpoint as a rudimentary means of verifying the request was followed, and 4DDRs were planned for more formal examination. The sub-optimal standard of returned 4DDRs, which may reflect the burden of time required to complete one correctly, prevented our nutritional analyses so participants' dietary behaviors and other living routines cannot

be confirmed as constant. For example, negligible weight increases within all three groups may reflect the Hawthorne effect where an individual's eating behavior organically changes in a clinical trial.[54]

Another limitation is that participants were free to mix the supplements in foods or fluids of their choice to optimize their adherence. This is consistent with Kumar et al.,[20] but Bongers et al.[26] advised participants to mix their supplements in water. Walrand et al.[55] have found when 10 g doses of CH were ingested with fermented milk versus water, that milk improved the bioavailability of Pro and Hyp within their participants' plasma. Therefore, the medium chosen by participants for ingesting their supplements may have been an additional dietary source of Pro for supporting collagen biosynthesis.[12,29,30]

Finally, the PROMs were collected in a survey modelled on KOOS.[24] The addition of "n/a" responses in the ADL and sport domains was a cause of missing data, despite the validity of participants' reasons for selecting "n/a" (e.g. the inability to squat, run or jump, not owning a bath, or avoiding activities for the fear of causing pain). Selecting "n/a" more often than scored alternatives means the participants' raw scores were invalid for conversion onto the scale out of 100.[32] Similarly, some participants either did not complete all their surveys at home or they skipped more questions than answered. Ultimately, the sample size of each group was unbalanced which impacts on the statistical power of hypothesis testing, but the Greenhouse-Geisser correction was applied to all split-plot ANOVA to overcome unequal group sizes, and the Expectation Maximization (EM) technique was used to impute missing values for ITT analysis to compare with PP data in a sensitivity analyses protocol which optimized the validity of the findings.[42,43]

4.6 Conclusion and future directions

This study makes an important contribution to the literature on CH as a potential nutraceutical for knee pain. Daily consumption of 15 g protein as CH (bovine hide) was not any more effective than MP and MDx in providing women aged ≥50 years with relief from knee pain. There were clinically and statistically significant improvements in pain perceptions within all three supplement groups, without any of those supplements having a superior effect. The subjective improvements may be attributable

to the contextual effect (i.e. affect heuristics and/or the placebo effect), but more research with objective and subjective data is needed before such a conclusion. For instance, CH may have improved functional performance in the Stair Climb Test, and there were more participants from the CH group than the comparators with bilateral pain at baseline who reported unilateral pain at endpoint. Therefore, the CH supplement may have impacted on milder forms of pain so a study to examine that notion would be worthwhile.

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In which chapter is the manuscript/published work?	Chapter 5

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CHAMP study conception and planning: JC, Janet Weber (JW) and Katie Schraders (Study Coordinator);
Data collection: DG, JC, KS;
Analysis and interpretation of results then drafting of the chapter and preparation of its revisions for a final version: DG;
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Chapter 5: Collagen hydrolysate was ineffective on cartilage turnover and inconclusive for inflammation in a randomized double-blind controlled trial on women aged ≥50 years with knee pain

This chapter achieved objective 3 of the research project. It is a report on secondary outcomes of the CHAMP study examining the effect of CH and comparator groups on biomarkers of cartilage turnover and inflammation.

5.1 Abstract

Preclinical data show collagen hydrolysate (CH) can promote collagen synthesis in cartilage affected by knee osteoarthritis (KOA). Human studies with objective outcomes are scant, so this RDBCT of four months examined biomarkers of cartilage turnover and inflammation. Females ≥50 years who self-reported knee pain not necessarily due to KOA were recruited, and they consumed either 15 g protein as collagen hydrolysate (from bovine hide) or comparators of milk protein (MP), or maltodextrin (MDx). It was hypothesized CH would increase collagen synthesis (PIIANP), whilst reducing the biomarkers of breakdown (COMP, and urinary CTX-II) and inflammation (IL-1β and TNF-α). Fasted blood samples were collected, and the data were analyzed with split-plot ANOVA using Bonferroni corrections for pairwise comparisons. Per protocol data were reported as mean, standard error, and 95% confidence intervals after verification with intention-to-treat analyses. Eighty-two participants were allocated to the isoproteic, and isoenergetic supplements respectively. Eight dropped out and two were excluded (adherence <75%). There were no interactions between groups over time for PIIANP (F(2.000, 64.000) = 0.836, $p=0.438$, η_p^2 = 0.025), COMP (F(2.000, 64.000) = 0.446, $p=0.642$, η_p^2 = 0.014) or CTX-II (F(2.000, 63.000) = 0.645, $p=0.528$, η_p^2 = 0.020). Mean PIIANP and COMP levels increased within each group (all $p<0.001$), but CTX-II levels were stable. The biomarkers of inflammation were below their limits of detection at baseline and endpoint so were not compared. Ultimately, the three supplements performed similarly over time, without having any impact on slowing an early stage of dysregulation in cartilage turnover.

Trial Registration: Australia and New Zealand Clinical Trials Registry (ACTRN 12622000779774).

Keywords: Knee osteoarthritis, collagen hydrolysate, bovine, inflammation, biomarkers of collagen/cartilage turnover

5.2 Introduction

Knee osteoarthritis (KOA) is a degenerative disease, causing pain and affecting the quality of life among sufferers worldwide.[1] It is characterized by articular cartilage degradation (i.e. the rate of cartilage breakdown exceeding synthesis), and is exacerbated by chronic inflammation which is also associated with excess body fat stores.[2,3] Postmenopausal women are at greatest risk through an effect from estrogen depletion, if not from other sex-related differences which are yet to be fully understood.[4-8]

Type II collagen fibrils are the main organic component of cartilage.[9] They stem from procollagen polymers which are tightly coiled triple helices formed during the development phase of the cartilage extracellular matrix (ECM).[10] In that time, C- and N- termini propeptides (PIICP and PIINP) are cleaved as the ECM matures[9,10]; thus PIICP and PIINP reflect collagen synthesis as measured in the synovial fluid (SF), or once they reach the circulatory system for eventual elimination in urine.[11] However, PIINP exists in two isoforms (PIIANP and PIIBNP), and PIIANP is only expressed by articular chondrocytes during embryogenesis or responding to cytokines and growth factors promoting ECM repair in adulthood.[12] As such, PIIANP has potential as a biochemical marker (biomarker) for detecting the "silent" phase of KOA so treatment can commence before it becomes more advanced.[13,14] Similarly, fragments of the ECM such as C-telopeptide Type II (CTX-II) and cartilage oligomeric protein (COMP) represent its breakdown by proteases, while cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) are markers of the inflammatory response.[3] Altogether, these soluble biomarkers can evaluate interventions aimed at effecting change in the ECM before structural or functional improvements may have had time to develop and become observable.[1,15,16]

Collagen hydrolysates (CH) may attenuate (but not arrest) the disruptions to cartilage turnover synonymous with KOA. Also known as collagen peptides, they are derived from undenatured Type I collagen fibers (UC-I) which are highly concentrated in repeating motifs of Glycine-Proline-Hydroxyproline (Gly-Pro-Hyp). The composition of prolyl-hydroxyproline (Pro-Hyp) in native UC-I varies by animal source, and terrestrial mammals offer a richer reservoir of these postulated bioactive

peptides (BAPs) than avians or fish.[17] For instance, Pro-Hyp can promote collagen synthesis *in vitro* and in mice,[18] but there is a general shortage of longitudinal research on humans for soluble biomarkers of cartilage turnover and inflammation.[19]

Most of the existing human studies are epidemiological, aiming to identify or validate biomarkers of KOA for diagnostic or prognostic purposes.[1,19,20] Of the few studies on CH derived from terrestrial mammals which have measured soluble biomarkers,[21,22] the results are inconclusive and non-transferable to cohorts at high risk of KOA such as peri- and postmenopausal women.[5,15] Furthermore, the maltodextrin (MDx) placebos were matched by weight rather than energy, and without isoproteic comparators such as cow's milk protein. Therefore, any observed effects on study outcomes to date are not necessarily attributable to CH due to study design limitations with the comparators.[23-25] For example, milk protein is rich in Pro, so it may have as much potential as CH in supporting endogenous collagen biosynthesis.[10,26,27] Therefore, the aim of this study was to examine cartilage turnover and inflammation among women aged ≥50 years with self-reported knee pain consuming an oral supplement of CH versus energy, and protein matched comparators using a set of soluble biomarkers. It was a report of secondary outcomes from the collagen hydrolysate and milk protein (CHAMP) study which measured pain as a primary outcome.[28]

5.3 Methodology

The randomized double-blind controlled trial (RDBCT) of four months (17 weeks) was prospectively registered with the Australia and New Zealand clinical trials registry (ACTRN 12622000779774). It was conducted according to the guidelines laid down in the Declaration of Helsinki, with all procedures involving humans approved by the New Zealand Health and Disability Ethics Committee (2022 exp 12925). Written informed consent was obtained from all participants.

5.3.1 Participants

Females aged ≥50 years with self-reported knee pain not necessarily due to KOA were recruited between January and October 2023. Advertisements appeared on social media and at community groups within the Manawatū region of New Zealand. The study was also promoted on

national radio, in print media and by word of mouth. Participants completed an online survey (Qualtrics XM 2023), adapted from the pain domain of the Knee Injury and Osteoarthritis Outcome Score (KOOS) before it became licensed.[29,30] The nine screening questions were self-administered on a 6-point scale rather than the usual 5-points. "Not applicable" ("n/a") was added so respondents could indicate not having engaged in the activity in question (e.g. ascending/descending stairs), but any "n/a" response was considered missed rather than scored as zero (i.e. indicating no pain during the activity). Raw scores ≥8/36 were included, but the criteria were refined midway through recruitment as several respondents with pain scores <8/36 reported changing their living routines for pain-related reasons (e.g. avoiding stairs because they evoke pain). Thereafter, respondents with pain scores of 6-7/36 were provided with the quality of life (QoL) questions from KOOS, and raw scores ≥7/16 for that domain were included.[29] All autoimmune conditions (except diet-controlled Coeliac Disease) were excluded, as were hepatic and renal diseases, and anyone who sustained knee trauma or underwent surgery in the previous six months. Similarly, any respondents who commenced hormone replacement therapy (HRT), or used oral nutrition supplements containing chondroitin, glucosamine, or collagen (undenatured or hydrolyzed) within the previous six months were excluded (Appendix 3: Supplementary Table 9-2).

5.3.2 Intervention

The participants were randomized with an equal representation ratio (1:1:1) for every nine admitted to the study using a computer-generated system (calculator.net). They were allocated on arrival at baseline to receive 15 g protein as CH (extracted from bovine hide), 15 g milk protein (MP), or the isoenergetic comparator of 19.2 g MDx (Table 5-1). To achieve the isoenergetic and isoproteic state, 4.2 g MDx was added to CH. The identically packaged supplements were presented to participants at the end of their baseline visit accompanied by verbal and written guidance to consume one sachet each day as a single dose or spread over the day in foods or fluids of each participant's choice to optimize adherence. They were also given a supply of multivitamins for daily consumption to reduce the possibility that micronutrients required for collagen synthesis were rate-limiting

(Appendix 3: Supplementary Table 9-3). The participants were asked to maintain their habitual diet and exercise patterns over the intervention. All researchers, biomarker analyses staff and participants were blind to the supplement groups until statistical analyses were complete.

Table 5-1. Nutritional information panel for the three isoenergetic supplements per sachet.

	Collagen Hydrolysate	Milk Protein	Maltodextrin
Energy (kJ)	325.8	325.8	325.8
Protein (g)	15	15	0
Fat (g)	0	1.2	0
Carbohydrate (g)	4.2	3.6	19.2
Total weight (g)	20.3	21.5	19.2

5.3.3 Data collection

Written informed consent was given at baseline. The baseline and endpoint visits were hosted at the Human Nutrition Research Unit (HNRU) of Massey University in Palmerston North, New Zealand. They started between 0730-0930 hours when participants arrived after an overnight fast of ≥12 hours. The first baseline visit was in February 2023, and the last endpoint visit was in February 2024. All participants completed a self-administered monthly online questionnaire starting from baseline (Qualtrics XM 2023 and 2024). It was one aspect of the CHAMP study evaluating the effect of CH on pain and functional performance,[28] and it included the KOOS pain domain so each participant's most painful knee at baseline could be quantified on a worst-best scale from 0-100.[30]

5.3.3.1 Soluble biomarkers

Fasted whole blood venipuncture samples were collected at baseline and endpoint before 10 am by a certified phlebotomist. All blood samples were methodically processed (Appendix 4: Supplementary Table 9-7), with duplicate aliquots of 500 µL serum and plasma stored upright in cryo-tubes at -80°C until analyses with enzyme-linked immunosorbent assay (ELISA) in 96-well plates by Canterbury Health Laboratories, using commercially available kits and protocols for plasma COMP (Invitrogen Human Thrombospondin-5/COMP ELISA Kit, Catalog # EH453RB / EH453RBX10; Thermo Fisher Scientific, Waltham, MA, USA), serum PIIANP (Catalog # EZPIIANP-53K, EMD Millipore, Burlington, MA, USA}, serum IL-1β (Invitrogen Human IL-β ELISA Kit, Catalog # BMS224-2 / BMS224-2TEN; Thermo Fisher Scientific), and serum TNF-α (Invitrogen Human TNF-α ELISA Kit, Catalog #

BMS223-4 / BMS223-4TEN; Thermo Fisher Scientific). First-pass, mid-stream urine samples were also collected on arrival at baseline and endpoint, and they were stored at -80°C until analyses in duplicates by research team members for urinary CTX-II values of spot samples, corrected for urinary creatinine concentration (Urine CartiLaps® (CTX-II) EIA, Immunodiagnostics Systems Limited, Boldon, UK; Product Code: AC-10F1). The reported coefficients of variation (CVs) and limits of detection for all the assays are presented in Supplementary Table 9-8 (Appendix 4).

5.3.3.2 Anthropometry

Overweight and obesity are associated with chronic inflammation and KOA progression,[3,31] so anthropometry measures were taken to compare the covariates between groups at baseline and endpoint. Body weight was measured in light clothes without shoes to the nearest 0.01 kg on digital scales (Tanita WB-110A, Japan), standing height to the nearest 0.1 cm with a stadiometer (Seca Medical Measuring Systems, Chino, CA, USA) and body mass index (BMI) calculated thereafter. Waist to hip ratios (WHR) were determined from hip and waist circumferences to the nearest 0.1 cm, and whole-body fat percentage was measured with Dual Energy X-ray Absorptiometry (DXA) (Horizon QDR Series, Hologic Inc., Bedford USA).

5.3.3.3 Adherence

All empty and unopened supplement sachets returned by participants were counted. A conservative threshold of <75% defined a protocol violation of non-adherence.

5.3.3.4 Adverse events

Participants were asked in their monthly surveys if they had any of the listed symptoms which have been experienced in similar studies on CH.[21,32] They could also report any ill effects with free text.

5.3.4 Statistical analyses

The statistical analyses were performed with SPSS (version 29, IBM SPSS statistics). A sample size of 27 per group was needed to attain power of 90% and a Type I error rate of 5% as calculated by G*Power (version 3.0.10) to detect change in the primary outcome measure of pain for the CHAMP

study. Changes in the biomarkers of cartilage turnover (PIIANP, COMP, CTX-II) and inflammation (IL-1β and TNF-α) were all secondary outcomes of the study.

Homogeneity of variance between groups was assessed with Levene's test. Distributions in variance within groups were evaluated with visual inspection of skewness (≤±1) and kurtosis (≤±2), Q-Q plots and the Shapiro-Wilk test. Violations in the assumptions of normality for the outcome of PIIANP were resolved by common (log10) transformation and removal of one extreme outlier from the MP group (Appendix 3: Supplementary Table 9-4). The data were examined with a two-way mixed-design (split-plot) ANOVA, and Greenhouse-Geisser correction was applied to overcome unequal group sizes. All analyses are presented as *F*-statistics, *p values* and partial eta (η_p^2) values to examine for statistical significance and interpret effect sizes.[33] Follow-up pairwise comparisons were made with Bonferroni corrections, presented as means, standard errors, and 95% confidence intervals with $p<0.05$ set for significance.

The biomarker outcome measures were checked in a sensitivity analysis model.[34] Intention-to-treat (ITT) data were analyzed before and after log10 transformation using the Expectation-Maximization (EM) method for imputation of missing values. The analyses were progressively repeated before and after log10 transformation for per protocol (PP) analyses without data from dropouts, without dropouts and participants with adherence rates <75%, and then without one extreme outlier of PIIANP in the MP group. Furthermore, the CTX-II data which caused an intra-assay CV above its acceptable threshold were removed to assess any impact on the results (Appendix 4: Supplementary Table 9-9).

5.4 Results

82 participants were allocated to supplements and 74 completed the study (Figure 5-1). The most common reasons for discontinuation were disliking the supplement taste (n=2) and private matters reported as unrelated to the study (n=2). Unless otherwise stated, the results are presented in the untransformed form for PP analysis of adherent completers (including the extreme outlier for PIIANP, and the CTX-II data which caused an elevated intra-assay CV), given the consistency of findings across all steps of sensitivity analysis examination.[34]

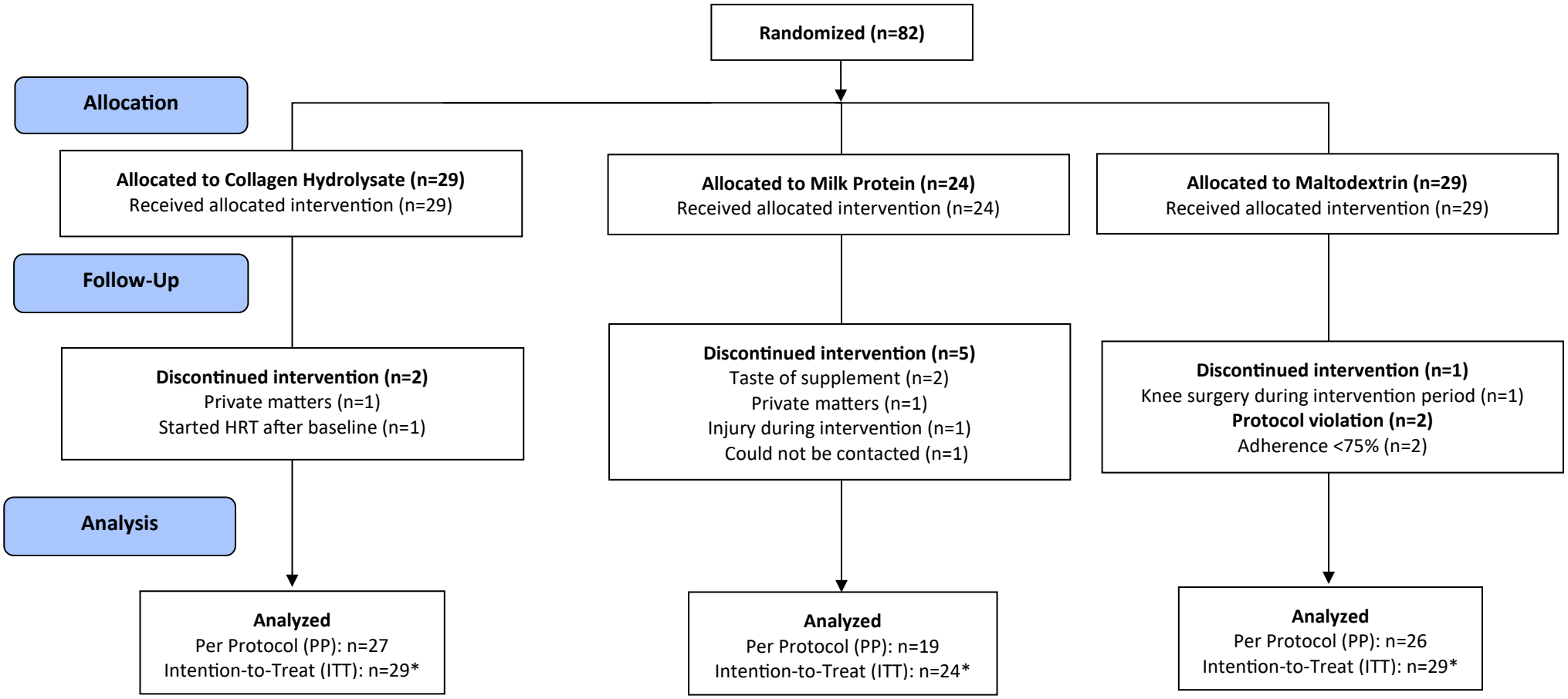

Figure 5-1. CONSORT diagram of participant flow from randomization to data analyses.

* Expectation-Maximation (EM) imputation for missing data for comparing ITT with PP in the untransformed and log10 transformed formats.

Baseline data are presented as means and standard deviations by supplement group and altogether (Table 5-2). The mean age of all participants was 63.5±8.3 years and the mean BMI was 29.2±5.6 kg/m^2. Each group's mean pain score was categorized as moderate.[30]

Table 5-2. Baseline characteristics of participants by supplement group and altogether.

	Supplement Group											
	Collagen Hydrolysate (CH)			Milk Protein (MP)			Maltodextrin (MDx)			Total		
	n=	Mean	SD	n=	Mean	SD	n=	Mean	SD	n=	Mean	SD
Age (years)	29	63.9	8.2	24	61.3	7.8	29	64.8	8.7	82	63.5	8.3
BMI (kg/m^2)	29	27.8	5.0	24	30.6	5.5	29	29.5	6.0	82	29.2	5.6
Waist-to-Hip Ratio	29	0.8	0.1	24	0.8	0.1	29	0.8	0.1	82	0.8	0.1
Total Body Fat (%)	29	43.0	6.2	24	45.5	4.4	29	44.6	5.3	82	44.3	5.4
Pain score (0-100)[A]	29	62.4	16.3	24	58.3	13.4	28[B]	58.4	15.9	81	59.8	15.3
Classification[30]	*Moderate (37.5-62.5)*			*Moderate (37.5-62.5)*			*Moderate (37.5-62.5)*			*Moderate (37.5-62.5)*		

[A] (0=extreme, 100 = absent),

[B] One participant missed 6 of 9 questions which invalidated their raw score for converting onto the scale out of 100.[30]

5.4.1 Cartilage turnover

Table 5-3 shows the changes in serum PIIANP, plasma COMP and urinary CTX-II by supplement group across time with their intra- and inter-assay CVs. There were no significant interactions between time and supplement groups for any of the outcome measures of collagen synthesis or cartilage breakdown (Table 5-3).

5.4.1.1 Collagen synthesis (PIIANP)

There was a significant change in serum PIIANP between baseline and endpoint ($F(1.000, 65.000) = 42.269$, $p<0.001$, $\eta_p^2 = 0.394$). Table 5-3 shows it significantly increased within each group compared to baseline (all $p<0.05$).

5.4.1.2 Cartilage breakdown (COMP and CTX-II)

There was a significant change in plasma COMP between baseline and endpoint ($F(1.000, 64.000) = 56.421$, $p<0.001$, $\eta_p^2 = 0.469$). It significantly increased within each group compared to baseline (all $p<0.001$), but there was no significant change in urinary CTX-II between baseline and endpoint ($F(1.000, 63.000) = 1.230$, $p=0.272$, $\eta_p^2 = 0.019$). It remained stable within all three groups (Table 5-3).

Table 5-3. Biomarkers of cartilage turnover at baseline (BL) and endpoint (EP) by supplement group.

	Supplement Group													
	Collagen Hydrolysate (CH)					Milk Protein (MP)					Maltodextrin (MDx)			
	n=	Mean	SE	95% CI		n=	Mean	SE	95% CI		n=	Mean	SE	95% CI	
PIIANP (ng/mL)	23					19					26				
Intra-assay: 4.4%															
Inter-assay: 1.46%															
Time (EP vs. BL)	$F(1.000, 65.000) = 42.269$, ***p<0.001***, $\eta_p^2 = 0.394$														
Time*Group	$F(2.000, 65.000) = 0.650$, *p=0.526*, $\eta_p^2 = 0.020$														
Baseline		587.7	64.0	459.9	715.4		579.8	70.4	439.2	720.4		416.7	60.2	296.5	536.8
Endpoint		818.8	74.1	670.7	966.9		922.1	81.6	759.1	1085.0		761.2	69.7	621.9	900.5
Within-group p value[A]		***p=0.005***					***p<0.001***					***p<0.001***			
COMP (ng/mL)	24					18					25				
Intra-assay: 6.8%															
Inter-assay: 10.9%															
Time (EP vs. BL)	$F(1.000, 64.000) = 56.421$, ***p<0.001***, $\eta_p^2 = 0.469$														
Time*Group	$F(2.000, 64.000) = 0.190$, *p=0.827*, $\eta_p^2 = 0.006$														
Baseline		3.72	0.33	3.06	4.38	*18*	3.69	0.38	2.92	4.46		3.35	0.33	2.70	4.00
Endpoint		5.33	0.43	4.47	6.19		5.01	0.50	4.02	6.00		4.92	0.42	4.08	5.76
Within-group p value[A]		***p<0.001***					***p<0.001***					***p<0.001***			
CTX-II (ng/mmol Cr)	24					18					24				
Intra-assay: 10.3%															
Inter-assay: 8.9%															
Time (EP vs. BL)	$F(1.000, 63.000) = 1.230$, *p=0.272*, $\eta_p^2 = 0.019$														
Time*Group	$F(2.000, 63.000) = 1.421$, *p=0.249*, $\eta_p^2 = 0.043$														
Baseline		497.9	54.6	388.8	607.0		534.9	63.0	408.9	660.9		549.9	54.6	440.8	659.0
Endpoint		532.9	54.2	424.6	641.2		474.3	62.6	349.2	599.4		475.9	54.2	367.6	584.3
Within-group p value[A]		*p=0.478*					*p=0.289*					*p=0.137*			

[A] EP versus BL

5.4.2 Inflammation

The limits of detection for IL-1β and TNF-α were not reached for almost all participants across all groups (Table 5-4). Inferential statistics were not performed given the extent of missing values at baseline.[14]

Table 5-4. Descriptive statistics on biomarkers of inflammation at baseline (BL) and endpoint (EP) by group.

	Supplement Group																			
	Collagen Hydrolysate (CH)					Milk Protein (MP)					Maltodextrin (MDx)					Total				
	n=	Mean	SD	Min.	Max.	n=	Mean	SD	Min.	Max.	n=	Mean	SD	Min.	Max.	n=	Mean	SD	Min.	Max.
BL: IL-1β (pg/mL)	1	6.6	-	6.6	6.6	2	39.8	19.7	25.9	53.7	2	21.6	2.2	20.1	23.2	5	25.9	17.2	6.6	53.7
EP: IL-1β (pg/mL)	6	32.0	27.9	9.3	80.0	5	21.1	21.5	10.3	59.5	5	11.0	3.0	6.4	14.1	16	22.0	21.6	6.4	80.0
BL: TNF-α (pg/mL)	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
EP: TNF-α (pg/mL)	5	10.9	4.4	5.2	15.5	5	11.3	7.0	4.1	18.9	5	11.2	4.8	5.6	17.2	15	11.1	5.1	4.1	18.9

5.4.3 Anthropometry

Table 5-5 shows the means for BMI, WHR and total body fat percentage by supplement group across time. There were no significant interactions between time and supplements (Time*Group) for any of these measures (all *p>0.05*).

Table 5-5. Anthropometry measures at baseline (BL) and endpoint (EP) by supplement group.

	Supplement Group													
	Collagen Hydrolysate (CH)					Milk Protein (MP)					Maltodextrin (MDx)				
	n=	Mean	SE	95% CI		n=	Mean	SE	95% CI		n=	Mean	SE	95% CI	
BMI (Kg/m^2)	27					19					26				
Time (EP vs. BL)	F(1.000, 69.000) = 5.867, ***p=0.018***, η_p^2 = 0.078														
Time*Group	F(2.000, 69.000) = 0.463, *p=0.632*, η_p^2 = 0.013														
Baseline		27.9	1.0	25.8	30.0	19	30.7	1.2	28.2	33.2	26	28.6	1.1	26.4	30.7
Endpoint		28.0	1.1	25.9	30.2		30.9	1.3	28.4	33.5		28.9	1.1	26.7	31.0
Within-group p value[A]		*p=0.418*					*p=0.212*					***p=0.035***			
Waist-to-Hip Ratio	27					19					26				
Time (EP vs. BL)	F(1.000, 69.000) = 1.601, *p=0.210*, η_p^2 = 0.023														
Time*Group	F(2.000, 69.000) = 0.718, *p=0.491*, η_p^2 = 0.020														
Baseline		0.8	0.0	0.8	0.9		0.9	0.0	0.8	0.9		0.8	0.0	0.8	0.9
Endpoint		0.8	0.0	0.8	0.9		0.8	0.0	0.8	0.9		0.8	0.0	0.8	0.9
Within-group p value[A]		*p=0.601*					*p=0.127*					*p=0.989*			
Total Body Fat (%)	27					19					26				
Time (EP vs. BL)	F(1.000, 69.000) = 5.363, ***p=0.024***, η_p^2 = 0.072														
Time*Group	F(2.000, 69.000) = 0.252, *p=0.778*, η_p^2 = 0.007														
Baseline		43.5	1.0	41.5	45.5		44.7	1.2	42.3	47.1		44.1	1.0	42.1	46.1
Endpoint		43.8	1.0	41.9	45.7		45.3	1.2	43.0	47.6		44.5	1.0	42.5	46.5
Within-group p value[A]		*p=0.365*					*p=0.095*					*p=0.181*			

[A] EP versus BL

5.4.3.1 Body mass index (BMI)

There was a significant change in BMI between baseline and endpoint (F(1.000, 69.000) = 5.867, *p=0.018*, η_p^2 = 0.078). It significantly increased within the MDx group (mean change: 0.3±0.1, 95% CI: 0.02, 0.60 *p=0.035)* but remained stable within the CH and MP groups (Table 5-5).

5.4.3.2 Waist-to-hip ratio (WHR)

There was no significant change in WHR over time (F(1.000, 69.000) = 1.601, *p=0.210*, η_p^2 = 0.023). It remained stable compared to baseline within all three groups (Table 5-5).

5.4.3.3 Body fat percentage (DXA)

There was a significant change in whole body fat percentage between baseline and endpoint (F(1.000, 69.000) = 5.363, *p=0.024*, η_p^2 = 0.072), although none of the within-group increases reached statistical significance (Table 5-5).

5.4.4 Adverse events

Supplementary Table 9-5 (Appendix 3) shows general gastrointestinal discomfort was noted once by a participant consuming CH, twice by one participant in the MP group and seven times by the MDx group (two participants in the MDx group reported that complaint on two occasions between baseline and endpoint). Heartburn was specifically reported by two participants who consumed CH, and one participant in the MDx group reported heartburn twice (i.e. at months 1 and 2). Weight gain was reported by two participants in the MP group and two from the MDx group. Presumably unrelated conditions including an itchy eyelid, Covid-19 cough, cholecystitis and gastrointestinal issues self-attributed to other foods or illnesses were reported by 13 participants (CH: n=6, MP: n=3, MDx: n=4).

5.5 Discussion

A secondary objective of the CHAMP study was to compare the oral intake of 15 g protein as CH (derived from bovine hide) with isoenergetic, and isoproteic supplements by evaluating their effects on biomarkers of collagen synthesis and cartilage degradation (i.e. cartilage turnover). Measuring both aspects of turnover enables a better assessment of the KOA trajectory within individuals, rather than inspecting either of the two parameters in isolation.[14,35] Women aged ≥50 years with self-reported knee pain not necessarily due to KOA were recruited for an intervention of four months. There were no statistically significant differences between groups for the outcomes of collagen synthesis (PIIANP) or cartilage breakdown (i.e. CTX-II and COMP), but there were statistically significant within-group elevations in PIIANP and COMP. Essentially, the three supplements performed similarly over time, without having any impact on slowing an early stage of cartilage turnover dysregulation.

These findings are important for adding to existing evidence on the BAP potential of CH as a nutraceutical. They add to the analyses of data from the same cohort of women who engaged in functional tests and completed patient reported outcome measures (PROMs).[28] In that aspect of the CHAMP study, participants in all three groups of CH, milk protein (MP), and maltodextrin (MDx) reported statistically and clinically significant improvements in their perceptions of pain, without there

being any differences between the groups.[28] Improvements in the objective functional tests were negligible considering they were below thresholds of clinical importance reported for similar cohorts in those tests (Appendix 3: Supplementary Table 9-6).[36] It is possible there was not enough time for performance differences to have developed,[13,28] but the existing CHAMP study data on biomarkers counter that notion. The data are consistent with Bongers et al.[21] who found PROMs significantly improved after 12 weeks of supplementation with CH and a placebo of MDx, without either of those supplements having an impact on the rate of collagen breakdown (i.e. CTX-II), or synthesis (measured with CPII) which increased in both groups. Therefore, the CHAMP study biomarker data reinforce a suggestion that previously reported improvements in PROMs after consuming CH from terrestrial mammals may be due to contextual factors (encompassing the placebo effect and participants' felt experiences interacting with clinicians) rather than any postulated bioactivity of CH.[28,37] Moreover, the biomarker analyses show the value of evaluating interventions with objective data alongside PROMs.[38,39]

The existing results also demonstrate a difficulty in monitoring soluble biomarkers of KOA over time.[14] Progression of KOA is non-linear, and cross-sectional biomarkers are unique to each person's stage of disease at that time of measurement (e.g. baseline and endpoint).[14] The outcome measures of inflammation (IL-1β and TNF-α) were below the assay levels of detection for almost all the participants at baseline, and this could be explained by a well-known issue of assays lacking sensitivity in evaluating KOA biomarkers.[14] Alternatively, the missing data may relate to the heterogeneous pathophysiology of KOA. For instance, low-grade inflammation is a hallmark sign of KOA,[1,31,40] although only some sufferers have elevations in proinflammatory markers compared to healthy controls.[41,42] There were similar proportions within the three groups with detectable levels at endpoint (Table 5-4), so that small sub-group may be exhibiting the inflammatory phenotype which only accounts for 16-30% of KOA cases according to a meta-analysis by Dell'Isola et al.[43]

The participants' stages of cartilage degradation are also a factor to consider. Serum PIIANP levels are expected as an early sign of damaged cartilage, as chondrocytes only produce PIIANP during

embryogenesis or during the attempted repair of cartilage in adulthood.[12] A study by Sharif et al.[44] found serum PIIANP and urinary CTX-II levels were significantly higher (*p<0.05*) among sufferers of medically diagnosed KOA with worsening of their signs and symptoms over five years (progressors) compared to those diagnosed with KOA showing relative stability over the same timeframe (non-progressors). The mean urinary CTX-II levels of the CHAMP study participants were stable in each of the three supplement groups, so their increases in PIIANP may indicate cartilage turnover at an early stage of disruption, especially since plasma COMP levels also increased within the three groups. Verma and Dalal[45] found serum COMP levels were negatively correlated with the duration of disease among cases of confirmed KOA (i.e. COMP stores are degraded before collagen). However, Sharif et al.[46] also found serum COMP increased from baseline (*p<0.001*) in the first year among cases with medically diagnosed KOA which worsened over 5 years (progressors), but they were unchanged in the non-progressors. Similarly, Petersson et al.[47] found COMP levels increased (*p<0.001*) among cases with radiographic evidence of KOA after three years of follow but were stable among those with normal radiographs at that timepoint. Inter-individual variance in COMP is also attributable to biology (e.g. age, ethnicity, BMI) and physical activity rather than KOA alone.[48,49] Therefore, the CHAMP study data must be interpreted with caution given the complexity of interpreting KOA biomarkers over time.

The intra- and inter-assay CVs in this study were all within acceptable reference ranges, except for the intra-assay CV of CTX-II (actual: 10.3%, acceptable: ≤7.8%). Reinspection of that data without outliers (thus an intra-assay CV of 7.0%) was consistent (which validates the precision of the CTX-II data presented and discussed), but participants with self-reported pain were recruited without imaging techniques (i.e. X-ray) to confirm a KOA diagnosis (i.e. KL Grade ≥II). Furthermore, they were not defined and stratified by the known KOA phenotypes, all of which may evoke different (non) responses to oral intervention trials such as CH.[43] Even if they were confirmed with KOA, the results could still be confounded by the inclusion of participants at various stages of KOA (i.e. early pre-radiographic stage versus more advanced radiographic changes), and from progressors or non-progressors of disease.[39] Moreover, the biomarkers were systemic (whole-body) measures from blood

and urine, rather than directly from the knee joint synovial fluid. Whole-body measures may not be truly representative of any changes in the knee,[14,39] but they are less invasive to collect and accepted in the research setting for monitoring soluble biomarkers.[39]

5.6 Conclusion and future directions

In conclusion, this facet of the CHAMP study examined objective data on cartilage turnover to complement its primary focus on the effect of CH on pain perception and functional performance measures.[28] Compared to the isoenergetic, and isoproteic comparator groups of milk protein and maltodextrin, daily oral intake of 15 g CH (bovine hide) for four months did not change the increased rate of collagen synthesis (i.e. PIIANP) any more or less among a cohort of women aged ≥50 years with self-reported knee pain not necessarily due to KOA, nor did it attenuate the rate of cartilage breakdown measured with COMP and urinary CTX-II. Overall, these results suggest CH from terrestrial mammals had no apparent effect as a nutraceutical for knee pain, but more research using the same objective measures of biomarkers over a longer timeframe, and with more participants stratified by KOA phenotype and stage of disease (confirmed by radiography) is warranted.

5.7 Chapter 5 references

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Conception and design of the scoping review: DG;
Design and execution of data extraction for the narrative syntheses: DG;
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Chapter 6: The effect of oral supplementation with collagen hydrolysate (CH) on skin condition: a systematic scoping review

Chapter Six shifts the research focus from the effect of CH on joint pain to skin condition. It is an overview of optimal skin function and the pathophysiology of deteriorating skin condition. Furthermore, it explores existing literature investigating the effect of CH from a terrestrial mammal on skin condition to inform the CHAMP study design in achieving objective four of the project.

6.1 Abstract

Background: Skin condition deteriorates with age, especially among postmenopausal women. It is characterized by collagen loss causing thin, dry skin with wrinkles. Collagen hydrolysate (CH) is marketed as a nutricosmetic to combat these effects, but its reputed effect is based on inconclusive research. Most reviews include CH from any animal source, despite variability in their amounts of prolyl-hydroxyproline (Pro-Hyp) which are postulated as bioactive peptides (BAPs) for improving skin condition.

Objectives: To identify studies using CH from terrestrial mammals and assess the internal validity of each study before exploring and appraising their methods for collecting data on skin condition parameters. The overarching goal is to identify opportunities for assisting the design of future studies.

Method: The review was designed in accordance with the PRISMA-ScR framework. Scopus, Medline and CINAHL were searched from inception to November 2024. Data extraction included the participants' characteristics (i.e. age, sex and skin type if available) and the intervention regimens of CH and its comparators (dose and duration). The data collection methods were noted along with the key findings for each study, and the National Heart, Lung and Blood Institute (NHLBI) Quality Assessment Tool for Controlled Intervention Studies was used to evaluate their internal validity.

Results: Twelve out of 124 studies met the inclusion criteria. Six were judged as low quality, four were fair, and two were good quality in reference to the NHLBI quality assessment checklist. The most common primary outcome measure was for wrinkles, followed by elasticity, hydration, and then thickness. These parameters were measured over several areas of the face or forearm with a range of techniques. Some studies reported improvements after oral intake of CH compared to a placebo, but other studies found improvements within both groups, or no effect in either group.

Conclusion: Research on the effect of CH from terrestrial mammals on skin condition is inconclusive. The data are compromised by a lack of good quality studies and heterogeneity in study designs. More robust research is needed, including more studies on skin thickness.

Keywords: Collagen hydrolysate, bioactive peptides (BAPs), bovine, porcine, skin condition

6.2 Introduction

Skin is the largest organ of the human body.[1,2] It holds ≈20% of the total body water supply, predominantly in the dermis, underneath its avascular epidermal layers.[3] The stratum corneum is the outermost layer of the epidermis in constant contact with the environment for defense against pathogens or allergens, and protecting the underlying layers from dehydration.[1,2,4,5] The dermis contains fibroblasts and blood vessels among other structures (e.g. sweat glands, hair follicles, lymphatic vessels and nerve endings), all embedded within an extracellular matrix (ECM) of water, hyaluronic acid (HA), elastin and collagen.[4,6-8] The dermis is divided into a thin papillary layer, adjacent to the epidermis, and a larger reticular region which makes up most of the total dermal thickness.[8-12]

More than ¾ of the dry weight of skin is accounted for by collagen fibers.[9] They are bundles of Type I collagen fibrils, coexisting with Type III fibrils and elastin primarily in the reticular dermis. The papillary layer contains thinner collagen fibers and most of the skin's HA stores, with the remaining HA in the stratum granulosum underneath the stratum corneum of the epidermis.[13] In concert with elastin and HA, collagen fibers endow skin with a viscoelastic property where collagen and elastin give skin its firmness and elasticity, while HA confers it with an affinity for water.[10,12-14] If external pressure is applied (i.e. touch), the collagen fibers will slide to align with the direction of tension until it is removed, and then recoil to their original configuration in parallel to the skin's surface.[1,6,12] However, optimal skin function relies on fibroblasts to moderate the dermal ECM in the same way chondrocytes control the integrity of articular cartilage.[5,8,10,15] As such, skin function (i.e. appearance and condition) naturally declines with age (intrinsic aging) due to fibroblast senescence.[5,10,13,16,17]

Intrinsic aging of skin is unavoidable.[18,19] Reactive oxygen species (ROS) progressively accumulate in fibroblasts due to mitochondrial dysfunction.[8,10,13,17] The ensuing oxidative stress causes the fibroblasts to upregulate their secretion of metalloproteinases (MMPs), leading to net collagen loss and fragmentation of the remaining cross-linked collagen fibers.[8,10,18,19] Concurrently, elastin fibers lose flexibility, and the epidermal HA stores lessen.[8] The main reserves of dermal HA are generally unchanged, but their aggregation with collagen decreases so the skin's water-holding

capacity (i.e. viscosity) falls.[8,10,13,20] Consequently, dermal fibroblasts collapse which impairs their ability to regenerate the ECM.[5,8,10,17,19]

The effects of intrinsic aging manifest as loose, thin and dry skin with the emergence of wrinkles.[8,10,21-23] They are pronounced among postmenopausal women due to an apparent effect from estrogen depletion,[6,24] and up to ≈30% of dermal collagen is lost in the first five years after menopause.[6,25,26] Modifiable external factors such as air pollution (i.e. tobacco smoke and exhaust fumes), ultraviolet (UV) radiation from sunlight (photoaging), and poor nutrition potentiate oxidative stress on dermal fibroblasts, thus activating an inflammatory response which amplifies degradation of the dermal matrix.[11,20,27-29] Consequently, a market for 'anti-aging nutricosmetics' has emerged.[7,11,30,31]

'Nutricosmetics' are food products formulated for beauty purposes,[3,30] and collagen hydrolysate (CH) has attracted interest as a potential ingredient due to its postulated bioactivity.[32,33] Also known as collagen peptides, CH are derived from undenatured collagen (UC) which is the main structural protein of vertebrates.[34-36] Type I fibrils (UC-I) are the main component of collagen fibers in the skin or hide,[37,38] and they are formed by fibroblasts in the same way Type II fibrils (UC-II) are made by chondrocytes in articular cartilage.[39-41] However, Type I fibrils are heterotrimers of two Type I, alpha-1 (α1) chains and one Type I, alpha-2 (α2) chain.[39-41] The α1 and α2 chains are repeating motifs of Gly-X-Y with proline (Pro) and hydroxyproline (Hyp) often occurring in the X and Y positions,[37,38,42,43] but Type I, α2 chains are more variable in their X-Y residues than the two identical Type I, α1 chains.[44,45] As such, the composition of peptides within collagen fibers of the skin or hide can differ between and within all classes of the animal kingdom.[7,35,39,46] For example, terrestrial mammals have a higher concentration of Gly-Pro-Hyp than avians and fish,[7,32,39,46] so they are a richer source of postulated bioactive peptides (BAPs).

Systematic reviews and meta-analyses regularly report that collagen peptides improve skin condition in humans.[47-52] Pu et al.[51] found in their meta-analysis of 26 studies (1,721 participants) that CH supplementation improved elasticity and hydration measures compared to controls, but they included studies using CH from any animal.(i.e. chicken, fish and terrestrial mammals). Furthermore,

the authors reported several biases in the studies they had included, which is consistent with findings from other meta-analyses.[50,53] However, Peres et al.[54] claim most systematic reviews and meta-analyses in the dermatology field have low internal validity, and publication bias is a recurring issue which impacts on the integrity of their conclusions.[55,56]

The aim of this scoping review is multifactorial. Its focus is on studies which investigated the effect of CH from terrestrial mammals on skin condition parameters affected by aging (e.g. viscoelasticity, hydration and dermal thickness). The primary objective is to assess the internal validity of those studies. Secondary objectives are to explore and appraise the key features of each study's design (i.e. participants' characteristics, intervention regimens, environmental conditions), including their methods of data collection (i.e. which outcome parameters were evaluated, and from what areas on the body they were taken), and to ascertain each study's main findings for those outcome measures. The overarching goal is to identify opportunities for assisting the design of future studies on CH from terrestrial mammals and skin condition to ultimately improve their quality of evidence.

6.3 Methodology

This scoping review was designed in reference to guidelines described by Peters et al.[57] and Munn et al.[58] It aligned with the Preferred Reporting Items for Systematic Reviews and Meta Analyses extension for scoping reviews (PRISMA-ScR),[59] but it was not registered on PROSPERO because scoping reviews are beyond the scope of that database at present.[60]

6.3.1 Search strategy

6.3.1.1 Primary

The Scopus, Medline and CINAHL databases were searched by one author (DG) on 30 November 2024 (Table 6-1). Each database was searched from its date of inception up until that day, and the strategy was saved as an ongoing alert on each database for any articles published until December 31, 2024 as the final cutoff.

Table 6-1. Primary search strategy for the scoping review.

Database	Terms
SCOPUS, Medline via Pubmed, CINAHL	skin OR derma* AND aging OR ageing OR photo* AND “collagen hydro*” OR “hydro* collagen” OR “collagen peptide*” AND elasticity OR thickness OR density OR hydration OR wrinkle*

6.3.1.2 Secondary

A secondary search was performed after a preliminary list of studies was compiled from the primary search. The reference lists of those studies were reviewed by one author (DG), and relevant titles not already found in the primary search were retrieved for subjecting to the selection process which the primary search results had undergone.

6.3.2 Selection process

6.3.2.1 Screening

The titles and abstracts of all records found in the primary search were screened on Endnote (version 20.6) between December 02 and 05, 2024. One reviewer (DG) compared the studies with predetermined eligibility criteria (Table 6-2), and a hierarchy of exclusion was applied to quantify the primary reason of exclusion when studies were ineligible for multiple reasons (i.e. preclinical research using CH sourced from fish). Pilot studies and case reports were considered to broaden the search, as were observational or non-blinded intervention trials, but only full articles in English were included. If the title or abstract was insufficient in detail (e.g. an unspecified animal source of CH), the full text was retrieved, and if that information was also missing then the corresponding author was contacted by email. Concurrently, online web searches for any CH interventions reported as a brand name were executed, but the studies were excluded after two weeks if a response was not received from the author, or the source could not be identified with the web search.

Table 6-2. Inclusion / exclusion criteria for screening in the scoping review.

***Inclusion criteria*:**
Collagen hydrolysate (CH) of terrestrial mammal origin
Randomized double-blind controlled trial
Pilot study / observational study / non-blinded study / case report
Primary or secondary outcome measure on the efficacy of CH on skin condition parameters (*i.e. hydration, elasticity, thickness)*
***Hierarchy of exclusion criteria*:** 1. Reviews, meta-analyses, editorials 2. Preclinical (i.e. animal or *in vitro*) studies 3. Undenatured collagen or non-oral CH 4. Primary or secondary outcome measure not on the efficacy of CH on skin condition 5. Non-terrestrial mammal source of CH (i.e. avian or marine) 6. Other reason(s)

6.3.3 Data collection

A template was used to extract data for narrative syntheses. The authors' names, year of publication, trial registration number, sample size and participants' sex, mean age and skin type were recorded, as were CH source, dose, and duration of intervention. The comparator (composition and dose), outcome measures and key findings were also extracted. Furthermore, the instruments used to collect data in each study were noted.

6.3.4 Quality of evidence assessment

The 14-item checklist for the quality assessment of controlled intervention studies created by the US National Heart Lung and Blood Institute (NHLBI) was used for grading the internal validity of included studies.[56] A scoring system was adapted from the suggestion of Zareen[61]; one point was awarded for every yes, but 0.5 points were deducted if the answer could not be determined (i.e. not reported). Scores of 0-5 were classed as poor, 6-10 were fair, and 11-14 were considered good quality.

6.4 Results

6.4.1 Primary search

A total of 124 records were found in the Scopus (72 records), Medline (46 records), and CINAHL databases (6 records). After uploading the results to Endnote, 52 duplicates were automatically removed, and 72 records were screened (Figure 6-1).[62]

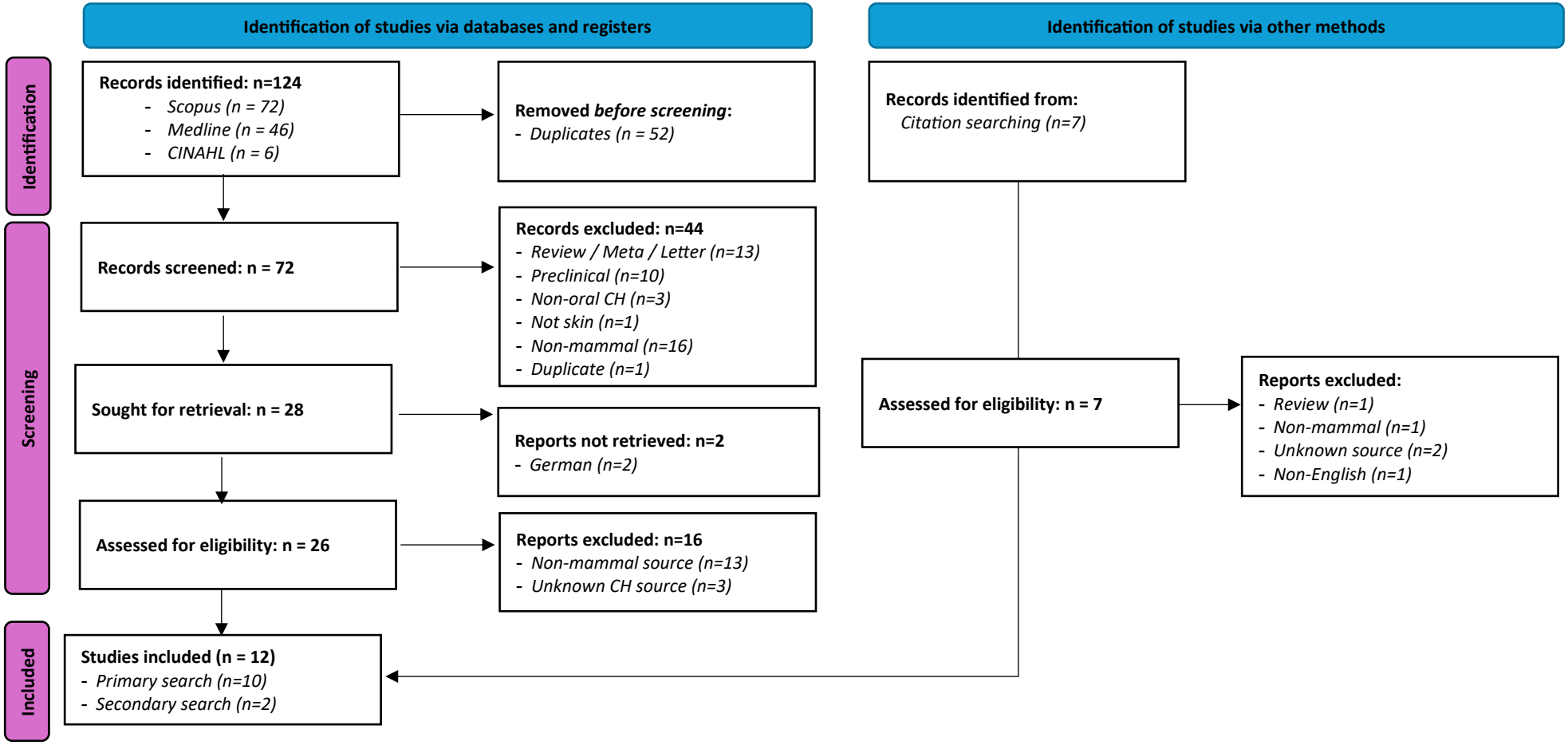

Figure 6-1. PRISMA diagram of the scoping review screening process (adapted with permission by creative commons license from Page et al.[62]

6.4.1.1 Screening phase

Titles and abstracts

A total of 44 records were excluded after screening the primary search titles and abstracts (Figure 6-1). The most common reason for exclusion was the CH source being non-terrestrial mammal (fish/marine: n=14, chicken: n=2) followed by review articles (n=13) and preclinical research (n=10). Overall, 28 articles were sought for retrieval, but two were unavailable in English[63,64] so 26 full articles were assessed for eligibility (Figure 6-1).

Full text

Nine studies were included after retrieving the full articles. The authors clearly stated the CH source as bovine or porcine.[65-73] Thirteen studies were excluded because the CH source was not of terrestrial mammal origin (fish/marine: n=12, synthetic: n=1).

Email enquiries

Three emails were sent to corresponding authors to seek information about the origins of CH in their intervention.[74-76] Two emails were not replied to,[74,76] and the requested information was not found with a web search so the studies were excluded. One reply was received stating the CH source as porcine so the study was included.[75] There was not an email address for a corresponding author for one study by Nomoto et al.,[77] and the website for 'V Cresc CP10'[78] was non-specific about the animal source of CH so the study was excluded. Therefore, 10 studies were included from the primary search.

6.4.2 Secondary search

Seven articles of interest were identified from reference lists of primary search results.[29,79-84] One was included after retrieving the full article,[79] and three were excluded because the CH source was fish,[83] the article was a review,[29] or it was unavailable in English.[82]

Three emails were sent to corresponding authors to identify the CH source,[80,81,84] but one of those emails failed and the author could not be contacted.[84] The website for the CH intervention in that study by Choi et al.[84] states the source as fish (i.e. exclusion) or porcine (i.e. inclusion),[85] so the study was excluded on the grounds of uncertainty. Similarly, the website for the product used by Miyanaga et al.[81] states it is marine so it was excluded.[86] The study by Maia Campos et al.[80] was

included because it was referred to as bovine in a later study by Maia Campos et al.[79] Ultimately, 12 studies were included at the end of the selection process (Table 6-3). One study was a pilot,[65] one was an open-labelled trial without a comparator group,[75] and the remaining 10 were randomized controlled trials.

Table 6-3. Clinical trials assessing the effect of collagen hydrolysate from terrestrial mammal sources on parameters of skin condition.

<table>
<tr><th rowspan="2">Author(s), year, trial registration</th><th rowspan="2">Design</th><th rowspan="2">Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th rowspan="2">Outcome measure(s)</th><th rowspan="2">Key findings</th></tr>
<tr><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th></tr>
<tr><td>Asserin et al.[65]
2015
Clinical trial registration number: unreported</td><td>Parallel RDBCT study 1 [A]

<u>Environment</u>:
Temperature 22±1ºC
Relative humidity 50±10%</td><td><u>Total recruited</u>:
n=33 healthy Japanese females
40-59y

CH-porcine:
n=11

CH.fish:
n=11

Control group:
n=11

<u>Dropouts</u>:
n=0</td><td>Porcine (CH-por)
OR
fish (CH-fsh)
MW [B] = 2-5KDa

10g/d in a formulated drink at bedtime</td><td>Maltodextrin (MDx)
10g/d in a formulated drink at bedtime</td><td>8w</td><td>PRIMARY
Hydration [C]
3 unspecified points per Subject

SECONDARY
Transepidermal water loss [D] (TEWL)
3cm from the earlobe on a line to the mouth</td><td>NHLBI quality assessment: 5 / 14 – Poor
<table><tr><th colspan="5">HYDRATION (arbitrary units, a.u.)</th></tr><tr><td></td><td>CH-por</td><td>CH-fsh</td><td>MDx</td><td></td></tr><tr><td>0w</td><td>≈50</td><td>≈50</td><td>≈50</td><td></td></tr><tr><td>4w</td><td>≈59
p>0.05 (vs 0w)</td><td>≈55
p>0.05 (vs 0w)</td><td>≈50
p>0.05 (vs 0w)</td><td>NS</td></tr><tr><td>8w</td><td>≈65
p<0.001
(vs 0w)</td><td>≈55
p<0.05
(vs 0w)</td><td>≈50
p>0.05 (vs 0w)</td><td>p=0.003*</td></tr></table>NS = not significant between groups
* CH-porcine vs placebo

TEWL data not shown but reported as not statistically significantly different between all three groups.</td></tr>
<tr><td colspan="8">[A] Study 2: n=106 healthy Caucasian females consuming 10g CH (fish) vs 10g placebo for 12 weeks, [B] Molecular weight, [C] Corneometer device, [D] Tewameter device</td></tr>
<tr><td>Bolke et al.[66]
2019
Clinical trial registration number:
<u>DRKS00015664</u></td><td>Parallel RDBCT with randomization via alternating allocation

<u>Environment:</u>
Temperature 20ºC
Relative humidity 40-60%</td><td><u>Total recruited</u>:
n=72 healthy females
35 – 73y

CH group:
n=36
50.6±11.2y

Control group:
n=36
52.4±8.3y
<u>Dropouts</u>:
n=0</td><td>Bovine, 2.5g / d within 25mL of Elasten®[A] before or with a meal</td><td>2.5g / d Elasten®[A] (without CH)</td><td>16w total
12w with + 4w washout (CH group)</td><td>PRIMARY
Hydration[B]
Stratum corneum (mean of 3 unspecified forearm sites)
Elasticity[C]
Forearm (mean of 3 sites unspecified)
Roughness[D]
Facial site Unspecified
Density[E]
Thigh site Unspecified

Continued overleaf</td><td>NHLBI quality assessment: 7.5 / 14 – Fair
<table><tr><th colspan="4">Hydration (arbitrary units, a.u.)</th></tr><tr><td></td><td>CH</td><td>Placebo</td><td>BG</td></tr><tr><td>0w</td><td>35.0±4.8</td><td>33.7±5.1</td><td>p=0.2405</td></tr><tr><td>12w
WG</td><td>44.5±4.4
p≤0.0001</td><td>36.6±5.7
p≤0.0001</td><td>p<0.0004</td></tr><tr><td>16w
WG</td><td>40.1±4.9
p≤0.0001</td><td></td><td></td></tr><tr><th colspan="4">Elasticity – R2 (arbitrary units, a.u.)</th></tr><tr><td></td><td>CH</td><td>Placebo</td><td></td></tr><tr><td>0w</td><td>0.69±0.05</td><td>0.71±0.06</td><td>p=0.3240</td></tr><tr><td>12w
WG</td><td>Increased by 0.81±0.04
p≤0.0001</td><td>Increased by 0.75±0.06
p≤0.0001</td><td>p<0.0004</td></tr><tr><td>16w
WG</td><td>0.76±0.07[G]
p≤0.0001</td><td></td><td></td></tr></table>WG = within group (vs. 0w)
BG = between group (CH vs. placebo)

Continued overleaf</td></tr>
</table>

<table>
<tr><th>Author(s), year, trial registration</th><th>Design</th><th>Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th>Outcome measure(s)</th><th>Key findings</th></tr>
<tr><th></th><th></th><th></th><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th><th></th><th></th></tr>
<tr><td>Bolke et al.[66] continued</td><td></td><td></td><td></td><td></td><td></td><td>SECONDARY
Self-assessment at w12 and w16 (efficacy, odor, taste, consistency, skin appearance)</td><td>
<table>
<tr><th colspan="4">Roughness (wrinkle depth)</th></tr>
<tr><td></td><td>CH</td><td>Placebo</td><td>BG</td></tr>
<tr><td>0w</td><td>161.6 ± 11.4µm</td><td>161.7 ± 13.0µm</td><td>p=0.9702[B]</td></tr>
<tr><td>12w
WG</td><td>118.0 ± 16.4µm
p<0.0001 [A]</td><td>151.4 ± 15.9µm
p<0.0001 [A]</td><td>p<0.0004 [B]</td></tr>
<tr><td>16w
WG</td><td>131.6 ± 21.9µm
p<0.0001 [A]</td><td></td><td></td></tr>
<tr><th colspan="4">Density (Epidermal Thickness)</th></tr>
<tr><td></td><td>CH</td><td>Placebo</td><td>BG</td></tr>
<tr><td>0w</td><td>35.7±7.2µm</td><td>36.9±8.6µm</td><td></td></tr>
<tr><td>12w
WG</td><td>44.0±7.6µm
p<0.0001 [A]</td><td>39.0±8.7µm
p<0.0109 [A]</td><td>p<0.0004 [B]</td></tr>
<tr><td>16w
WG</td><td>36.7±6.9µm
p=0.0008 [A]</td><td></td><td></td></tr>
</table>
WG = within group vs. 0w, [B] BG = between group (CH vs. placebo)
</td></tr>
<tr><td colspan="8">[A] Multi-ingredient oral ampoule containing 666mg acerola fruit extract, 80mg vitamin C, 3mg zinc, 2.3mg vitamin E and 50 mcg biotin,
[B] Corneometer CM 825, [C] Cutometer MPA 580, [D] PRIMOS Compact for wrinkle depth, [E] SkinScanner DUB Simple (sonography),</td></tr>
<tr><td>Demir-Dora et al.[67]
2024
Clinical trial registration number:
<u>NCT05235997</u></td><td>Parallel RDBCT
(1:1 allocation by a statistician)

<u>Environment</u>:
Not reported</td><td><u>Total recruited</u>:
n=120 healthy females
35-57y
(44±5.9y)

CH group:
n=57

Control group:
n=55
<u>Dropouts</u>: n=8[A]

n=8[A]</td><td>Bovine, 10g/d
(CollaSell Pro®)</td><td>10g/d maltodextrin</td><td>12w (8w treatment + 4w washout)</td><td>PRIMARY
Hydration[B]
Stratum corneum of the inner forearm (unspecified site) And crow’s feet region of the face

Elasticity[B]
Inner forearm (unspecified site) & crow’s feet
Roughness[B]
Crow’s feet region of face</td><td>NHLBI quality assessment: 9 / 14 – Fair
<table>
<tr><th colspan="4">Hydration (g/m³)</th></tr>
<tr><td></td><td>CH</td><td>Placebo</td><td>CH vs Pla</td></tr>
<tr><td>0w</td><td>52.2 ±16.1</td><td>54.8 ±14.2</td><td>p=0.367</td></tr>
<tr><td>8w
WG (vs 0w)</td><td>65.8 ±18.9
p<0.001</td><td>53.1 ±14.9
p=0.467</td><td>p<0.001</td></tr>
<tr><td>12w
WG (vs 0w)</td><td>64.6 ±14.4
p<0.001</td><td>60.1 ±15.8
p=0.017</td><td>p=0.115</td></tr>
<tr><th colspan="4">Elasticity (mPa)</th></tr>
<tr><td></td><td>CH</td><td>Placebo</td><td>CH vs Pla</td></tr>
<tr><td>0w</td><td>39.9±4.7</td><td>39.9±6.3</td><td>p=0.971</td></tr>
<tr><td>8w
WG (vs 0w)</td><td>43.0±7.4
p=0.009</td><td>40.3±3.3
p=0.571</td><td>p=0.017</td></tr>
<tr><td>12w
WG (vs 0w)</td><td>41.8±4.3
p=0.001</td><td>40.2±3.3
p=0.693</td><td>p=0.027</td></tr>
</table>
WG = within group

Continued overleaf</td></tr>
</table>

<table>
<tr><th>Author(s), year, trial registration</th><th>Design</th><th>Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th>Outcome measure(s)</th><th>Key findings</th></tr>
<tr><td></td><td></td><td></td><td>Source and dose</td><td>Placebo and dose</td><td>Duration</td><td></td><td></td></tr>
<tr><td>Demir-Dora et al.[67] continued</td><td></td><td></td><td></td><td></td><td></td><td></td><td>
<table>
<tr><th colspan="4">Roughness (µg/cm²)</th></tr>
<tr><td></td><td>CH</td><td>Placebo</td><td>CH vs Pla</td></tr>
<tr><td>0w</td><td>24.8
±18.2</td><td>22.0
±13.8</td><td>p=0.371</td></tr>
<tr><td>8w

WG (vs 0w)</td><td>40.2
±20.4
p<0.001</td><td>24.9
±20.9
p=0.235</td><td>p<0.001</td></tr>
<tr><td>12w

WG (vs 0w)</td><td>53.1
±24.1
p<0.001</td><td>35.4
±25.6
p<0.001</td><td>p<0.001</td></tr>
</table>
</td></tr>
<tr><td colspan="8">[A] Eight were excluded from analysis due to pregnancy (n=2), Covid (n=2), pneumonia (n=1), nausea (n=1), and unspecified dropout (n=2) [B] Callegari Soft-Plus device</td></tr>
<tr><td>Gibson et al.[75]
2024
Clinical trial registration number:
Unreported/ not applicable</td><td>Open-labelled

<u>Environment</u>:
Temperature 20-24ºC, Relative humidity - 35-50%</td><td><u>Total recruited</u>:
n=135 healthy females
45-65yy
(54.5±6.2y)

<u>Dropouts</u>:
<u>n=19</u>
(n=116 Completers)

Fitzpatrick skin type: I-VI</td><td>Porcine, 2.5g/d via 3x 1,500mg tablets (Richelet Skin Renewal[A]) taken together with a glass of water</td><td>Nil</td><td>3 months</td><td>PRIMARY
Global score (0-9) of wrinkles from expert grader

SECONDARY
Facial elasticity[B] (middle malar region),

Self-assessment (26 items),

serum antioxidant status</td><td>NHLBI quality assessment: 5 / 14 - Poor
<table>
<tr><th colspan="2">Wrinkle score (expert grader assigned)</th></tr>
<tr><td colspan="2">0m</td><td>5.9</td></tr>
<tr><td colspan="2">3m</td><td>5.0</td></tr>
<tr><td colspan="2"></td><td>p<0.001</td></tr>
</table>
<table>
<tr><th colspan="3">Elasticity - R2 (a.u.)</th></tr>
<tr><td></td><td>n=</td><td></td></tr>
<tr><td>0m</td><td>135</td><td>0.67±0.14</td></tr>
<tr><td>1m</td><td>127</td><td>0.67±0.14
p=0.7904</td></tr>
<tr><td>2m</td><td>122</td><td>0.69±0.13
p=0.1743</td></tr>
<tr><td>3m</td><td>116</td><td>0.74±0.10
p<0.001</td></tr>
</table>
<u>Self-assessment</u>
<table>
<tr><th colspan="2">Improved Hydration at</th></tr>
<tr><td>0m</td><td>Baseline not shown</td></tr>
<tr><td>1m</td><td>17.6±24.1</td></tr>
<tr><td></td><td>p<0.001</td></tr>
<tr><td>2m</td><td>22.5±26.8</td></tr>
<tr><td></td><td>p<0.001</td></tr>
<tr><td>3m</td><td>31.0±26.9</td></tr>
<tr><td></td><td>p<0.001</td></tr>
</table>
Continued overleaf</td></tr>
</table>

<table>
<tr><th rowspan="2">Author(s), year, trial registration</th><th rowspan="2">Design</th><th rowspan="2">Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th rowspan="2">Outcome measure(s)</th><th rowspan="2">Key findings</th></tr>
<tr><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th></tr>
<tr><td>Gibson et al.[75] continued</td><td></td><td></td><td></td><td></td><td></td><td></td><td>
<table>
<tr><th colspan="2">Improved overall condition at:</th></tr>
<tr><td>0m</td><td>Baseline not shown</td></tr>
<tr><td>1m</td><td>15.1±23.3</td></tr>
<tr><td></td><td>p<0.001 (vs 0m)</td></tr>
<tr><td>2m</td><td>27.8±25.2</td></tr>
<tr><td></td><td>p<0.001 (vs 0m)</td></tr>
<tr><td>3m</td><td>31.0±28.5</td></tr>
<tr><td></td><td>p<0.001 (vs 0m)</td></tr>
</table>
</td></tr>
<tr><td colspan="8">[A] 600µg RE vitamin A, 1.4mg riboflavin, 8.0mg niacin, 450µg biotin, 120mg ascorbic acid, 5.0µg vit D3, 18mg α-TE vit E, 1.0mg Cooper, 150µg iodine, 1.0mg manganese, 55µ selenium, 14mg zinc, 120mg hyaluronic acid, [B] Cutometer MPA 580</td></tr>
<tr><td>Guadanhim et al.[68]
2023
Clinical trial registration number: unreported</td><td>Parallel RDBCT
(computer randomization with factorial allocation)

Environment:
Not reported</td><td><u>Total recruited</u>:
n=56 females with Stage I forearm dermatoporosis
60-93y
(69.5±7.3y)

<u>Dropouts</u>:
n=3</td><td>Bovine & porcine (MW [A]=2KDa)
5g/d (CH)
AND
1x / d of 2.5% CH topical (ch)
OR
placebo (pl)</td><td>Maltodextrin, 5g / d (PL)
AND
1x / d of 2.5% CH topical (ch)
OR
placebo (pl)</td><td>6 months</td><td>PRIMARY
Thickness [B]
Inner forearm (mean of 2 readings taken 7 & 10cm from the antecubital fossa)

Elasticity [C]
outer forearm (mean of 3 readings taken 5, 7 & 10cm from the antecubital fold)

Dermal collagen Content [D]

Self-Assessment: quality of life [E]</td><td>NHLBI quality assessment: 12 / 14 - Good
<table>
<tr><th colspan="5">Epidermal Thickness (µm)</th></tr>
<tr><th></th><th>CH + ch</th><th>CH + pl</th><th>PL + ch</th><th>PL + pl</th></tr>
<tr><td>0m</td><td>94.7 ± 37.0</td><td>93.3 ± 37.0</td><td>92.6 ± 28.0</td><td>93.2 ± 36.2</td></tr>
<tr><td>6m
WG</td><td>91.2 ± 20.4
p>0.05*</td><td>87.2 ± 30.1
p>0.05*</td><td>90.8 ± 30.3
p>0.05*</td><td>96.9 ± 33.2
p>0.05*</td></tr>
<tr><th colspan="5">Dermal Collagen (%)</th></tr>
<tr><th></th><th>CH + ch</th><th>CH + pl</th><th>PL + ch</th><th>PL + pl</th></tr>
<tr><td>0m</td><td>47.2±4.6</td><td>47.0±3.1</td><td>44.3±6.5</td><td>45.3±5.0</td></tr>
<tr><td>6m
WG</td><td>44.4±4.1
p<0.05*</td><td>44.3±3.6
p<0.05*</td><td>45.3±4.3
p>0.05*</td><td>44.2±4.4
p>0.05*</td></tr>
<tr><th colspan="5">Total Dermal Thickness (mm)</th></tr>
<tr><td>0m</td><td>1.16 ±0.20</td><td>1.17 ±0.18</td><td>1.14 ±0.16</td><td>1.16 ±0.17</td></tr>
<tr><td>6m
WG</td><td>1.17 ±0.20
p>0.05*</td><td>1.18 ±0.22
p>0.05*</td><td>1.14 ±0.17
p>0.05*</td><td>1.14 ±0.19
p>0.05*</td></tr>
<tr><th colspan="5">Elasticity – R2 (a.u.)</th></tr>
<tr><td>0m</td><td>0.62 ±0.11</td><td>0.62 ±0.11</td><td>0.65 ±0.10</td><td>0.60 ±0.10</td></tr>
<tr><td>6m
WG</td><td>0.64 ±0.10
p>0.05*</td><td>0.66 ±0.11
p>0.05*</td><td>0.65 ±0.09
p>0.05*</td><td>0.65 ±0.08
p>0.05*</td></tr>
<tr><th colspan="5">Elasticity – R5 (a.u.)</th></tr>
<tr><td>0m</td><td>0.70 ±0.21</td><td>0.69 ±0.21</td><td>0.73 ±0.25</td><td>0.68 ±0.19</td></tr>
<tr><td>6m
WG</td><td>0.71 ±0.25
p>0.05*</td><td>0.74 ±0.24
p>0.05*</td><td>0.77 ±0.17
p>0.05*</td><td>0.74 ±0.17
p>0.05*</td></tr>
</table>
* WG = within group (vs. 0m)

Continued overleaf</td></tr>
</table>

<table>
<tr><th rowspan="2">Author(s), year, trial registration</th><th rowspan="2">Design</th><th rowspan="2">Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th rowspan="2">Outcome measure(s)</th><th rowspan="2">Key findings</th></tr>
<tr><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th></tr>
<tr><td>Guadanhim et al.[68]
continued</td><td></td><td></td><td></td><td></td><td></td><td></td><td>
<table>
<tr><th colspan="5">Elasticity – R6 (a.u.)</th></tr>
<tr><th></th><th>CH + ch</th><th>CH + pl</th><th>PL + ch</th><th>PL + pl</th></tr>
<tr><td>0m</td><td>0.80
±0.17</td><td>0.80
±0.20</td><td>0.88
±0.28</td><td>0.85
±0.22</td></tr>
<tr><td>6m
WG</td><td>0.87
±0.23
p>0.05*</td><td>0.83
±0.23
p>0.05*</td><td>0.87
±0.17
p>0.05*</td><td>0.87
±0.17
p>0.05*</td></tr>
<tr><th colspan="5">Elasticity – R7 (a.u.)</th></tr>
<tr><td>0m</td><td>0.39
±0.10</td><td>0.37
±0.11</td><td>0.38
±0.08</td><td>0.36
±0.09</td></tr>
<tr><td>6m
WG</td><td>0.37
±0.11
p>0.05*</td><td>0.39
±0.11
p>0.05*</td><td>0.41
±0.10
p>0.05*</td><td>0.39
±0.08
p>0.05*</td></tr>
</table>
*WG = within group (vs. 0m)
<u>Quality of Life</u>: Data not shown but reported as all groups significantly improved from baseline (p<0.01) without between group differences (p=0.65)</td></tr>
<tr><td colspan="8">[A] Molecular weight, [B] DermaScan-C, [C] Cutometer, R2 (gross elasticity), R5 (liquid elasticity), R6 (viscous component), R7 (viscoelastic component of the relaxation curve),
[D] Skin biopsy, outer forearm (7cm from antecubital fold), [E] Dermatology Life Quality Index [87]</td></tr>
<tr><td>Laing et al.[69]
2020
Clinical trial registration number:
<u>DRKS00017093</u></td><td>Parallel RDBCT
(unspecified methods of randomization and allocation to treatment)
Environment:
Temperature 21±1°C, Relative humidity 50±5%</td><td><u>Total recruited</u>:
n=60 healthy females
40 - 70y
(54.4±7.5y)

CH group:
n=30
Control group:
n=30

<u>Dropouts</u>:
n=0</td><td>Bovine, 2.5g / d within 25mL Elasten®[A] before or with a meal</td><td>2.5g / d placebo of Elasten®[A] (without CH)</td><td>12w (85d)</td><td>PRIMARY
Collagen fragmentation[B] (cheek epidermis & dermis)

SECONDARY
Self-Assessment of 26 items at w12</td><td>NHLBI quality assessment: 6.5 / 14 - Fair
<u>Primary</u>

Decreased fragmentation among treatment group (p=0.037) but not placebo (p=0.365) versus baseline and a significant difference between treatment vs placebo at endpoint (p=0.044) as determined by an expert grader.

<u>Secondary</u>
No significant differences between placebo versus CH except for self-reported improvements in:
Hydration (p=0.035)
Softness (p=0.043)
Suppleness (0.024)</td></tr>
<tr><td colspan="8">[A] Multi-ingredient oral ampoule containing 666mg acerola fruit extract, 80mg vitamin C, 3mg zinc, 2.3mg vitamin E and 50 mcg biotin,
[B] Confocal microscopy assessment by an expert grader blinded to treatment groups and using an ordinal scoring system</td></tr>
<tr><td>Maia Campos et al.[79]
2022
Clinical trial registration number: unreported</td><td>Continued overleaf</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

<table>
<tr><th rowspan="2">Author(s), year, trial registration</th><th rowspan="2">Design</th><th rowspan="2">Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th rowspan="2">Outcome measure(s)</th><th rowspan="2">Key findings</th></tr>
<tr><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th></tr>
<tr><td>Maia Campos et al.[79]
continued</td><td>Parallel RDBCT
(unspecified methods of randomization and allocation to treatment)

Environment:
Not reported</td><td><u>Total recruited</u>:
n=60 healthy Brazilian females
45-60y
(53.4±4.2y)

Fitzpatrick skin type: II-III

<u>Dropouts</u>:
n = unspecified</td><td>Bovine, 5g/d and study sunscreen (SPF 60) for use ad libitum</td><td>Maltodextrin (MDx), 5g/d
+
study sunscreen (SPF 60) for use ad libitum</td><td>90d</td><td>Scaliness [A];
Smoothness [A];
Roughness [A];
(6x8mm2 within facial malar region)

Wrinkles [B]
(nasolabial & periorbital region)

Dermis Echogenicity [C]:
(6x8mm2 within facial malar region)</td><td>NHLBI quality assessment: 3 / 14 - Poor
<table>
<tr><th></th><th>CH</th><th>MDx</th></tr>
<tr><td colspan="3">Scaliness*</td></tr>
<tr><td>0d</td><td>≈0.30</td><td>≈0.28</td></tr>
<tr><td>90d
WG</td><td>≈0.21
p<0.05**</td><td>≈0.23
p>0.05**</td></tr>
<tr><td colspan="3">Smoothness*</td></tr>
<tr><td colspan="3">Reported as not significant (data not shown)</td></tr>
<tr><td colspan="3">Roughness*</td></tr>
<tr><td>0d
90d
WG</td><td>≈1.05
≈0.75
p>0.05**</td><td>≈1.05
≈0.90
p>0.05**</td></tr>
<tr><td colspan="3">Wrinkles – periorbital (0 – 5)</td></tr>
<tr><td>0d
90d
WG</td><td>≈2.3
≈2.0
p<0.05**</td><td>≈2.2
≈2.0
p>0.05**</td></tr>
<tr><td colspan="3">Wrinkles – nasolabial (0 – 5)</td></tr>
<tr><td>0d
90d
WG</td><td>≈2.3
≈2.0
p<0.05**</td><td>≈2.5
≈2.5
p>0.05**</td></tr>
<tr><td colspan="3">Echogenicity</td></tr>
<tr><td colspan="3">Presented as a change in the echogenicity ratio between 0d and 90d. Increased dermis echogenicity in the CH group without statistically significant difference in change score vs. MDx</td></tr>
</table>
*unspecified units of measurement and data presented in graphs without clear error estimates
**WG = within group (vs.0d)</td></tr>
<tr><td colspan="8">[A] Surface Evaluation of the Living Skin (SELS) with Visioscan VC98 and SELS 2000 software,
[B] High-resolution photography with Visioface digital photography imaging graded on a 5-point scale, [C] Dermascan Combo</td></tr>
<tr><td>Maia Campos et al.[80]
2015
Clinical trial registration number unreported

Continued overleaf</td><td>Parallel RDBCT
(unspecified methods of randomization and allocation to treatment)</td><td><u>Total recruited</u>:
n=60 healthy Females
40–60y
CH group:
n=30
Control group:
n=30</td><td>Bovine 10g/d
WITH
vitamins A (600µg), C (45mg),
E (10mg), and
zinc (7mg)
Dissolved in any liquid</td><td>Maltodextrin (MDx) 10g/d
Dissolved in any liquid</td><td>90d</td><td>Either the forehead, periorbital or nasolabial facial regions (left or right) at random for:

Continued overleaf</td><td>NHLBI quality assessment: 4 / 14 – Poor
Continued overleaf</td></tr>
</table>

<table>
<tr><th rowspan="2">Author(s), year, trial registration</th><th rowspan="2">Design</th><th rowspan="2">Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th rowspan="2">Outcome measure(s)</th><th rowspan="2">Key findings</th></tr>
<tr><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th></tr>
<tr><td>Maia Campos et al.[80]
continued</td><td>Environment:
Temperature 21.5±1°C, Relative humidity 50±5%</td><td>Fitzpatrick skin type: II-IV

<u>Dropouts</u>:
n = unspecified</td><td></td><td></td><td></td><td>Net elasticity[A] (R5)

Hydration[B]
stratum corneum (mean of 5 measures)

Wrinkle count[C]

Dermis echogenicity[D]</td><td>
<table>
<tr><th colspan="4">Elasticity (R5 – net elasticity)***</th></tr>
<tr><th></th><th>CH</th><th>MDx</th><th></th></tr>
<tr><td>0d</td><td>≈0.30</td><td>≈0.33</td><td></td></tr>
<tr><td>90d</td><td>≈0.51
p<0.05</td><td>≈0.45
p>0.05</td><td></td></tr>
<tr><th colspan="4">Hydration</th></tr>
<tr><td colspan="4">Data not shown. Reported as not significant within groups except forehead (p value not shown) but without significant difference versus MDx (p value not shown).</td></tr>
<tr><th colspan="4">Wrinkle count</th></tr>
<tr><td colspan="4">Data not shown. Reported as a reduction in wrinkle count in CH group without quantification.</td></tr>
<tr><th colspan="4">Dermis Echogenicity</th></tr>
<tr><td colspan="4">Presented as improved within CH group at endpoint vs baseline for forehead (p<0.05) & nasolabial (p<0.05) regions but not periorbital. Reported as showing increased fibroblast density and enhanced formation of collagen fibrils.</td></tr>
</table>
***Data presented in graphs without clear definition of error estimates</td></tr>
<tr><td colspan="8">[A] Cutometer SEM 575, [B] Corneometer CM 825, [C] High-resolution photography with Visioface digital photography imaging, [C] Dermascan Combo</td></tr>
<tr><td>Proksch et al.[71]
2014
Clinical trial registration number: unreported</td><td>Parallel RDBCT
(unspecified methods of randomization and allocation to treatment)

Environment:
Temperature 21.5°C, Relative humidity 50%</td><td><u>Total recruited</u>:
n=114 healthy females
45.0 – 65.4y (55.6±6.0y)

CH group:
n=57
Control group:
n=57

Fitzpatrick skin type: I-III

<u>Dropouts</u>:
n=6</td><td>Porcine (Verisol®) (MW[A]=2KDa) 2.5g/d in any fluid</td><td>Maltodextrin (MDx) 2.5g/d in any fluid</td><td>12w total (8w + 4w washout)</td><td>PRIMARY
Wrinkle volume[B]
Mean of 3 Measures of crow’s feet (left eye, lateral canthus) area

SECONDARY
Subgroup T I procollagen (blister biopsy: inner forearm, unspecified region (n=48)</td><td>NHLBI quality assessment: 5.5 / 14 Poor
<table>
<tr><th colspan="4">Wrinkle Volume (mm³)*</th></tr>
<tr><td></td><td>CH</td><td>MDx</td><td></td></tr>
<tr><td>0w</td><td>0.38
±0.36</td><td>0.38
±0.26</td><td>p=0.93</td></tr>
<tr><td>8w</td><td>0.33
±0.38</td><td>0.41
±0.25</td><td>p<0.01</td></tr>
<tr><td>12w</td><td>≈0.33
±0.02[C]</td><td>≈0.36
±0.05[C]</td><td>p<0.01</td></tr>
</table>
*Mean ± SEM

Type I Procollagen Analysis
CH: n=24 (n=20 completers)
MDx: n=24 MDx (n=20 completers)
Type I procollagen increased ≈1.6-fold from baseline for the CH group at 8 weeks (p<0.05) and remained constant in the MDx group</td></tr>
<tr><td colspan="5">[A] Molecular weight, [B] Primos Compact device imaging, [C] Approximation estimated from results presented in figures</td><td></td><td></td></tr>
<tr><td>Proksch et al.[70]
2014
Clinical trial registration number: unreported</td><td>Continued overleaf</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

<table>
<tr><th rowspan="2">Author(s), year, trial registration</th><th rowspan="2">Design</th><th rowspan="2">Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th rowspan="2">Outcome measure(s)</th><th rowspan="2">Key findings</th></tr>
<tr><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th></tr>
<tr><td>Proksch et al.[70]
continued</td><td>Parallel RDBCT
(unspecified methods of randomization and allocation to treatment)

Environment:
Temperature 21.5±1°C, Relative humidity 50±5%</td><td><u>Total recruited</u>:
n=69 healthy females
35-55y

CH group A: n=23
CH group B: n=23
Control group: n=23

Fitzpatrick skin type: I-IV

<u>Dropouts</u>:
n=7</td><td>Porcine (Verisol®) (MW[A]=2KDa):
Group A: 2.5g/d
Group B: 5g/d
in any fluid</td><td>Maltodextrin (MDx) 2.5g / d in any fluid</td><td>12w total (8w + 4w washout[B])</td><td>PRIMARY
a. Elasticity[C]
Inner left forearm (R5 value)

SECONDARY
a. Hydration[D]:
Stratum corneum (inner left forearm)
b. TEWL[E]: Inner left forearm
c. Roughness[F]:
Inner right forearm

(5x5cm area of unspecified site on inner forearm)</td><td>NHLBI quality assessment: 5 / 14 - Poor
Elasticity - R5 (a.u.)
(relative to MDx group at endpoint)
<table>
<tr><th></th><th>2.5g CH</th><th>5g CH</th><th>MDx</th></tr>
<tr><td>0w[G]</td><td colspan="3">0.85-0.89 ± 0.11-0.13 (p=0.46)</td></tr>
<tr><td>8w</td><td>↑ ≈1.10x vs MDx (p<0.05)</td><td>↑ ≈1.07x vs MDx (p<0.05)</td><td></td></tr>
<tr><td>12w</td><td colspan="3">Data not shown or reported</td></tr>
</table>
Hydration (a.u.)
(relative to MDx group at endpoint)
<table>
<tr><th></th><th>2.5g CH</th><th>5g CH</th><th>MDx</th></tr>
<tr><td>0w[G]</td><td colspan="3">27.9-30.0 ± 4.3-6.1 (p=0.31)</td></tr>
<tr><td>8w</td><td>↑ ≈0.95x vs MDx (p=0.96)</td><td>↑ ≈0.95x vs MDx (p=0.96)</td><td></td></tr>
</table>
TEWL (g/m²/h)
(relative to MDx group at endpoint)
<table>
<tr><th></th><th>2.5g CH</th><th>5g CH</th><th>MDx</th></tr>
<tr><td>0w[G]</td><td colspan="3">8.0 ± 1.2–1.3 (p=0.99)</td></tr>
<tr><td>8w</td><td>↑ ≈1.0x vs MDx (p=0.90)</td><td>↑ ≈1.0x vs MDx (p=0.90)</td><td></td></tr>
</table>
Roughness (µm)
(relative to MDx group at endpoint)
<table>
<tr><th></th><th>2.5g CH</th><th>5g CH</th><th>MDx</th></tr>
<tr><td>0w*</td><td colspan="3">18.3-19.9 ± 4.2-6.9 (p=0.59)</td></tr>
<tr><td>8w</td><td>↑ ≈0.95x vs MDx (p=0.63)</td><td>↑ ≈0.95x vs MDx (p=0.63)</td><td></td></tr>
</table></td></tr>
<tr><td colspan="8">[A] Molecular weight, [B] Elasticity parameter only, [C] Cutometer MPA 580, [D] Corneometer CM 825, [E] Dermalab, [F] PRIMOS Compact, [G] baseline mean of all groups combined</td></tr>
<tr><td>Vleminckx et al.[72]
2024
Clinical trial registration number: unreported

Continued overleaf</td><td>Parallel RDBCT
(Randomized and allocated using SPSS software without the ratio of</td><td><u>Total recruited</u>:
n=85 healthy East Asian Females
43-65y
with crow's feet grade ≥ 2</td><td>Porcine (Peptan®), 5g/d in 200mL water before Breakfast
AND
2x/d application of standard face</td><td>Maltodextrin (MDx), 5g/d in 200mL water before breakfast
AND
2x/d standard</td><td>12w (84d)</td><td>Dermal density / Echogenicity[C]
Temple (random area)
Hydration[D]
Cheekbone (left or right)</td><td>NHLBI quality assessment: 9 / 14 – Fair
<table>
<tr><th colspan="4">Density[C]
(Gray scale: 0-255)[G]</th></tr>
<tr><th></th><th>CH</th><th>MDx</th><th>BG</th></tr>
<tr><td>0d</td><td>≈115</td><td>≈110</td><td>p>0.05</td></tr>
<tr><td>84d
WG</td><td>≈120
p<0.05</td><td>≈110
p>0.05</td><td>p<0.05</td></tr>
</table></td></tr>
</table>

<table>
<tr><th rowspan="2">Author(s), year, trial registration</th><th rowspan="2">Design</th><th rowspan="2">Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th rowspan="2">Outcome measure(s)</th><th rowspan="2">Key findings</th></tr>
<tr><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th></tr>
<tr><td>Vleminckx et al.[72]
continued</td><td>allocation specified)

Environment:
Temp: 21±1°C, Relative humidity 45±5%</td><td>on the Skin Aging Atlas volume 2[A]

CH group: n=43
Control group: n=42
<u>Dropouts</u>: n=0[B]</td><td>cream from 28 days before baseline (0d) to endpoint (84d)</td><td>face cream 28 days before baseline (0d) to endpoint (84d)</td><td></td><td>Elasticity[D]
Cheekbone (left or right)

Wrinkle volume[E]
Crow's Feet

Self assessment[F]</td><td>
Elasticity
(Grader scale: 0-none, 9-high)
<table>
<tr><th></th><th>CH</th><th>MDx</th><th>BG</th></tr>
<tr><td>0d</td><td>≈2.6</td><td>≈2.6</td><td>p>0.05</td></tr>
<tr><td>84d</td><td>≈3.7</td><td>≈3.6</td><td>p>0.05</td></tr>
<tr><td>WG</td><td>p<0.05</td><td>p<0.05</td><td></td></tr>
</table>
Hydration
(Grader scale: 0-none, 9-high)
<table>
<tr><th></th><th>CH</th><th>MDx</th><th>BG</th></tr>
<tr><td>0d</td><td>≈2.6</td><td>≈2.7</td><td>p>0.05</td></tr>
<tr><td>84d</td><td>≈3.7</td><td>≈3.6</td><td>p<0.05</td></tr>
<tr><td>WG</td><td>p<0.05</td><td>p<0.05</td><td></td></tr>
</table>
Wrinkle Depth[E] (a.u.)
<table>
<tr><th></th><th>CH</th><th>MDx</th><th>BG</th></tr>
<tr><td>0d</td><td>≈2.35</td><td>≈2.40</td><td>p>0.05</td></tr>
<tr><td>84d</td><td>≈2.20</td><td>≈2.25</td><td>p>0.05</td></tr>
<tr><td>WG</td><td>p<0.05</td><td>p<0.05</td><td></td></tr>
</table>
Self-Assessment – skin beauty
(0-not, 9-very beautiful)
<table>
<tr><th></th><th>CH</th><th>MDx</th><th>BG</th></tr>
<tr><td>0d</td><td>≈4.0</td><td>≈4.0</td><td>p>0.05</td></tr>
<tr><td>84d</td><td>≈5.4</td><td>≈5.5</td><td>p>0.05</td></tr>
<tr><td>WG</td><td>p<0.05</td><td>p<0.05</td><td></td></tr>
</table>
BG = between group (CH vs MDX)
WG = within group (vs 0d)
</td></tr>
<tr><td colspan="8">[A] Assessed by a dermatologist investigator in the research team member, [B] n=1 from placebo excluded from analysis of the echogenicity (dermis density) parameter due to missing data,
[C] DUB® SkinScanner (ultrasound for echogenicity measure of dermal density quantified on a gray scale of 0-255, with higher scores indicating greater density,
[D] Visual and tactile scale (0-none to 9-high) graded by the dermatologist, [E] Photographic imaging (COSDERMA Photo Bench), expressed in arbitrary units, [F] Skin beauty (10-pont scale)</td></tr>
<tr><td>Zmitek et al.[73]
2024
Clinical trial registration number: <u>NCT05730517</u></td><td>Parallel RDBCT
(Computer generated randomization & 1:1:1 group allocation)</td><td><u>Total recruited</u>: n=90 healthy Caucasian women 40-65y</td><td>Bovine (MW[A]: 2-4kDa) 5g/d WITH 80mg vitamin C AND 30mg hyaluronic acid (CPHA) OR without hyaluronic acid (CP) taken as a 15mL syrup [B] at mealtime</td><td>Placebo syrup [B] 15mL (minus CH, vit C, HA)</td><td>16w</td><td>PRIMARY
Thickness [C]
Right cheek Set 4cm² area (mean of 2 reads)</td><td colspan="2">NHLBI quality assessment: 11.5 / 14 – Good
<table>
<tr><th colspan="6">Thickness [E] (μm)</th></tr>
<tr><th></th><th>CPHA</th><th>CP</th><th>Pla</th><th>p=</th></tr>
<tr><td>0w</td><td>979.8 ± 149.7</td><td>978.0 ± 116.5</td><td>972.3 ± 154.3</td><td>0.98</td></tr>
<tr><td>16w</td><td>991.2
95% CI:
960.1, 1022.2</td><td>990.3
95% CI:
960.4, 1020.2</td><td>949.8
95% CI:
919.4, 980.2</td><td>0.99</td></tr>
<tr><th colspan="6">Viscoelasticity [E] (MPa)</th></tr>
<tr><th></th><th>CPHA</th><th>CP</th><th>Pla</th><th>p=</th></tr>
<tr><td>0w</td><td>4.2 ± 1.2</td><td>4.5 ± 1.3</td><td>4.5 ± 1.6</td><td>0.96</td></tr>
<tr><td>16w</td><td>5.46
95% CI:
4.60, 6.32</td><td>5.60
95% CI:
4.77, 6.43</td><td>4.81
95% CI:
3.96, 5.65</td><td>NS</td></tr>
</table>
Continued overleaf</td></tr>
</table>

<table>
<tr><th rowspan="2">Author(s), year, trial registration</th><th rowspan="2">Design</th><th rowspan="2">Study population</th><th colspan="3">Collagen hydrolysate (CH) intervention</th><th rowspan="2">Outcome measure(s)</th><th rowspan="2">Key findings</th></tr>
<tr><th>Source and dose</th><th>Placebo and dose</th><th>Duration</th></tr>
<tr><td>Zmitek et al.[73]
continued</td><td>Environment:
Temperature 20-25°C,
Rel humidity 40-60%</td><td>CPHA group:
n=28
51.3±7.3y

CP group:
n=30
52.7±6.3y

Control group:
n=29
52.5±6.2y

Fitzpatrick skin type: I-IV

<u>Dropouts</u>:
n=3</td><td></td><td></td><td></td><td>SECONDARY
Viscoelasticity
Right cheek[C]
Set 4cm² area (mean of 3 reads)

Hydration
Stratum corneum of an unspecified forearm [C],
Set 4cm² area (mean of 8 measures)

Wrinkle depth[D]
lateral periorbital area on the left or right side of face with more obvious wrinkles at Baseline</td><td>
Hydration [E] (µS)
<table>
<tr><th></th><th>CPHA</th><th>CP</th><th>Pla</th><th>p=</th></tr>
<tr><td>0w</td><td>49.1 ± 22.0</td><td>47.3 ± 17.4</td><td>48.5 ± 14.0</td><td>0.93</td></tr>
<tr><td>16w</td><td>101.5
95% CI:
86.3, 116.8</td><td>98.6
95% CI:
83.9, 113.4</td><td>85.8
95% CI:
70.9, 100.7</td><td>NS</td></tr>
</table>
Roughness[E] (µm)
<table>
<tr><th></th><th>CPHA</th><th>CP</th><th>Pla</th><th>p=</th></tr>
<tr><td>0w</td><td>8.05 ± 0.68</td><td>7.98 ± 0.53</td><td>8.07 ± 0.48</td><td>0.76</td></tr>
<tr><td>16w</td><td>7.79**
95% CI:
7.62, 7.96</td><td>7.82**
95% CI:
7.65, 7.98</td><td>8.59
95% CI:
8.42, 8.75</td><td></td></tr>
</table>
Wrinkles – volume [E] (mm³)
<table>
<tr><th></th><th>CPHA</th><th>CP</th><th>Pla</th><th>p=</th></tr>
<tr><td>0w</td><td>7.43 ± 0.25</td><td>7.51 ± 0.24</td><td>7.44 ± 0.29</td><td>0.46</td></tr>
<tr><td>16w</td><td>6.63**
95% CI:
6.27, 7.00</td><td>6.64**
95% CI:
6.29, 6.99</td><td>7.67
95% CI:
7.31, 8.02</td><td></td></tr>
</table>
Wrinkles – depth [E] (mm)
<table>
<tr><th></th><th>CPHA</th><th>CP</th><th>Pla</th><th>p=</th></tr>
<tr><td>0w</td><td>0.12 ± 0.02</td><td>0.13 ± 0.02</td><td>0.13 ± 0.03</td><td>0.18</td></tr>
<tr><td>16w</td><td>0.110**
95% CI:
0.102, 0.118</td><td>0.113*
95% CI:
0.106, 0.121</td><td>0.135
95% CI:
0.127, 0.142</td><td></td></tr>
</table>
Wrinkles – indentation index [E] (a.u.)
<table>
<tr><th></th><th>CPHA</th><th>CP</th><th>Pla</th><th>p=</th></tr>
<tr><td>0w</td><td>43.10 ± 3.30</td><td>41.96 ± 3.11</td><td>42.01 ± 2.30</td><td>0.26</td></tr>
<tr><td>16w</td><td>39.53**
95% CI:
38.57, 40.50</td><td>39.73**
95% CI:
38.81, 40.66</td><td>43.33
95% CI:
42.39, 44.27</td><td></td></tr>
</table>
*p<0.05 vs. Placebo, ** p<0.001 vs Placebo,
NS: not significant (between group comparisons)
</td></tr>
<tr><td colspan="7">[A] Molecular weight. [B] Lactic, malic, and phosphoric acids, flavorings, xylitol, sucralose, sodium benzoate, potassium sorbate, caramel coloring, xanthan gum and gellan gum. [C] DermaLab SkinLab Combo – ultrasound/elasticity/flat hydration probes, [D] Antera 3D CS,
[E] Analysis of covariance (ANCOVA) accounting for baseline variation as a covariate</td></tr>
</table>

6.4.3 Quality of evidence assessment

Six of the 12 studies were poor quality according to the scoring criteria for the NHLBI quality assessment checklist.[65,70,71,75,79,80] Four were graded fair,[66,67,69,72] and two were of good quality.[68,73] Four studies reported trial registration numbers,[66,67,69,73] but eight did not so their protocols were not retrieved for their quality assessments.[65,68,70-72,75,79,80]

6.4.4 Study design factors

6.4.4.1 Participant characteristics

Six studies did not provide information on how their participants were randomized or allocated to groups.[65,69-71,79,80] Five studies gave some information about randomization and allocation,[66-68,72,73] but Demir-Dora et al.[67] did not specify how their randomization was achieved other than saying by a statistician. Vleminckx et al.[72] gave no detail about their ratio of allocation to groups, and the open-labelled trial by Gibson et al.[75] did not have a control group so randomization and allocation to treatment was not applicable.

All 12 studies exclusively studied female participants. Baseline comparisons of their characteristics were not made by five,[65,68,69,79,80] while seven provided some information either in a table or written in text. Gibson et al.[75] reported their participants' mean age and the number within each skin phototype category according to the Fitzpatrick scale.[88] Bolke et al.[66] reported the mean age of participants in their supplement groups without any further baseline data, and Demir-Dora et al.[67] gave the mean age and BMI of all participants in their study without between group comparisons. Two studies presented the mean age of participants in a table without any other characteristics for comparison, but they did report non-statistically significant differences between groups at baseline for their primary and secondary outcome measures.[70,71] Likewise, Vleminckx et al.[72] reported similarities in baseline measures of their primary and secondary outcome measures after testing for significance, but without showing their data. One study presented all such baseline data in a table (i.e. age, BMI and outcome measures at baseline).[73]

The largest sample recruited was 135 with an age range of 45-65 years (mean 54.5±6.2).[75] Only 116 participants completed the study,[75] and the next largest sample allocated to treatment was

120 with an age range of 35-57 and mean of 54.5±6.2 years.[67] Eight of those participants were excluded from analysis as dropouts due to pregnancy (n=2), Covid-19 (n=2), pneumonia (n=1), nausea (n=1), and unspecified reasons (n=2). The smallest sample of 33, recruited for a pilot study by Asserin et al.[65] had an age range of 40-59 years without any dropouts. The remaining sample sizes ranged between 56 and 114 (Table 6-3), although only 108 completed the study of 114 participants by Proksch et al.[71] Their six dropouts were reportedly unrelated to the study supplement. There were three dropouts from the recruited sample of 56,[68] three from a sample of 90 aged between 40-65 years,[73] and 62 out of 69 completed a study by Proksch et al.[70] In that case, the seven dropouts were reported as reasons unrelated to the treatment.[70] Two studies gave no information about their participants' attrition rates.[79,80]

Participants' skin phototypes, as determined by the Fitzpatrick scale[88] were reported by six studies. All skin types (i.e. I-VI) were included in one study,[75] whereas participants were only included if their skin type was I-III,[71] I-IV,[70,73] II-III,[79] or II-IV[80] in the other studies. Meanwhile, six studies did not report their participants' skin types,[65-69,72] and one of those studies recruited participants with stage I dermatoporosis of the forearm.[68]

6.4.4.2 Intervention regimens

The lowest oral daily dose of CH was 2.5 g in five studies.[66,69-71,75] The highest dose was 10 g in three studies,[65,67,80] and four studied a daily dose of 5 g.[68,70,72,73] Five studies compared a placebo of maltodextrin (MDx) with CH supplements containing other potentially active ingredients such as zinc, HA, or vitamins A, C, and E.[66,69,73,75,80] Conversely, seven studies compared a placebo with supplements of CH without any other ingredients.[65,67,68,70-72,79] However, one of those studies compared the topical application of CH in a cream versus a placebo lotion alongside their oral intake of CH versus MDx in a factorial design.[68]

The shortest intervention was eight weeks in four studies.[65,67,70,71] The longest trial was 26 weeks in the study by Guadanhim et al.,[68] and the remaining studies employed periods between 12[66,69,72,75,79,80] to 16 weeks.[73] Four studies also collected data after four weeks of washout from their

intervention.[66,67,70,71] but only three presented or reported data for that timepoint.[66,67,71] None of the 12 studies reported results on their participants' adherence to their interventions.

6.4.4.3 Environmental factors

The climatic conditions (i.e. temperature and relative humidity) of data collection visits were reported by nine studies.[65,66,69-73,75,80] At least 30 minutes were given to participants for acclimatization before data collection in six studies,[65,69-71,73,75] or between 15-20 minutes for two.[72,80] Bolke et al.[66] did not state any timeframe, and three studies did not report any information about their indoor testing conditions.[67,68,79]

6.4.5 Outcome measures

6.4.5.1 Wrinkles

The most common outcome measure was for wrinkles. Also referred to as roughness, this skin topography parameter was a primary outcome in seven studies,[66,67,71,72,75,79,80] and a secondary outcome for two.[70,73]

There were several methods and instruments used for capturing and reporting data for wrinkles. Bolke et al.[66] measured wrinkle depth (in units of µm) of an unspecified facial region using phase-shift rapid in vivo measurement of skin (PRIMOS). They found significant reductions in the CH and control groups after 12 weeks of intervention (Table 6-3). The decrease was more profound in the CH group compared to control at end point ($p<0.0004$), but the authors did not show both groups' means after their washout of four weeks.[66] Proksch et al.[70,71] used PRIMOS in their studies to assess wrinkle volume (in units of mm^3) in the crow's feet (lateral periorbital) area of the face (i.e. around the eye) as a primary outcome,[71] or the inner right forearm as a secondary outcome measure in units of µm.[70] There was a significant difference between 2.5 g doses of CH and the control at endpoint for wrinkle volume in the crow's feet which was sustained after four weeks of non-treatment from CH (Table 6-3),[71] but there were not any significant effects on wrinkles at the inner forearm with doses of 2.5 g or 5 g CH versus placebo after four weeks ($p=0.61$) or eight weeks ($p=0.63$) of intake.[70] In that instance, the authors presented their data in a figure showing the two doses of CH on a scale of wrinkle

volume relative to the MDx group (i.e. "x-fold of placebo") without showing the mean volumes of wrinkles for any of the three groups.[70]

Demir-Dora et al.[67] used a different tool to assess depth and width of wrinkles in the crow's feet. They reported a significant improvement for the CH group but not placebo after their eight-week intervention compared to baseline (*p<0.001*), as detected by increased readings with the Callegari device in units of μg/cm^2 (Table 6-3). The authors[67] also reported significant within group improvements for the CH and control after a washout of four weeks compared to baseline, with the effect more pronounced for the CH group compared to placebo (*p<0.001*). Vleminckx et al.[72] found crow's feet wrinkle depth decreased (i.e. improved) after 12 weeks of their 5 g CH and MDx treatments (both *p<0.05*), but the difference between groups was not significant at endpoint (Table 6-3). Their data were captured with the Cosderma Photo Bench and presented as a change in the luminosity intensity of photographs of wrinkle segments relative to the surrounding skin area. Žmitek et al.[73] used an Antera 3D camera to assess their participants' crow's feet for volume of wrinkle depressions (mm^3), the maximum volume of wrinkles (mm), and a wrinkle indentation index measured in arbitrary units. After accounting for baseline group means as covariates, all three parameters improved significantly for the groups consuming supplements of CH with or without HA compared to the MDx group (Table 6-3).

The Visioface digital photography imaging system was used to assess wrinkles in two studies by Maia Campos et al.[79,80] In both studies, the number of wrinkles in the nasolabial (nose to mouth), periorbital (crow's feet) and forehead regions of the face were counted.[79,80] Maia Campos et al.[80] did not show their data despite reporting a reduction in wrinkles in the forehead region for the CH group versus baseline. They did not make any comments on their findings at the nasolabial and periorbital regions. Meanwhile, Maia Campos et al.[79] presented their data as figures and showed significant improvements at endpoint compared to baseline in the CH treatment but not the control group for the nasolabial and periorbital regions (*p<0.05*). The authors did not compare the CH group at endpoint with the placebo group (Table 6-3).

Gibson et al.[75] reported a significant reduction in wrinkles in their open-label trial using a subjective method. High-resolution photographs of the frontal and sidewise aspects of participants' faces were taken, and a grader reviewed those images to assign a score between 0-9 representing a total (global) count of wrinkles.

6.4.5.2 Hydration

Hydration was a primary outcome measure in five studies[65-67,72,80] and a secondary measure for two studies.[70,73] The Corneometer was the most common device used for data acquisition,[65,66,70,80] while the Callegari Soft Plus device was used once,[67] as was the Dermalab Combo.[73] Vleminckx et al.[72] compared scores from 0-9 derived from a dermatologist investigator's visual and tactile assessment of the parameter using an Evalux Bench mirror and lighting system.

The hydration measurements were made on the inner forearm by two studies,[67,70] a standardized but unspecified area of the forearm by Žmitek et al.,[73] and several areas on the face (i.e. cheek, crow's feet/periorbital, forehead, nasolabial) in three studies.[67,72,80] The pilot study by Asserin et al.[65] did not report where their hydration measures were made on the body.

Two studies found a clear difference between CH and the comparator. Asserin et al.[65] found hydration (measured in arbitrary units) significantly improved from baseline to the eight-week endpoint in groups who consumed CH sourced from fish (CH-fish) and porcine hide (CH-porcine) but not MDx (Table 6-3). The difference between MDx and CH-fish at endpoint was not significant, but it was between CH-porcine and the MDx comparator (*p=0.003*). Demir-Dora et al.[67] reported a significant within-group improvement in hydration (measured in g/m^3) for the CH group but not placebo after their eight-week intervention compared to baseline (*p<0.001*). After a washout of four weeks, they also found significant within-group improvements for the CH and placebo groups compared to baseline (Table 6-3), but there was no difference between the two groups at that point (*p=0.115*). The authors did not specify if their data were from the face, inner forearm, or if it were pooled from both sites.

Three studies reported hydration was significantly improved within their CH and comparator groups at the end of their 12 or 16-week interventions.[66,72,73] In two of those studies, the improvements were greater (i.e. statistically significant) for the CH compared to MDx groups,[66,72] while Žmitek et al.[73] found the differences between treatments of 5 g CH with or without HA were not statistically different to each other or the comparator in their analysis of covariance which accounted for baseline mean hydration values of the forearm (unspecified inner or outer).

Maia Campos et al.[80] did not show their data on hydration assessment. They reported no significant differences in measures of the periorbital and nasolabial regions of the face compared to baseline, and a significant difference between baseline and endpoint in the forehead for the CH group. They reported no significant difference in this parameter compared to the placebo at endpoint without showing p values.[80] Proksch et al.[70] assessed epidermal hydration of the inner forearm as a secondary outcome measure with the Corneometer in arbitrary units, and they presented their findings by showing the two doses of 2.5 g and 5 g CH in a figure with a scale of hydration changes over the intervention relative to the MDx group (e.g. "x-fold of placebo"). They did not show mean values for any of the three groups and reported no significant difference in hydration levels between the treatment and comparator groups (Table 6-3).

6.4.5.3 Elasticity

Elasticity was a primary outcome for six studies[66-68,70,79,80] and a secondary outcome for two.[73,75] Measurements were made with a Cutometer device in five studies; with three of those studies presenting data for the R2 (gross elasticity) parameter,[66,68,75] and three presenting R5 (net elasticity) data.[68,70,80] Guadanhim et al.[68] also presented their findings for the R6 (viscoelastic ratio) and R7 (biological elasticity) variables. One study measured viscoelasticity with a Dermalab Combo,[73] and the Callegari Soft Plus device was used once for elasticity assessment.[67] Vleminckx et al.[72] did not use a device and relied on graded scoring by a dermatologist to assess elasticity as they had done for hydration.

Elasticity was most commonly measured in the middle malar area of the face (i.e. cheekbone).[72,73,75] Two studies measured elasticity of the inner forearm,[67,70] one focused on a defined area of the outer forearm[68] and one did not specify which area of the forearm was measured.[66] One study chose at random anywhere from the forehead, periorbital (around the eyes) or nasolabial (between nose and mouth) areas,[80] while Demir-Dora et al.[67] focused on elasticity at the crow's feet (i.e. periorbital).

6.4.5.4 Thickness

Skin density or thickness was a primary outcome for three studies.[66,68,73] It was unspecified as a primary or secondary measure by one.[72] Two studies used a SkinScanner DUB device[66,72] and two used a DermaScan.[68,73] Dermal layer echogenicity was also assessed with the DermaScan-C in two studies by Maia Campos et al.[79,80]

Bolke et al.[66] measured epidermal (i.e. outer skin layer) thickness of the thigh with a SkinScanner DUB. They reported statistically significant increases in the CH and comparator groups compared to baseline (Table 6-3), with a greater increase for CH than the placebo ($p<0.0004$). Vleminckx et al.[72] also used the SkinScanner DUB, and they found density of the dermal layer (i.e. underneath the epidermal layer and highest concentration of Type I collagen) improved within the CH group compared to baseline ($p<0.05$) and compared to the placebo ($p<0.05$). Vleminckx et al.[72] measured an unspecified area of the temple, and the SkinScanner DUB images were graded on a Gray scale ranging from 0-255 rather than quantified in metric units as per Bolke et al.[66]

Guadanhim et al.[68] used the DermaScan-C to measure dermal layer thickness of a specific site on the inner forearm. They did not find any change from baseline to endpoint across their CH and placebo groups which also included topical application of CH or placebo. Conversely, Žmitek et al.[73] assessed dermal thickness of a specific area of the cheek with the DermaScan, and they found mean thickness increased in both CH treatment groups (i.e. with or without HA) but the placebo group mean decreased. The 95% confidence intervals were wide, and the two CH groups' means were not significantly different to baseline or compared to the placebo.[73]

Maia Campos et al.[80] examined echogenicity of the forehead, eye (periorbital), or mouth (nasolabial) regions at random. They presented their data as a change in echogenicity (calculated by counting pixels) from baseline to endpoint without showing their baseline and endpoint means. No differences were found for the placebo, but CH intake was reported to have improved echogenicity in the forehead and nasolabial (mouth) regions compared to baseline ($p<0.05$), but not the periorbital area.[80] Maia Campos et al.[79] measured echogenicity of the malar facial region (cheek), and they reported it was improved for the CH group without reaching a significant difference from baseline or the placebo group. The authors did not show the groups' mean values for baseline and endpoint and instead presented differences between endpoint and baseline values for the CH and placebo groups' echogenicity ratios.

6.4.5.5 Other outcome measures

Collagen content or the extent of fragmentation (breakdown) was measured by three studies either as a primary[68,69] or secondary outcome.[71] Laing et al.[69] assessed fragmentation of the epidermal and dermal layers of the cheek with confocal microscopy, as graded by an expert operator blinded to the treatment groups. Proksch et al.[71] conducted a sub-group analysis on 48 of their 114 participants to compare Type I collagen content in skin blister biopsies from the inner forearm, and Guadanhim et al.[68] measured collagen content in the epidermis from skin biopsies taken from the outer forearm, 7 cm away from the antecubital fold. Patient reported outcome measures (i.e. self-assessment) were captured in five studies as secondary outcomes,[66,68,69,72,75] but only Guadanhim et al.[68] used a validated tool to capture the data.[87] Demir-Dora et al.[67] stated their participants self-completed diaries at each follow up, but they gave no detail on what data was collected nor did they show any of their results.

6.5 Discussion

The primary objective of this review was to assess the quality (internal validity) of studies using CH from terrestrial mammals to examine its effect on skin condition parameters. In contrast to avian and fish, terrestrial mammals offer a richer (i.e. more concentrated) source of postulated BAPs (i.e. Pro-Hyp).[7,32,39,46] The review's secondary objectives were to explore and appraise features of the

included studies' designs (i.e. participant characteristics and intervention regimens) and identify methods used for elucidating their data. The overall goal was to identify opportunities for assisting the design of future studies to ultimately improve their quality of evidence.

6.5.1 Quality of evidence assessment

Half of the studies included were of poor quality in reference to the NHBLI 14-item checklist for the quality assessment of controlled intervention studies.[56] None of the studies reported their participants' adherence to intervention, and two did not provide data on their participants' attrition rates.[79,80] A common method of monitoring adherence is counting returned supplements, but only one study mentioned doing so in their methods without reporting any findings.[73] Similarly, two studies reported their intent of tracking adherence by self-report at each follow up, but the authors never gave any data on their findings.[67,75] Studies that report adherence rates are more likely to report negative findings,[89] while not reporting attrition rates prevents readers from determining if an appropriate statistical analysis plan (i.e. intention-to-treat) was executed.[56,90] Therefore, omitting these data impacts on the transparency of a study which introduces bias to their results.[56,89,90]

6.5.2 Study design factors

Authors of the studies infrequently presented baseline data. Doing so prevented their included participants' characteristics being compared by treatment group to determine the effectiveness of randomization in mitigating selection bias.[91] All studies specifically recruited females, most of whom were of the peri and postmenopausal age, but only half of those studies reported their participants' Fitzpatrick skin phototypes.[88] Of the six which did, the range of skin types varied considerably as one study included all skin types while others were more selective and only included skin types II-III.[75,79] Skin phototyping allows the level of melanin in the participants' epidermis to be estimated.[88] The pigment absorbs UV radiation to provide natural protection from photoaging.[2,3,92,93] Applying sunscreen is an artificial means of protection, and one study gave their participants a sunscreen with a standardized sun protection factor (SPF) for using *ad libitum* over their intervention.[79] Participants across all other studies (i.e. numerous geographical locations) may have

used their own sunscreen with differing SPFs if they used any at all. Therefore, the level of sun-exposure (i.e. photoaging) was uncontrolled as an exposure variable.

The composition of supplements studied also created a potentially confounding factor. Five out of the 12 studies included a combination of active ingredients[30] in their CH supplement for either comparing with a placebo,[66,69,73,80] or not.[75] Most of those studies reported a superior effect of CH compared to the placebo for parameters of elasticity, hydration, thickness, wrinkles or collagen structure (i.e. ameliorating its fragmentation), but the improvements are not necessarily attributable to an effect exerted by CH considering the effect of oxidative stress on skin condition.

Oxidative stress on dermal fibroblasts is inevitable with intrinsic aging. It is exacerbated by photoaging,[31,94] but micronutrients such as zinc, selenium, and vitamins A, C and E, all of which have antioxidant properties, can attenuate its deleterious effects.[28,30,31,95] Vitamin C is also essential for collagen biosynthesis,[37] and like HA it is hydrophilic which means supplements with vitamin C and HA have the potential to improve skin hydration.[31] Therefore, the inclusion of micronutrients and/or HA with CH supplements may have contributed to positive effects on the skin condition measures observed in the treatment groups.[96]

Climatic conditions at data collection were generally controlled and reported on, except for three studies.[67,68,79] Several studies reported a relative humidity range of 40-60% which is reasonably wide considering how seasonal differences in air temperature and humidity influence skin condition measurements.[97,98] Relative humidity is lower in cooler months of autumn and winter when the air is dry and can absorb more water from skin than the warmer months, especially when the air is heated for warmth indoors (which explains why dry skin is a common complaint in winter).[97,99,100] Nonetheless, topical application of skin lotion improves hydration which correlates with an increase in elasticity.[101-104] Vleminckx et al.[72] provided a moisturizing face cream for participants to use twice daily during their intervention, but no other studies reported doing so. The participants were generally advised to avoid applying any lotion directly before data collection visits, but outside of those times

this was another uncontrolled covariate akin to the heterogenous use of sunscreen which impacts on the comparability of results.

6.5.3 Outcome measures

Skin condition is a multi-parametric variable to examine due to its anisotropic structure.[105,106] Wrinkles, hydration, elasticity and dermal thickness were the predominant primary outcomes in the studies reviewed, but there are several ways of assessing each parameter.[107,108]

Hydration of the epidermis is often assessed by non-invasive electrical-based methods of capacitance, conductance or impedance,[103,109,110] if not more intensively with a thermal technique of differential scanning calorimetry.[108] Transepidermal water loss (TEWL) is also examined in some studies,[65,70] but TEWL is more representative of skin barrier function than hydration.[4,111] Meanwhile, dermal thickness is generally determined from ultrasound,[112-114] although it can also be estimated from biopsies or using skinfold calipers.[112]

Elasticity can be quantified by deformation of skin either along its plane (i.e. resistance to torsion) or horizontal to it with suction using stress-strain and strain-time curves.[14,108] The Cutometer is widely used to measure strain as a function of time in arbitrary units.[14] The R2 (gross elasticity) or R5 (net elasticity) values are primarily presented in research as they provide information about the elevation (stretching of collagen and elastin) and retraction phases.[14] Both phases are important to assess as healthy young skin and older loose skin are easy to elevate, but the time to retract is prolonged for older skin with less elasticity than young skin which retracts quickly.[115] Irrespectively, the Cutometer is criticized for not factoring in dermal thickness, despite that variable being influential in the elevation and retraction phases.[104,105,108,115] Suction devices which account for dermal thickness (e.g. Callegari Soft Plus or Dermalab Combo) are used in some studies, and they capture data in units of megapascal (MPa).[67,73] However, the precision (i.e. variance) of results from the Cutometer, Callegari Soft Plus and Dermalab devices can differ which impacts on the comparability of results from these devices.[106,107,116]

Anatomical sites of measurement are also important to consider when comparing study results. Skin condition parameters such as thickness, hydration and elasticity vary by region within and between individuals.[92,106,114] Chopra et al.[117] found in their study that the dermal layer of the face was thinnest at the eyelid and thickest at the lower nose region. Jeong et al.[112] also reported the dermis was thickest in the nasolabial area (i.e. between the nose and mouth) but thinnest at the lateral forehead region. Ryu et al.[118] found elasticity of the sun-exposed cheek was less than areas on the upper arm (i.e. partially protected from sunlight) and lower back (i.e. fully protected from sunlight), and hydration of the epidermis was greater in the face compared to the mid-forearm. The face and dorsal (outer) forearm are generally exposed to sunlight (i.e. extrinsic and intrinsic aging regions) while the ventral (inner) forearm is more protected (i.e. intrinsic aging predominates). It has been suggested that sun-protected areas may be less responsive to treatment compared to areas undergoing photoaging,[50] so that notion may warrant further investigation.

6.5.4 Limitations

A limitation of this review is that it was performed as a rapid review.[58] The search period was cutoff at the end of 2024, so any new articles published in 2025 were not considered. Due to time constraints, one reviewer conducted the quality of evidence assessments, but each study was assessed twice on different occasions for intra-rater reliability to align with guidance on conducting systematic scoping reviews as best possible.[57] Overall, the design and execution of this systematic scoping review are strengths considering it included an examination of each study's internal validity when most such reviews do not. Therefore, these findings make a valuable contribution to the existing evidence, despite its limitations.

6.6 Conclusion and future recommendations

This scoping review identified an opportunity for further research on oral CH supplements and skin condition. Most studies to date have used CH sourced from fish, but terrestrial mammals have a higher concentration of Pro-Hyp which is postulated as a bioactive peptide for improving the skin condition parameters affected by aging (i.e. elasticity, hydration and dermal layer thickness). Half of

the included studies were judged as low quality according to the NHLBI Quality Assessment Tool for Controlled Intervention Studies, and a recurring issue was that baseline characteristics of participants were missing, as were details on adherence and attrition rates. Furthermore, there were a variety of methods used for collecting data on parameters of elasticity, hydration, and dermal thickness (i.e. the tools used for collecting data and the body sites examined), and how the data were presented. Significant improvements were reported at endpoint for CH and comparators in some studies but not others, and this may relate to the inclusion of active ingredients with antioxidant properties. Overall, the existing evidence is inconclusive and more research under more tightly controlled conditions is needed. Future studies would be improved in quality (i.e. internal validity and reliability) by applying the following recommendations:

- Data collection:
  - Focus on a specific cohort of participants at high risk of deteriorating skin condition (e.g. women ≥50 years)
  - Conduct testing within an environmental chamber, after ≥30 minutes of acclimatization
  - Include skin phototype assessment with the Fitzpatrick scale
  - Measure dermal thickness alongside hydration and viscoelasticity
  - Assess hydration status at baseline and endpoint (via urine specific gravity)
  - Assess patient reported outcome measures to complement objective measures
  - Collect self-reported data of weekly: sun exposure/time outdoors, use of standard moisturizers/sunscreens (administered at baseline for *ad libitum* use)
- Intervention regimen
  - Allow at least eight weeks of intervention
  - Compare a placebo to CH with and without antioxidant ingredients (i.e. three groups)
- Minimum reporting standards
  - Baseline participant data (e.g. skin phototype)
  - Adherence and dropout rates
  - Specify the body region(s) measured and with what devices

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Student name:	Drew Gordon (DG)
Name and title of main supervisor:	Professor Jane Coad (JC)
In which chapter is the manuscript/published work?	Chapter 7

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

JC conceived the CHAMP study and oversaw its overall direction and planning with Janet Weber (JW) and Katie Schraders (Study Coordinator). The analysis of results was performed by DG before drafting this chapter as a manuscript and preparing its final version. JC and JW contributed to critical review of the manuscript drafts and all authors approve of its contents. Final approval of the version submitted for publication was given by JC.

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Chapter 7: The effects of collagen hydrolysate and milk protein on skin condition in a randomized double-blind controlled trial on women aged ≥50 years with knee pain

This chapter achieved the fourth and final objective of the research project. It is a report on secondary outcomes of the CHAMP study examining the effect of CH on skin condition (thickness, hydration and viscoelasticity) and patient reported outcomes.

7.1 Abstract

Collagen hydrolysates (CHs) are advertised as 'nutricosmetics' for improving skin condition which naturally declines with age, especially among postmenopausal women and with regular sunlight exposure. Evidence for the 'anti-aging' efficacy of CH is inconclusive, so the aim of this study was to examine the effect of 15 g protein as CH (bovine hide) versus isoenergetic, and isoproteic comparators of milk protein and maltodextrin on dermal thickness (DT), epidermal hydration (EH) and viscoelasticity (VE). The randomized, double-blind controlled trial of four months recruited females aged ≥50 years with self-reported knee pain. The DT, EH and VE variables were measured at baseline and endpoint with a Dermalab Combo, from participants' ventral (sun-protected) and dorsal (sun-exposed) forearms, and cheeks (sun-exposed). DT was the outcome of primary interest, while EH and VE were secondary. All data were examined with split-plot ANOVA, presented as *F*-statistics, *p values* and partial eta (η_p^2). Pairwise comparisons were made with Bonferroni corrections, and per protocol analyses were reported as mean, standard error, and 95% confidence intervals after verification through sensitivity analyses. Eighty-two participants were allocated to the three isoenergetic supplements. Eight dropped out and two were excluded (adherence <75%). The ventral forearm significantly increased in DT within the CH, but not MP or MDx groups. Dorsal forearms significantly increased in DT for CH and MDx but not MP, while cheek DT significantly increased in all three groups. There were no differences between or within groups for EH or VE. Collectively, these exploratory results are inconclusive and warrant further investigation.

Trial Registration: Australia and New Zealand clinical trials registry (ACTRN 12622000779774).

Keywords: Dermal thickness, hydration, elasticity, collagen hydrolysate, bovine, aging skin

7.2 Introduction

Chronological (intrinsic) skin aging is a natural phenomenon involving cellular senescence. Reactive oxygen species (ROS) accumulate in dermal fibroblasts (DFs), causing an upregulation of metalloproteinases (MMPs) which are deleterious to the dermal matrix of collagen, elastin and hyaluronic acid (HA).[1-3] Over time, skin becomes thin, dry and loses its ability to stretch then return to shape (viscoelasticity).[2,4-6] These effects are amplified by an apparent effect from estrogen depletion among peri and postmenopausal women,[6-8] and further intensified by sunlight (photoaging) which means body parts often exposed to the sun (e.g. face and outer forearm) can show signs of age faster than the areas more typically protected (e.g. inner forearm).[1,2,6,8-10] Consequently, strategies to counter the cosmetic effects of aging in general are sought after by consumers and researchers.[3,8]

Collagen peptides (hydrolysates) are advertised as 'nutricosmetics' for improving skin condition.[11-13] These fragments are derived from undenatured Type I collagen, after being extracted from a host animal and hydrolyzed.[14-16] The native collagen fibers are comprised of repeating Glycine-X-Y (Gly-X-Y) sequences, with prolyl-hydroxyproline (Pro-Hyp) often occurring in the X-Y positions.[14,17] However, the concentration of Pro-Hyp varies by animal source, and terrestrial mammals have a higher concentration of these potentially bioactive peptides (BAPs) in their hides than avian or fish skin/scale alternatives.[18-20]

Early i*n vitro* research found Pro-Hyp dipeptides are chemoattractants for DFs.[21] The DFs are responsible for repairing the dermal matrix,[2,22,23] and Pro-Hyp is readily absorbed intact for distribution to skin cells more than other areas of the body such as chondrocytes in cartilage.[24-26] Some studies on mice, rats and pigs have found CH supplementation was associated with an increase in DF density and synthesis of Type I collagen compared to placebos,[27-30] but this is not a consistent finding considering the same studies or other authors have found CH supplementation had no effect on those parameters or similar measures such as dermal layer thickness.[28,30,31] Overall, preclinical evidence on the effect of CH as an 'anti-aging nutricosmetic' is inconclusive, but meta-analyses of human studies

regularly report CH supplements are effective for improving skin condition parameters such as epidermal hydration (EH) and viscoelasticity (VE).[32-36]

The integrity of an individual study design can affect the reliability of outcome measures being reported. A recent meta-analysis by Myung and Park.[37] found 10 out of their 23 included studies were classified with some concern for, if not having a high risk of bias according to the Cochrane RoB 2.0 or Jadad scale scoring systems.[38,39] Those 10 studies tended to report an effect from CH whereas the high-quality studies did not.[37] Moreover, several intervention studies included in earlier meta-analyses have overestimated treatment effects from CH due to biases or limitations in their designs.[35,36] Therefore, robust research is needed to improve the accuracy of future meta-analyses examining the effect of CH on skin condition.

Existing human studies on skin condition have predominantly used CH sourced from fish.[37,40] The studies which used CH from terrestrial mammals, with a higher concentration of Pro-Hyp than avian or fish, included placebos matched by weight rather than energy,[41-43] and they did not include protein comparators despite earlier animal studies having done so.[28,29,31] Furthermore, the effect of CH on dermal thickness (DT) was investigated less often than VE or EH, even though DT affects VE which is also influenced by EH.[44-47] Essentially, any observed effects on the study outcomes are not necessarily attributable to CH,[48] so the aim of this study was to examine the effect of CH sourced from bovine hide on skin condition (DT, EH and VE) versus energy and protein matched comparators among women aged ≥50 years with self-reported knee pain. It was a report on secondary outcomes of the collagen hydrolysate and milk protein (CHAMP) study,[49] and DT was of primary interest considering its modulatory effect on EH and VE.

7.3 Methodology

The randomized double-blind controlled trial (RDBCT) of four months (17 weeks) was registered with the Australia and New Zealand clinical trials registry (ACTRN 12622000779774). It

adhered to standards laid down in the Helsinki Declaration (1964, and its amendments) and was approved by the New Zealand Health and Disability Ethics Committee (2022 exp 12925).

7.3.1 Participants

Females aged ≥50 years with self-reported knee pain (with or without a medically confirmed diagnosis of knee osteoarthritis) were recruited between January and October 2023. Advertisements appeared on social media and at community groups within the Manawatū region of New Zealand. The study was also promoted on national radio, in print media and by word of mouth. Participants completed an online screening survey (Qualtrics XM 2023), adapted from the pain domain of the Knee Injury and Osteoarthritis Outcome Score (KOOS) before it became licensed.[50,51] Raw scores ≥8/36 were included, but the criteria were refined midway through recruitment as several respondents with pain scores <8/36 reported changing their living routines for pain-related reasons (e.g. avoiding stairs which evoke pain). Thereafter, respondents with pain scores of 6-7/36 were provided with the quality of life (QoL) questions from KOOS, and raw scores ≥7/16 for the QoL domain were included.[50] All autoimmune conditions (except diet-controlled Coeliac Disease) were excluded, as were hepatic or renal diseases, and anyone who sustained knee trauma or underwent surgery in the previous six months. Likewise, respondents who commenced hormone replacement therapy (HRT), or used oral nutrition supplements containing chondroitin, glucosamine, or collagen within the previous six months were excluded (Appendix 3: Supplementary Table 9-2).

7.3.2 Intervention

The participants were randomized with an equal representation ratio (1:1:1) for every nine admitted to the study using a computer-generated system (calculator.net). They were allocated on arrival at baseline to receive 15 g protein as CH (extracted from bovine hide), 15 g milk protein (MP), or 19.2 g maltodextrin (MDx). To achieve its isoenergetic and isoproteic state, 4.2 g MDx was added to CH (Table 7-1). The identically packaged supplements were presented to participants at the end of their baseline visit, accompanied by verbal and written guidance to consume one sachet of powder each day as a single dose or spread over the day in foods or fluids of each participant's choice. The

participants were given a supply of multivitamins for daily consumption to reduce the possibility that micronutrients required for collagen synthesis were rate-limiting (Appendix 3: Supplementary Table 9-3), and they were asked to maintain their usual diet and exercise patterns over the intervention. All stakeholders were blind to the supplement groups until statistical analyses were complete.

Table 7-1. Nutritional information panel for the three isoenergetic supplements per sachet.

	Collagen Hydrolysate	Milk Protein	Maltodextrin
Energy (kJ)	325.8	325.8	325.8
Protein (g)	15	15	0
Fat (g)	0	1.2	0
Carbohydrate (g)	4.2	3.6	19.2
Total weight (g)	20.3	21.5	19.2

7.3.3 Data collection

Baseline and endpoint visits were hosted at the Human Nutrition Research Unit (HNRU) of Massey University in Palmerston North, New Zealand. They started between 0730-0930 and finished by 1130-1300. The first baseline visit was in February 2023, and the last endpoint visit was in February 2024.

7.3.3.1 Patient-Reported Outcome Measures

After providing written informed consent at baseline, participants completed a self-administered monthly online questionnaire (Qualtrics XM 2023 and 2024). The survey was one aspect of the CHAMP study evaluating the effect of CH on pain.[49] It included 12 statements to evaluate the participants' opinions about their skin condition on an 11-point ordinal scale (0=disagree, 10=agree), and they were prompted by email to complete the survey each month at home until their endpoint visit at the HNRU.

7.3.3.2 Skin Condition

Dermal thickness (DT), EH and VE were measured in that order with the Dermalab Combo.[45] The left ventral (inner) forearm was chosen to assess the effect of CH on intrinsic aging without photoaging. It was marked for DT and EH assessment with a water-based ink pen, 5 cm from the radiocarpal joint and 5 cm from the antecubital fossa. The corresponding dorsal (outer) forearm areas were marked for DT and EH readings so the cumulative effect of photoaging could be compared with

the ventral forearm. The sun-exposed middle malar region of the left cheek was chosen by sight for measuring DT and EH, while the same approximate area on the right cheek was used for VE. The DT and EH parameters were measured in duplicate or triplicate, and the two closest values (aiming for identical as a benchmark) were averaged. Lastly, VE was measured once on the left ventral forearm (midway between the upper and lower markings) and once on the right cheek.

Participants were seated during their skin condition assessments after ≥30 minutes acclimatization to environmental conditions of the HNRU. They were asked to avoid movement or talking,[45] and to refrain from wearing makeup or applying moisturizing lotion before their arrival to avoid erroneous readings of EH and VE.[52]

Dermal Thickness (DT)

The Dermalab standard ultrasound probe was used at its optimal bandwidth of 20 MHz and scan length of 17.6 mm. A thin layer of gel was applied and massaged into the area as per the manufacturer's instructions,[45,53] and an amplification (gain) setting of 0 was used for all scans of the forearm and cheek.

Epidermal Hydration (EH)

The eight-pin probe was used to assess EH.[45] All measurements were taken adjacent to where the gel had been to measure DT before being removed.

Viscoelasticity (VE)

The VE variable was measured because it encompasses the elevation and retraction phases of a strain/time curve.[45] It was measured last to avoid a false increase of DT,[54] and repeat measures were not made because ≥45 minutes rest is recommended between measurements to avoid elastic hysteresis.[46] A 'normal' skin setting of 400 mbar negative pressure was used on all participants,[45] with the default 1 mm skin thickness, and five elevation/retraction cycles in all cases. The left ventral forearm measurement was made midway between the radiocarpal joint and antecubital fossa markings, and the right cheek measurement replicated where EH and DT were measured on the left cheek.

7.3.3.3 Adherence

All empty and unopened supplement sachets returned by participants were counted.[55] A conservative threshold of <75% defined a protocol violation of non-adherence.

7.3.3.4 Adverse events

Participants were asked in their monthly survey if they had any of the listed symptoms which have been experienced in similar studies on CH.[56-58] They could also report any ill effects with free text.

7.3.4 Statistical analyses

The statistical analyses were performed with SPSS (version 29, IBM SPSS statistics). A sample size of 27 per group was needed to attain power of 90% and a Type I error rate of 5% as calculated by G*Power (version 3.0.10) to detect change in the main outcome of pain for the CHAMP study.[49]

The data were inspected for violations in assumptions of normality with a data cleaning and sensitivity analysis protocol to identify and manage outliers (Appendix 5: Supplementary Table 9-10). Distributions in variance within groups were evaluated with visual inspection of skewness (≤±1) and kurtosis (≤±2), Q-Q plots and the Shapiro-Wilk tests, then homogeneity of variance between groups was assessed with Levene's test. Outliers of continuous data (i.e. DT, EH and VE) were considered for inclusion or removal after common (log10) transformation of data to assess the magnitude of any violation of assumptions in normality. For example, an outlier was removed if it remained an outlier after log10 transformation and was outside a reference range of published norms in its untransformed format (Appendix 5: Supplementary Table 9-11). In such instances, a measurement error was assumed to reflect a technical issue or non-adherence to the request of arriving without wearing makeup or lotions.[22,52]

The cleaned data were examined with a two-way mixed-design (split-plot) ANOVA. The Greenhouse-Geisser correction was applied to overcome unequal group sizes,[59] and the analyses were presented as *F*-statistics, *p values* and partial eta (η_p^2) to check for statistical significance and interpret effect sizes.[60] Follow up pairwise comparisons were made with Bonferroni corrections, presented as means, standard errors, and 95% confidence intervals with $p<0.05$ representing statistical significance.

The ordinal data on patient reported outcome measures were assessed with non-parametric ANCOVA (Quade's test), adjusting for baseline values as covariates.

7.4 Results

82 participants with a mean age of 63.5±8.3 years were allocated to supplements and 74 completed the study (Figure 7-1). The most common reasons for discontinuation were disliking the supplement taste (n=2) and private matters reported as unrelated to the study (n=2). Unless otherwise stated, the exploratory results are reported in the untransformed form following per protocol data analyses of adherent completers (without any data assumed to be erroneous and removed during data cleaning), based on the consistency of findings after executing the sensitivity analysis protocol.[61]

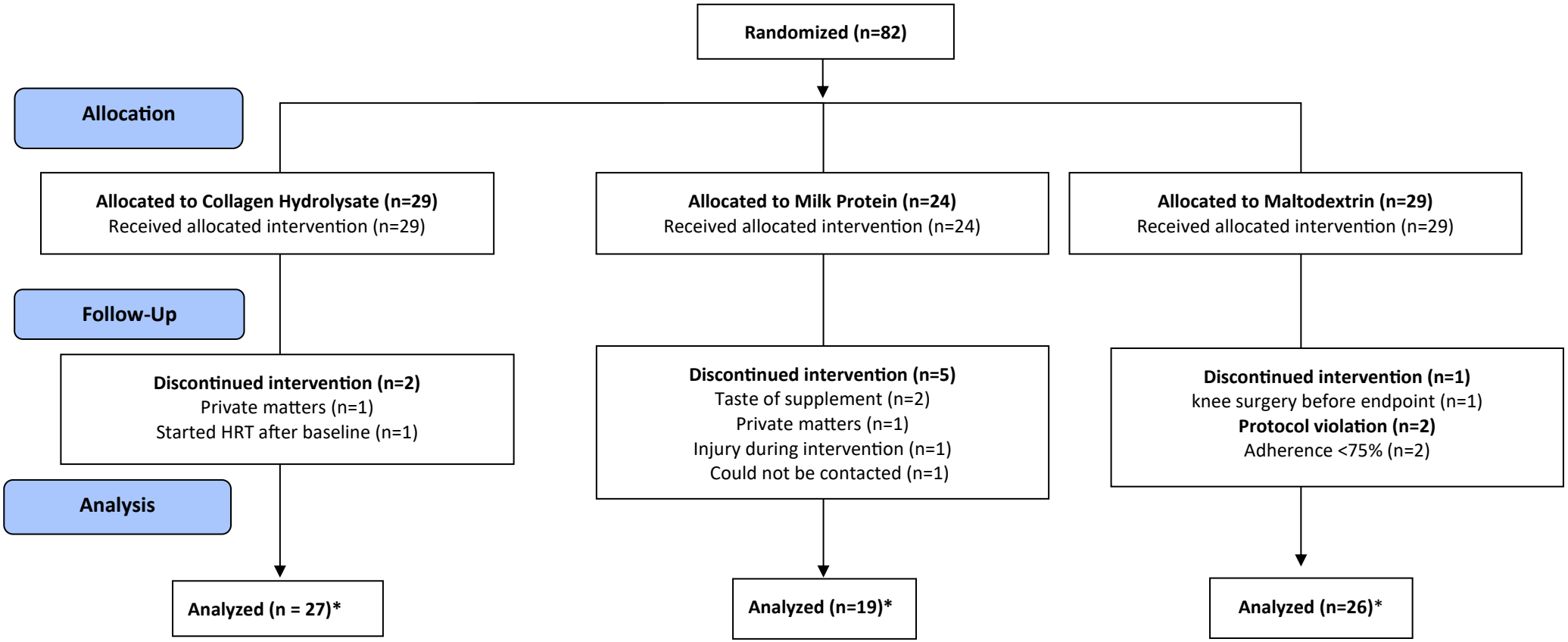

Figure 7-1. CONSORT diagram of participant flow from randomization to data analyses.

* Not all of the per protocol data were examined due to missing data and outlier removals as indicated in the ensuing results tables.

7.4.1 Dermal Thickness (DT)

7.4.1.1 Ventral (inner) forearm

Table 7-2 shows a statistically significant interaction between time and supplement groups for the upper ventral forearm DT (F(2.000, 62.000) = 3.593, $p = 0.033$, η_p^2 = 0.104), but not the lower ventral forearm (F(2.000, 64.000) = 2.590, $p=0.083$, η_p^2 = 0.075). There were statistically significant changes in DT between baseline and endpoint for both regions (upper ventral forearm: F(1.000, 62.000) = 5.325, $p=0.024$, η_p^2 = 0.079, lower ventral forearm: F(1.000, 64.000) = 9.335, $p=0.003$, η_p^2 = 0.127), but the increased means within each group were only significant for CH (Table 7-2).

7.4.1.2 Dorsal (outer) forearm

There was an interaction between time and supplement groups for the upper dorsal forearm DT (F(2.000, 62.000) = 3.336, $p=0.042$, η_p^2 = 0.097). It was not significant with ANOVA of the log10 transformed data (Table 7-2), nor was there an interaction between time and supplement groups for the lower dorsal forearm (F(2.000, 58.000) = 1.191, $p=0.311$, η_p^2 = 0.039). Statistically significant changes in DT between baseline and endpoint were observed at the upper (F(1.000, 62.000) = 13.217, $p<0.001$, η_p^2 = 0.176) and lower dorsal forearm (F(1.000, 58.000) = 11.364, $p=0.001$, η_p^2 = 0.164), but the increased means for the upper dorsal forearm within each group were only significant for CH (mean change: 87.9±20.6, 95% CI: 46.6, 129.1 $p<0.001$) and MDx (mean change: 53.6±22.4, 95% CI: 8.7, 98.4 $p=0.002$). The increase in means within each group for the lower dorsal forearm were significant for CH (mean change: 65.5±31.9, 95% CI: 1.6, 129.5 $p=0.045$) and MDx (mean change: 106.2±32.7, 95% CI: 40.8, 171.6 $p=0.002$), but only MDx after ANOVA of the log10 transformed data during sensitivity analysis as indicated in Table 7-2.

7.4.1.3 Left cheek

There was no interaction between time and supplement groups for the cheek DT (F(2.000, 52.000) = 0.144, $p=0.866$, η_p^2 = 0.006). There was a significant change in DT between baseline and endpoint (F(1.000, 52.000) = 28.351, $p<0.001$, η_p^2 = 0.353), and the increased means within all three supplement groups were statistically significant (Table 7-2).

Table 7-2. Mean dermal thickness (DT) for each supplement group by measurement site.

Dermal Thickness (µm)	Supplement Group													
	Collagen Hydrolysate (CH)					Milk Protein (MP)				Maltodextrin (MDx)				
	n=	Mean	SE	95% CI		n=	Mean	SE	95% CI	n=	Mean	SE	95% CI	
Lower Ventral Forearm	24					17				26				
Time*Group	$F(2.000, 64.000) = 2.590$, *p=0.083*, $\eta_p^2 = 0.075$													
Time	$F(1.000, 64.000) = 9.335$, ***p=0.003***, $\eta_p^2 = 0.127$													
Baseline		1056.63	34.76	987.19	1126.06		1110.26	41.30	1027.76	1192.76		1072.83	33.39	1006.12	1139.54
Endpoint		1185.82	47.09	1091.75	1279.89		1116.07	55.95	1004.30	1227.84		1135.12	45.24	1044.74	1225.50
Within-group p value		***p<0.001***					*p=0.890*					*p=0.071*			
Upper Ventral Forearm	25					15				25				
Time* Group	$F(2.000, 62.000) = 3.593$, ***p=0.033***, $\eta_p^2 = 0.104$													
Time	$F(1.000, 62.000) = 5.325$, ***p=0.024***, $\eta_p^2 = 0.079$													
Baseline		889.54	27.56	834.45	944.63		955.02	35.58	883.91	1026.14		923.77	27.56	868.69	978.86
Endpoint		983.29	35.15	913.02	1053.55		986.18	45.38	895.47	1076.90		916.93	35.15	846.66	987.19
Within-group p value		***p<0.001***					*p=0.370*					*p=0.799*			
Lower Dorsal Forearm	23					16				22				
Time*Group	$F(2.000, 58.000) = 1.191$, *p=0.311*, $\eta_p^2 = 0.039$													
Time	$F(1.000, 58.000) = 11.364$, ***p=0.001***, $\eta_p^2 = 0.164$													
Baseline		1153.51	36.50	1080.45	1226.58		1223.27	43.76	1135.67	1310.86		1127.84	37.32	1053.14	1202.55
Endpoint		1219.06	44.39	1130.21	1307.91		1252.48	53.22	1145.95	1359.00		1234.08	45.38	1143.23	1324.92
Within-group p value		***p=0.045***[A]					*p=0.449*					***p=0.002***			
Upper Dorsal Forearm	26					17				22				
Time*Group	$F(2.000, 62.000) = 3.336$, ***p=0.042***[B], $\eta_p^2 = 0.097$													
Time	$F(1.000, 62.000) = 13.217$, ***p<0.001***, $\eta_p^2 = 0.176$													
Baseline		1167.53	44.43	1078.72	1256.34		1253.16	54.94	1143.33	1362.99		1141.61	48.30	1045.06	1238.16
Endpoint		1255.39	46.27	1162.90	1347.88		1256.25	57.22	1141.87	1370.64		1195.17	50.30	1094.62	1295.72
Within-group p value		***p<0.001***					*p=0.904*					***p=0.020***			
Cheek	19					14				22				
Time*Group	$F(2.000, 52.000) = 0.144$, p=0.866, η_p^2 =0 .006													
Time	$F(1.000, 52.000) = 28.351$, ***p<0.001,*** $\eta_p^2 = 0.353$													
Baseline		1267.81	34.37	1198.84	1336.78		1274.82	40.04	1194.47	1355.16		1252.63	31.94	1188.53	1316.72
Endpoint		1399.48	35.46	1328.32	1470.64		1421.53	41.31	1338.63	1504.43		1366.94	32.96	1300.81	1433.07
Within-group p value[A]		***p=0.002***					***p=0.003***					***p=0.004***			

[A] *p=0.126* (log10 data mean change: 0.02±0.01, 95% CI: -0.01, 0.04). [B] $F(2.000, 62.000) = 2.633$, *p=0.080*, $\eta_p^2 = 0.078$ (log10 data)

7.4.2 Epidermal Hydration (EH)

There were not any interactions between time and supplement groups for the EH measurement sites on the forearm and cheek (Table 7-3), nor were there any statistically significant changes in EH between baseline or endpoint.

Table 7-3. Mean epidermal hydration (EH) for each supplement group by measurement site.

Epidermal Hydration (µS)	Supplement Group													
	Collagen Hydrolysate (CH)					Milk Protein (MP)					Maltodextrin (MDx)				
	n=	Mean	SE	95% CI		n=	Mean	SE	95% CI		n=	Mean	SE	95% CI	
Lower Ventral Forearm	23					16					24				
Time*Group	$F(2.000, 60.000) = 1.726$, *p=0.187*, $\eta_p^2 = 0.064$														
Time	$F(1.000, 60.000) = 1.063$, *p=0.307*, $\eta_p^2 = 0.017$),														
Baseline		125.43	11.29	102.85	148.00		138.61	13.53	111.55	165.68		139.44	11.05	117.34	161.54
Endpoint		135.68	10.08	115.52	155.85		118.64	12.09	94.47	142.81		127.91	9.87	108.17	147.65
Within-group p value		*p=0.363*					*p=0.142*					*p=0.296*			
Upper Ventral Forearm	22					16					24				
Time*Group	$F(2.000, 59.000)=0 .295$, *p=0.746*, $\eta_p^2 = 0.010$														
Time	$F(1.000, 59.000)=0 .180$, *p=0.673*, $\eta_p^2 = 0.003$														
Baseline		147.01	8.36	130.27	163.74		139.07	9.81	119.45	158.70		150.49	8.01	134.47	166.52
Endpoint		155.94	9.56	136.81	175.07		135.45	11.21	113.01	157.88		153.39	9.15	135.07	171.70
Within-group p value		*p=0.405*					*p=0.772*					*p=0.777*			
Lower Dorsal Forearm	24					16					21				
Time*Group	$F(2.000, 58.000) = 1.712$, *p=0.189*, $\eta_p^2 = 0.056$														
Time	$F(1.000, 58.000) = 0.266$, *p=0.608*, $\eta_p^2 = 0.005$)														
Baseline		112.30	10.91	90.46	134.13		90.53	13.36	63.79	117.27		133.59	11.66	110.25	156.93
Endpoint		111.31	9.97	91.35	131.28		104.80	12.22	80.35	129.25		106.24	10.66	84.89	127.58
Within-group p value		*p=0.945*					*p=0.418*				.	*p=0.078*			
Upper Dorsal Forearm	24					19					22				
Time*Group	$F(2.000, 62.000) = 0.432$, *p=0.651*, $\eta_p^2 = 0.014$														
Time	$F(1.000, 62.000) = 1.357$, *p=0.247*, $\eta_p^2 = 0.022$)														
Baseline		114.50	10.92	92.67	136.32		114.22	12.27	89.69	138.76		132.60	11.41	109.80	155.39
Endpoint		111.58	9.04	93.50	129.66		109.25	10.16	88.93	129.57		114.25	9.45	95.37	133.13
Within-group p value		*p=0.813*					*p=0.719*					*p=0.157*			
Cheek	18					11					16				
Time*Group	$F(2.000, 42.000) = 1.735$, *p=0.189*, $\eta_p^2 = 0.076$														
Time	$F(1.000, 42.000) = 0.003$, *p=0.958*, $\eta_p^2 < 0.001$														
Baseline		182.35	12.47	157.17	207.52		190.55	15.96	158.34	222.75		202.24	13.23	175.53	228.94
Endpoint		181.01	13.76	153.25	208.77		214.48	17.60	178.97	249.99		181.14	14.59	151.70	210.59
Within-group p value		*p=0.927*					*p=0.206*					*p=0.179*			

7.4.3 Viscoelasticity (VE)

Table 7-4 shows there were not any interactions between time and supplements for VE in the ventral forearm (F(2.000, 56.000) = 0.580, $p=0.563$, η_p^2 = 0.020) or cheek (F(2.000, 53.000) = 0.492, $p=0.614$, η_p^2 = 0.018).

There was a statistically significant change in ventral forearm VE between baseline and endpoint (F(1.000, 56.000) = 4.837, $p=0.032$, η_p^2 = 0.080), but the decreased means within each group were not statistically significant (Table 7-4). There was also a significant change in cheek VE between baseline and endpoint (F(1.000, 53.000) = 6.816, $p=0.012$, η_p^2 = 0.114). The decrease in means within each group was statistically significant for CH (mean change: -1.6±0.8, 95% CI: -3.2, -0.1 $p=0.039$) but not MP or MDx as shown in Table 7-4.

Table 7-4. Mean viscoelasticity (VE) for each supplement group by measurement site (ventral forearm and right cheek).

Viscoelasticity (MPa)	Supplement Group													
	Collagen Hydrolysate (CH)					Milk Protein (MP)					Maltodextrin (MDx)				
	n=	Mean	SE	95% CI		n=	Mean	SE	95% CI		n=	Mean	SE	95% CI	
Ventral Forearm	23					15					21				
Time*Group	$F(2.000, 56.000) = 0.580$, *p=0.563,* $\eta_p^2 = 0.020$														
Time	$F(1.000, 56.000) = 4.837$, ***p=0.032***, $\eta_p^2 = 0.080$)														
Baseline		8.88	0.78	7.31	10.45		7.91	0.97	5.97	9.85		7.89	0.82	6.25	9.53
Endpoint		7.73	0.79	6.15	9.31		6.09	0.98	4.13	8.04		7.47	0.83	5.82	9.13
Within-group p value		*p=0.162*					*p=0.074*					*p=0.621*			
Cheek	21					15					20				
Time*Group	$F(2.000, 53.000) = 0.492$, *p=0.614*, $\eta_p^2 = 0.018$														
Time	$F(1.000, 53.000) = 6.816$, ***p=0.012***, $\eta_p^2 = 0.114$														
Baseline		4.14	0.78	2.57	5.70		4.57	0.92	2.72	6.43		5.98	0.80	4.37	7.58
Endpoint		2.50	0.67	1.16	3.85		4.03	0.79	2.44	5.62		4.41	0.69	3.03	5.79
Within-group p value		***p=0.039***					*p=0.554*					*p=0.053*			

7.4.4 Patient-Reported Outcome Measures

After controlling for baseline values, there were no significant differences within, or between groups for any of the 12 self-reported statements about skin condition (Table 7-5).

Table 7-5. Patient reported outcome measures of the 12 statements for self-assessment of skin condition at baseline (BL) and endpoint (EP).

Statement (0 = disagree, 10 = agree)	**Supplement Group**														
	Collagen Hydrolysate					**Milk Protein**					**Maltodextrin**				
	n=	Mean	SD	Min.	Max.	n=	Mean	SD	Min.	Max.	n=	Mean	SD	Min.	Max.
BL: My skin feels flexible	19	7.2	2.3	3	10	18	5.8	2.6	2	10	22	5.9	2.4	2	10
EP: My skin feels flexible	19	6.7	2.2	3	10	18	6.9	1.7	4	10	22	6.3	1.9	3	9
Quade ANCOVA[A]	*F*(2, 58)= 1.478 *p=0.237*														
BL: My skin feels hydrated	23	5.7	2.8	0	10	17	5.24	2.49	2	10	24	4.8	2.1	1	10
EP: My skin feels hydrated	23	6.4	2.3	2	10	17	5.94	1.92	3	9	24	5.5	1.9	2	9
Quade ANCOVA[A]	*F*(2, 63) = 0.274 *p=0.761*														
BL: My skin looks droopy/saggy	22	5.0	3.2	0	10	19	6.3	2.0	3	10	22	7.0	2.4	2	10
EP: My skin looks droopy/saggy	22	5.0	2.9	0	10	19	5.1	2.2	2	9	22	5.7	2.5	0	10
Quade ANCOVA[A]	*F*(2,61) = 0.266 *p=0.767*														
BL: My skin feels firm	22	5.1	2.0	2	10	19	4.5	2.0	3	10	20	4.3	2.3	0	8
EP: My skin feels firm	22	5.0	2.1	2	10	19	5.7	1.5	4	8	20	4.6	1.9	0	8
Quade ANCOVA[A]	*F*(2, 60) = 2.457 *p=0.094*														
BL: I have obvious crow's feet	24	6.9	2.6	0	10	17	7.0	2.1	2	10	24	8.3	1.7	5	10
EP: I have obvious crow's feet	24	6.2	2.3	1	10	17	6.2	2.6	1	10	24	7.3	1.8	3	10
Quade ANCOVA[A]	*F*(2, 63) = 0.338 *p=0.714*														
BL: I have obvious facial wrinkles	24	6.6	2.9	0	10	16	6.8	2.0	3	10	24	7.96	1.85	5	10
EP: I have obvious facial wrinkles	24	6.5	2.7	1	10	16	5.8	2.3	1	10	24	7.17	1.76	3	10
Quade ANCOVA[A]	*F*(2,63) = 1.548 *p=0.221*														
BL: My skin looks smooth	20	4.4	2.7	0	9	19	4.6	2.5	1	10	23	3.3	2.2	0	10
EP: My skin looks smooth	20	5.1	2.7	1	10	19	5.5	1.7	3	10	23	4.4	2.1	0	8
Quade ANCOVA[A]	*F*(2, 61) = 0.414 *p=0.663*														
BL: My skin appearance is good	22	5.7	2.7	1	10	15	4.7	2.2	2	10	24	4.2	2.3	0	10
EP: My skin appearance is good	22	6.3	2.2	2	10	15	6.1	1.8	3	10	24	5.1	2.1	0	8
Quade ANCOVA[A]	*F*(2, 60) = 0.971 *p=0.384*														
BL: My skin complexion is even	22	4.9	2.6	1	10	17	4.4	2.8	1	10	22	3.3	2.0	0	8
EP: My skin complexion is even	22	5.2	2.5	2	10	17	4.8	2.2	1	10	22	4.5	2.2	1	10
Quade ANCOVA[A]	*F*(2, 60) = 0.284 *p=0.754*														
BL: My skin feels soft	20	7.1	1.9	3	10	17	5.5	2.4	2	10	24	6.0	2.1	0	10
EP: My skin feels soft	20	6.5	2.3	2	10	17	6.7	1.8	3	10	24	6.3	1.6	3	9
Quade ANCOVA[A]	*F*(2, 60) = 1.348 *p=.268*														
BL: My skin looks younger than expected	20	5.0	2.4	0	9	17	5.1	2.6	1	10	21	4.6	2.6	0	9
EP: My skin looks younger than expected	20	5.9	2.3	1	10	17	5.5	2.1	2	10	21	4.9	2.4	1	10
Quade ANCOVA[A]	*F*(2, 57) = 0.844 *p=0.435*														
BL: I am happy with my skin's appearance	21	5.8	3.0	0	10	18	4.9	2.5	2	10	21	4.1	2.5	0	10
EP: I am happy with my skin's appearance	21	6.0	2.5	2	10	18	5.6	2.4	3	10	21	5.7	2.3	1	10
Quade ANCOVA[A]	*F*(2, 59) = 0.947 *p=0.394*														

[A] *Analysis performed with complete data only (i.e. baseline and endpoint values), adjusting for baseline.*

7.4.5 Adverse events

Supplementary Table 9-5 (Appendix 3) shows general gastrointestinal discomfort was noted once by a participant consuming CH, twice by one participant in the MP group and seven times by participants in the MDx group (two participants in the MDx group reported that complaint on two occasions between baseline and endpoint). Heartburn was specifically reported by two participants who consumed CH, and one participant in the MDx group reported heartburn twice (i.e. at months 1 and 2). Weight gain was reported by two participants in the MP group and two from the MDx group. Presumably unrelated conditions including an itchy eyelid, Covid-19 cough, cholecystitis and gastrointestinal issues self-attributed to other foods or illnesses were reported by 13 participants (CH: n=6, MP: n=3, MDx: n=4).

7.5 Discussion

7.5.1 Primary objective

The primary objective in this exploratory aspect of the CHAMP study was to report on the effect of 15 g protein as CH on dermal thickness (DT). Evaluating the effect of CH on epidermal hydration (EH) and viscoelasticity (VE) were secondary objectives as DT affects VE which is also influenced by EH.[22,62] Sun-protected (ventral) and exposed (dorsal) areas of the upper and lower forearm, and the sun-exposed cheek were compared between groups consuming CH or the isoenergetic and isoproteic comparators of milk protein (MP), and maltodextrin (MDx). A key finding is that DT significantly increased at the upper and lower ventral forearm regions within participants consuming CH, but not MP or MDx. Meanwhile, DT of the upper and lower dorsal forearm significantly increased within the CH and MDx groups, and DT of the cheek significantly increased within all three groups. Collectively, these results are inconclusive and warrant further investigation.

Research on the effect of CH from terrestrial mammals on DT is scant.[43,63] Guadanhim et al.[63] studied women with dermatoporosis which is a recognized medical condition of thin, fragile skin. They found no effect from consuming CH on DT at the inner forearm,[63] nor did Žmitek et al.[43] for DT of the cheek among their healthy female participants aged 40-65 years. The oral doses of CH consumed by

participants in both studies were approximately 3-fold lower than the present CHAMP study, and they were either taken for six months alongside topical application of a lotion containing CH or placebo,[63] or taken for 16 weeks with 80 mg vitamin C and 30 mg HA as extra ingredients.[43] The CHAMP study participants received a supply of multivitamins for daily consumption over their 17-week intervention, but its vitamin C content was approximately half of what participants consumed in the study by Žmitek et al.[43]

Vitamin C is a rate-limiting cofactor for collagen synthesis.[17] It is also a powerful antioxidant,[8,64,65] and oxidative stress on dermal fibroblasts (DF) is more profound within sun-exposed regions of the body (e.g. dorsal forearm and cheek) than sun-protected areas such as the ventral forearm.[1,2,6,8-10] It has been speculated that sun-protected regions are less responsive to interventions,[35] but the existing CHAMP study data does not support that notion. For instance, DT only increased at the upper and lower ventral (sun-protected) forearm regions for the group who consumed CH. The finding aligns with animal research where CH supplementation increased the diameter and density of collagen fibrils in the dermal layers of pigs and mice.[28,30] However, significant improvements in DT of the sun-exposed forearm within the CH and MDx groups, and sun-exposed cheek within the CH and both comparator groups are perplexing alongside the secondary objectives investigating the effect of CH on EH and VE.

7.5.2 Secondary objectives

There were not any significant differences between or within groups for EH or VE. These parameters have reportedly improved with supplementation of CH sourced from terrestrial mammals versus placebo in some studies,[41,42,66-68] but not others.[41-43,63,67,68] Moreover, CH supplements and comparators have both evoked significant improvements compared to baseline too.[66-69] Overall, the existing study results add to this inconsistent body of evidence on the effect of CH on EH and VE which may demonstrate the sensitivity of these variables to confounding factors.

Most of the studies which measured elasticity as a variable used a Cutometer.[41,42,66] Unlike the Dermalab used in the existing study to measure VE, the Cutometer does not account for DT, even

though DT affects the elevation and retraction phases of the strain/time response being measured.[44-47] As such, the elasticity metric from a Cutometer may be less reliable than VE measured from a Dermalab device.[22] Žmitek et al.[43] used a Dermalab in their study, and they found no difference in EH or VE between the CH supplement and placebo which is consistent with the results observed in the existing CHAMP study data. Therefore, these secondary outcomes of EH and VE may suggest the three supplements were ineffective at slowing the effects of aging (intrinsic and photoaging) on the ventral or dorsal forearm and cheek, but the data also illustrates the complexities of monitoring skin condition over time due to confounding factors.

7.5.3 Confounding factors

Skin condition is a multi-parametric variable due to its anisotropic structure.[54,70] Its VE property depends on DT and EH,[44-47] but EH is also affected by environmental conditions,[71-73] and lifestyle factors including an individual's use of moisturizers (promoting hydration), cleaning detergents and laundry powders (causing skin dehydration), and their total daily fluid intake.[64,74-76] Essentially, VE and EH are more sensitive to confounding factors than DT, and they are not always controlled or monitored which may help explain the mixed results from research to date, including the existing CHAMP study data. Therefore, its limitations are important to acknowledge as a caution before interpreting the results which could be useful for guiding future research on this topic.

7.5.4 Limitations

Room temperature and relative humidity (RH) during data collection of the CHAMP study were not measured as variables or controlled within a chamber as suggested by Tagami.[77] The participants' baseline and endpoint visits to the HNRU occurred at the same time of day, but in different seasons considering the intervention was four months (17 weeks). Palmerston North has a mean monthly air temperature of 8-18^{o}C and RH of 77-88%,[78] but outdoor readings do not correlate with those taken indoors.[79] Furthermore, skin is drier in cooler months when RH is lower and the air will absorb more moisture from skin when the air is artificially heated for comfort.[79,80] Consequently, seasonal variation plausibly impacted on the collection of EH and VE data.[72,73,80,81]

Participants' skin types (i.e. melanin content) were not assessed nor were their ethnicity data collected.[82] Most participants appeared to be Caucasian, presumably with skin types I-III on the Fitzpatrick scale indicating a lower concentration of melanin than skin types IV to VI.[82] Melanin provides natural protection from the sun, but the participants' use of sunscreen (i.e. artificial protection) or their time spent outdoors were not monitored so their levels of photoaging over the intervention were uncontrolled.[2] Moreover, their use of moisturizers were not monitored or controlled in accordance with earlier research,[46,63] even though moisturizer improves EH and an individual's habitual use (i.e. brand, amount and days of use) is highly variable and unique to that person.[74] The CHAMP Study participants were asked to avoid using makeup or lotions on the mornings of their data collection visits, but in cases of non-adherence, they were given alcohol-free makeup removal wipes for cleaning their skin before the DT, EH and VE measurements (i.e. during their ≥30 minutes acclimatization period so the cleaned skin could dry). This protocol risked unintentional invalidation of data, considering EH can be expected to change before and after using wet pads on skin, even after drying off any visible leftover moisture.[52,70]

Finally, the per protocol data analyses with split-plot ANOVA were affected by missing data. Outliers were removed during data cleaning, and it were assumed as erroneous (reflecting non-adherence to the guideline of arriving without wearing makeup or lotions) if it fell outside a reference range of published norms.[22,43,46,83-85] Missing data also arose through technical issues with the Dermalab device which have been encountered in similar studies.[46] The machine has good inter-rater reliability,[46] and measurements were made on the dorsal and ventral forearm sites guided by a template for standardized readings at baseline and endpoint. However, the cheek region was measured by sight, so future studies would benefit by designing and using a template for more standardized measurements there too.

7.6 Conclusion

This was a report on secondary outcomes of the CHAMP study. Its aim was to examine the effect of CH sourced from bovine hide on skin condition versus isoproteic and isoenergetic

comparators. Dermal thickness (DT) was of primary interest because it moderates epidermal hydration (EH) and viscoelasticity (VE). There were no interactions between the supplements and time for the secondary measures of EH or VE, but there were significant interactions between the supplements and time for DT at the upper ventral (inner) and dorsal (outer) forearm regions. Only CH was associated with a statistically significant increase in DT of the sun-protected ventral forearm, but all three supplements increased DT of the cheek regions, while CH and MDx increased DT at the sun-exposed forearm. There were no changes in patient-reported outcome measures, and the three supplements generally performed similarly over time within each group for the secondary outcome measures of EH and VE. Overall, the CH supplement may have shown a superior effect of increasing DT in photoprotected skin regions, but more research is warranted before a conclusion can be made about the efficacy of CH as a 'nutricosmetic' for skin condition. Future studies would benefit from evaluating the same objective measures under tightly controlled environmental conditions to reduce the impact of confounding factors for EH and VE.

7.7 Chapter 7 references

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Chapter 8: Thesis discussion

This chapter summarizes the CHAMP study. It discusses the main findings of Chapters Four, Five and Seven in relation to existing evidence, and it outlines limitations with the study design to identify future research opportunities before drawing a conclusion to the overall research project.

8.1 Overview

The Collagen Hydrolysate and Milk Protein (CHAMP) study examined the bioactive peptide (BAP) potential of collagen hydrolysate (CH) among women aged ≥50 years with self-reported knee pain (not necessarily due to KOA) and signs of aging skin (Appendix 1). Its primary focus was to evaluate the effect of CH on signs and symptoms of knee pain using subjective and objective data (PROMs, functional tests and biomarkers), while exploring the effect of CH on skin condition (thickness, hydration and viscoelasticity and PROMs) was of secondary importance.

8.1.1 The effects of collagen hydrolysate and milk protein on knee pain and functional performance in a randomized double-blind controlled trial on women aged ≥50 years

Chapter Four reported CH supplementation was no more effective than the two comparators of milk protein (MP) or maltodextrin (MDx) for improving pain or functional performance. Within-group improvements in pain perceptions assessed with KOOS were observed in all three groups, without there being any between-group differences. The finding contrasts with previous research which only assessed PROMs and reported CH supplements were more effective than a placebo for relieving pain.[1-4] There were statistically significant improvements in three out of the five functional tests observed in the CHAMP study data (Timed Up and Go, 30s-Chair Stand and Stair Climb), but the magnitudes of change were less than MDC values for those tests on similar cohorts (Appendix 3: Supplementary Table 9-6). As discussed in Chapter Four, MDC values are the smallest amount of change attributable to interventions,[5-9] so the observed improvements in functional tests were plausibly due to random error rather than true (i.e. objective) change. In that case, the data align with previous research which found no difference between CH and placebo for subjective PROMs and objective outcome measures of soluble biomarkers of inflammation and cartilage turnover or thickness.[10-12] However, the CHAMP study results raised a question of whether four months was enough time for performance measures to have fully developed. Therefore, the chapter validated a pre-planned intention to analyze additional objective data on soluble biomarkers of cartilage turnover (i.e. collagen synthesis and breakdown) and inflammation for a comprehensive investigation.[13,14]

8.1.2 Collagen hydrolysate was ineffective on cartilage turnover and inconclusive for inflammation in a randomized double-blind controlled trial on women aged ≥50 years with knee pain

Monitoring soluble biomarkers allows for the detection of molecular changes in cartilage before structural changes (i.e. cartilage thickness) or performance improvements (i.e. functional tests) may eventuate.[15,16] Chapter Five reported there were no significant differences between the CH, MP or MDx supplements on biomarkers of collagen synthesis (PIIANP) or cartilage breakdown (uCTX-II and COMP), and this is congruent with Bongers et al.[10] who measured uCTX-II and PIICP among relatively healthy men and women aged 56-68 years. Those authors also assessed inflammation markers of TNF-α and IL-6 (but not IL-1β), and they found no effect from CH or placebo which is consistent with research on CH from chicken or fish which measured similar biomarkers of inflammation or cartilage turnover.[17,18] However, that research cannot be compared with the present CHAMP study data.

Almost all of the CHAMP study participants' biomarkers of inflammation (IL-1β and TNF-α) at baseline were below detectable limits for the ELISA assays used in the analyses. As discussed in Chapter Five, possible reasons are that the participants did not have the inflammation phenotype for KOA,[19,20] or the systemic biomarkers lacked specificity considering they were not drawn from the SF of participants' knee joints (which is more invasive and difficult to do in clinical trials).[13,21] Therefore, more research into the effect of CH from terrestrial mammals on biomarkers of inflammation is indicated as discussed in the upcoming recommendations for future research.

8.1.3 The effects of collagen hydrolysate and milk protein on skin condition (thickness, hydration and viscoelasticity) in a randomized double-blind controlled trial on women aged ≥50 years

The research focus shifted to the effect of CH on skin condition in Chapters Six and Seven. Chapter Seven presented evidence that CH may be more effective than MP and MDx for improving dermal layer thickness in areas generally protected from sunlight (i.e. inner/ventral forearm). The data contrasts with a speculation by de Miranda et al.[22] that photoprotected areas may be insensitive to interventions such as CH, but the results from skin regions more commonly exposed to sunlight (i.e. cheeks, outer/dorsal forearm) were less clear. All three supplements equally improved the primary

outcome measure of dermal thickness at the cheek (and outer/dorsal forearm for CH and MDx), but there were not any between or within group changes in the secondary outcomes of hydration or viscoelasticity in the photoprotected ventral forearm or photoexposed dorsal forearm and cheek. However, epidermal hydration affects viscoelasticity, and both parameters are sensitive to confounding factors (e.g. environmental conditions and use of skin lotions) which may explain why no effect was observed in the secondary outcome measures.[23-25] The inner/ventral (i.e. photoprotected) forearm is recommended for studies of hydration,[26] so future research may benefit by focusing on that area alone to measure outcomes of hydration and viscoelasticity.[24,25]

8.2 Limitations

There were limitations to the CHAMP study which are necessary to acknowledge before drawing a conclusion to this thesis.

8.2.1 Recruitment

8.2.1.1 Sample size and statistical analyses

The number of participants in the CHAMP study (Chapters Four, Five and Seven) was arguably small. A sample size of 27 per group was needed to attain power of 90% and a Type I error rate of 5% as calculated by G*Power (version 3.0.10) to detect change in the primary outcome measure of pain.[27] In total, 29 participants were recruited and randomly assigned to receive the CH and MDx supplements respectively, but only 24 were recruited and assigned to MP. After accounting for dropouts (CH: n=2, MP: n=5, MDx: n=1), exclusion of participants with adherence rates <75% (MDx: n=2), and missing data from partially completed monthly surveys, the final number within the MP (n=19) and MDx (n=26) groups with complete data were lower than necessary for statistical power.

Per protocol (PP) data analyses were originally performed with Linear Mixed Models (LMM). The method managed unequal group sizes and prevented the listwise deletion of cases using alternative options, such as the mixed-model (split-plot) analysis of variance (ANOVA),[28-32] but it avoids intention to treat (ITT) analyses. Therefore, the Expectation-Maximization (EM) technique was used to impute missing values in SPSS (version 29, IBM SPSS statistics), and a sensitivity analyses model was

applied for comparing ITT with PP data analyses to validate the results from split-plot ANOVA (with the Greenhouse-Geisser correction to overcome unequal group sizes) in Chapters Four and Five.[33-36] However, the skin condition data reported in Chapter Seven were based solely on PP analysis without imputing missing values with the EM technique. It was an exploratory aspect of the project, and the reliability of data was compromised by suspected erroneous measurements as discussed in that chapter (i.e. technical issues with the Dermalab device or participants failing to effectively remove any makeup they were wearing). Overall, the statistical analyses methods employed in this thesis were evidence-informed to manage the limitation of a marginally undersized sample size arising from human resource constraints during the recruitment and data collection window (e.g. sickness of research team members or participants).

8.2.1.2 Participant characteristics

Stage of disease

An issue with existing diagnostics is that KOA is well-established before it can be confirmed.[37,38] By that time, the capacity of chondrocytes to maintain homeostasis in cartilage turnover is diminished, and the cascade of deleterious events causing progression have become irreversible.[39-41] However, the pace of progression is non-linear,[21,42] and undiagnosed cases of KOA are prevalent among individuals with multiple risk factors for its onset such as peri- and postmenopausal women with excess body fat stores.[37,43] Therefore, including participants who self-reported knee pain, with or without a confirmed medical diagnosis of KOA (i.e. KL Grade ≥II), may have affected the integrity of data presented in Chapters Four and Five. For instance, the participants were probably at different stages of the disease,[37] but individuals at an early, pre-radiographic change-stage (i.e. KL Grade <II) may respond better to nutraceuticals than the later stages when chondrocyte viability has declined.[37,44] With circumstances permitting (i.e. time, finance, equipment, and sampling population), future research could focus on stricter eligibility criteria so only early stages of KOA are included and later stages excluded.[45] Doing so could provide a more homogenous cohort for investigating the effect of CH on cartilage turnover before the mechanisms of breakdown are too far advanced.[46-49]

Phenotype

Low-grade inflammation is universal in cases of KOA, but IL-1β is not the only driver of its progression.[50] This could explain why most of the CHAMP study participants did not have detectable levels of inflammation at baseline, if not because blood samples were analyzed rather than samples directly from the SF. For instance, there are at least six phenotypes of KOA, and they are each underpinned by their own biological endotype.[16,51,52] Phenotypes are observable characteristics,[46] and Dell'Isola et al.[19] found in their meta-analysis that the prevalence of participants with the inflammatory phenotype ranged from 16-30%. Of those afflicted participants, only a sub-group were observed as having over-expressed proinflammatory cytokines (i.e. IL-1β), reported higher baseline pain levels and progressed in disease severity faster than the participants who under-expressed the same pro-inflammatory cytokines.

The CHAMP Study participants were not assessed nor classified by KOA phenotype at baseline. Heterogeneity in phenotypes is likely,[19] and an unknown number of participants may have been defined by the chronic pain or minimal joint disease phenotypes which have different etiologies and may be more likely to respond to interventions of cognitive behavioral therapy or exercise than nutraceutical interventions.[19] As such, an additional criterion of recruitment for future research exploring the effect of CH on inflammation could be to exclude participants without detectable levels of inflammation biomarkers at baseline. A preliminary screening visit would be needed so blood samples could be drawn and analyzed to confirm inclusion or exclude further participation (e.g. in the preceding week of baseline), assuming all laboratory analyses could be completed within days of each potential participant's screening visit. However, this 'ideal' scenario would prolong the time to achieve recruitment of eligible participants (especially in smaller regions such as the Manawatū in NZ), and would place more strain on laboratory resources in contrast to testing all baseline and endpoint samples at the same time as suggested by the OARSI Biomarkers Working Group.[53,54] Therefore, a multi-center study would be worthwhile to optimize recruitment.

8.2.2 Data collection

Data for the CHAMP study (Chapters Four, Five and Seven) were collected from participants attending their baseline and endpoints visits at the Human Nutrition Research Unit (HNRU). There was an order of activities (stations) for time management and consistency (Appendix 6), but some of those stations were affected by limitations.

8.2.2.1 Subjective data

Patient Reported Outcome Measures (PROMs)

The PROMs were collected with questions adopted from KOOS.[55] It is a validated self-administered tool which offers more insight into a respondent's perceived burden from KOA than alternative tools such as WOMAC.[56] However, some questions are irrelevant to individuals with symptoms of KOA.[57,58] For example, a respondent may avoid activities in the sport and recreation domain (e.g. running or squatting) due to their fear of causing pain. Furthermore, they may own a shower rather than bath or live in a single-storey home without ever encountering stairs in their activities of daily living (ADLs). To account for these possibilities, the "n/a" response was added into the monthly CHAMP study survey, but doing so caused missing data as discussed in Chapter Four (i.e. non-scored "n/a" responses led to invalid scores). Ideally, KOOS would have an option for respondents to indicate not having engaged in certain activities rather than skipping the question or recording a score which indicates no pain was felt as per current scoring guidelines.[56] Therefore, future researchers may like to refine KOOS to capture PROMs more effectively when respondents did not do the activity in question for whatever reason.

Participants' opinions about their responses to treatment were not collected and evaluated. Comparing pain perceptions at endpoint with baseline through an anchor question allows estimation of an MCID psychometric (*e.g. compared to before the trial, how much pain do you feel now: 1=none, 2=much less, 3=a little less, 4=no difference, 5=a little more, 6=a lot more*).[59] Calculating an MDC value alongside the MCID improves data interpretation, as both values are useful for determining if the perceived changes in pain scores were likely to reflect true and meaningful change (i.e. MCID values exceed the MDC) or fall within the range of sampling error (i.e. MCID values are less than the

MDC).[6,7,59] A PASS (patient acceptable symptom score) could also be calculated to assess participants' feelings about their supplement being effective enough to consider it helpful or not.[60,61]

8.2.2.2 Objective data

Pain medication

'Pain diaries' were provided to monitor the participants' use of rescue medications over their intervention. Some of the returned diaries at endpoint were very detailed (e.g. days of use were marked with the medication, its dose and frequency with days of non-use struck out), but others were relatively unclear. For example, some were returned completely blank which could mean the participant did not use any rescue medication or they did not have time for whatever reason to indicate when they had. Similarly, some diaries may not have been returned because pain medications were never used (i.e. there was no felt need to record anything and the pain diary was disposed of), or they were misplaced at home before the end visit. Time and human resource pressures at the endpoint data visits (e.g. hosting and managing two-three participants on one day) prevented researchers from consistently being available to question each participant and clarify their pain medication data, so the recorded improvements in PROMs or functional tests discussed in Chapter Four may be partly attributable to the use of analgesics.[62] In future, an extra question in the monthly online survey could quantify this metric more reliably (*e.g. In the previous month, have you used any medication for you knee pain?).*

Dietary and lifestyle behavior

Omission of the 4DDR data impacted on the reliability of results for this project. The participants' food records were intended to verify a request to keep dietary and lifestyle behaviors constant was adhered to, as discussed in Chapter Four. Without any 4DDR data analyses, all participants were assumed to have maintained their usual diet and exercise patterns as requested, even though dietary intake can change when participating in a clinical trial (i.e. the Hawthorne effect).[63] In future, a research team member could be designated to conduct a diet history or several 24-hour diet recalls with each participant at baseline and endpoint to mitigate the time-commitment

of self-reporting a 4DDR at home. The researcher could also ask a closed-ended question to check that exercise remained the same. However, diet histories also have limitations including desirability bias and underreporting which means collecting dietary data in general is relatively unreliable and remains an estimate at best.[64]

A system for monitoring adherence to the multivitamin was also overlooked when planning for data collection. It was given to all participants for daily consumption to reduce the possibility that micronutrients required for collagen synthesis were rate-limiting (i.e. vitamin C and iron). Some of its ingredients have antioxidant or anti-inflammatory properties (i.e. vitamins A, C, E and minerals such as zinc and selenium),[65-67] so they may have partially alleviated oxidative stress which exacerbates degradation of collagen fibers in the skin and articular cartilage.[68-71] Therefore, it is possible that the multivitamin contributed to the statistically significant within-group but not between-group improvements observed in dermal thickness of the cheek for all three supplement groups as reported in Chapter Seven. If the CHAMP study were repeated, asking participants to return their supply of multivitamins and counting the number consumed versus unused would quantify the adherence rates to be considered a covariate in statistical analyses, rather than assuming all participants were 100% adherent (despite several informal reports of non-adherence at endpoint, with reasons including a belief their nutritional status was fine without any multivitamin, or they forgot to pack their supply when traveling away from home).

Biomarker analysis

The analyses of biomarkers for inflammation and collagen synthesis were performed by an accredited external laboratory. Collagen synthesis was measured with PIIANP, but ELISA assays for PIIBNP or CPII were unavailable. Monitoring PIIANP is complicated by the fact it depends on the stage of disease, and whether an individual is a progressor or non-progressor of KOA.[21] It does not reflect total collagen synthesis as PIIBNP needs to be measured too,[72] if not PIICP as an alternative to PIIANP and PIIBNP.[73,74] With circumstances permitting, future studies would use ELISA assays for PIIANP and

PIIBNP (or PIICP instead) to reevaluate the effect of CH on cartilage turnover compared to the MP and MDx controls more completely.

Skin condition assessment

Skin photoaging (unprotected exposure to sunlight) is the most prominent modifiable factor of extrinsic aging which exacerbates intrinsic aging.[65,67,69] Melanin within the epidermis provides the skin with natural protection from photoaging,[65,75-77] and sunscreen is an artificial means assuming an appropriate sun protection factor (SPF) is applied regularly.[78] Skin phototyping with the Fitzpatrick scale enables an estimation of melanin content,[79] but it was not used for categorizing the CHAMP study participants (Chapter Seven), nor was their use of sunscreen assessed or controlled in any way like Maia Campos et al.[80] attempted to do.

In their Brazilian study of 5 g/d CH versus MDx on skin condition, Maia Campos et al.[80] provided participants with a standard SPF 60 lotion for daily use during an intervention of 90 days. However, there was no certainty that each participant used the sunscreen, or that their level of sunlight exposure remained constant. Similarly, the levels of sun exposure for CHAMP study participants presumably varied depending on their ADLs (e.g. regularly indoors versus often outdoors for recreation or work) and their months of involvement (i.e. during autumn/winter versus spring/summer). As such, photoaging of their skin was uncontrolled as an exposure variable which means some participants may have experienced more oxidative stress induced by extrinsic aging of their skin than others.[81] Future research could assess skin types for presenting as baseline data by supplement group, and extra questions in the monthly survey could evaluate the participants' levels of sunlight exposure (e.g. usual hours spent outside in the past month, and their routine with applying sunscreen). Subsequently, participants could be stratified by skin type and level of sun exposure to enable more focused statistical (sub-group) analyses.[65,82]

The Dermalab Combo was used for the first time in this study and operator-errors occurred through inexperience. A 'gain' setting of '0' was incorrectly used for measuring dermal thickness of the cheek,[83] and the reliability of data was also affected by participants' non-adherence to the request

of arriving to the HNRU without makeup or lotions which distorted hydration measures.[23,84] In those instances, participants were given facial cleansing wipes for the ventral forearm and cheek before measurements were taken (Appendix 7), but the product contained emollients which influence capacitance readings by reducing water loss from the stratum corneum.[26,84,85] This unintended consequence may have contributed to the variance of hydration data which is also affected by seasonal variation in temperature and relative humidity.[26,86-89] In future, a gain setting of '6' would be set for measuring dermal thickness of the cheek,[83] and ideally all participants would arrive without wearing makeup or lotion as asked in their pre-baseline information. Furthermore, testing would be within a sealed chamber (20°C, 40-50% relative humidity), and participants would rest ≥15 minutes for acclimatization to those conditions before data collection as recommended by Tagami.[26] These steps could reduce the amount of data deemed erroneous during the cleaning process described in Chapter Seven.

8.3 Future directions

8.3.1 Contextual effect

The CHAMP study did not provide evidence of a superior effect from CH in relieving knee pain or improving skin condition compared to MP or MDx. All three supplements produced statistically significant improvements in PROMs associated with KOA (i.e. pain), suggesting the contextual effect was involved to some extent.[90,91] It is largely explained by the placebo effect,[90,92] but participants' experiences when interacting with clinicians or researchers and affect heuristics (i.e. participants' hopes and expectations of their intervention) also have an influence.[91,93] Therefore, the contextual effect is worthy of further study as a treatment option for KOA pain management (irrespective of the active ingredient being evaluated), assuming all ethical allowances are made for transparency between the clinicians/researchers and patients/participants.[90,91] Meanwhile, more research is needed to fully explore the potential mechanisms of action from CH as a nutraceutical for KOA or 'nutricosmetic' for skin condition.

8.3.2 Mechanisms of action

8.3.2.1 Collagen synthesis

The CHAMP study aimed to examine the BAP potential of CH for promoting collagen synthesis in cartilage and skin. This mechanism of action is postulated to occur in target cells (i.e. articular chondrocytes and dermal fibroblasts) after first-pass metabolism.[94] However, a study on mice showed Pro-Hyp preferentially accumulate in the skin rather than articular cartilage after ingestion and absorption.[95] Therefore, the dose of CH may be a rate-limiting factor for its BAP potential in the articular cartilage and skin.

A daily dose of 15 g CH was consumed by participants in the CHAMP Study. A follow-up study on six women aged ≥50 years who consumed one dose of the same oral CH supplement after an overnight fast confirmed Pro-Hyp dipeptides were absorbed intact, two hours after consumption, for presumed distribution to the target cells (Appendix 8). After the CHAMP study data were collected, Devasia et al.[96] reported their study of 115 men and women aged 30-65 years (n = 106 completers) with confirmed KOA (KL Grade II-III), and they compared three oral daily doses (2.5 g, 5 g and 10 g) of CH from a bovine source enriched in Pro-Hyp against a 10 g dose of conventional CH from bovine hide (without Pro-Hyp enrichment) or 10 g placebo over 90 days. The authors examined the effects of each dose on PROMs (WOMAC and VAS scores) and serum CTX-II (cartilage breakdown), and they found the CH supplements all evoked statistically significant ($p<0.05$) improvements in PROMs and serum CTX-II compared to placebo. The 2.5 g dose of enriched CH performed comparably to the 10 g dose of conventional CH in its effect (i.e. it's efficacy improved four-fold), and the magnitude of improvements increased as the doses of CH enriched with Pro-Hyp increased.[96] In that sense, the 10g dose of CH with Pro-Hyp enrichment was presumably higher in BAP potential (i.e. Pro-Hyp content) than the 15 g/d dose of conventional CH consumed orally for four months (117 days) in the CHAMP study (i.e. not enriched with Pro-Hyp). In other words, there may not have been enough Pro-Hyp in the supplement reaching the chondrocytes and dermal fibroblasts for them to exert their full effect of collagen synthesis in the cartilage and skin to override the cascade of deleterious effects of KOA and aging (i.e. senescence and oxidative stress) in these target cells. Therefore, a formal dose-response trial is

recommended to reevaluate the CHAMP Study outcomes.[97] Ideally, covariates of dietary and lifestyle behavior, sun exposure, and multivitamin adherence would be controlled or monitored more effectively than circumstances allowed in the CHAMP study too.[82]

Conducting a longer intervention is also recommended as an outcome of this thesis. The four-month intervention in the CHAMP study was possibly insufficient for functional performance measures to have developed as discussed in Chapters Four and Five.[98] A recent six-month pilot study of 16 participants with KOA (KL Grade II) in Romania found daily oral intake of 10 g CH (unknown animal source and containing 100 mg hyaluronic acid and 230 mg vitamin C) significantly improved ($p<0.001$) range-of-motion (ROM) measured through knee flexion (bending).[99] There was no placebo group for comparison, but Salinas-Camargo et al.[100] also reported recently that ROM significantly ($p<0.05$) worsened within a placebo group of participants with exercise-associated knee pain after their six-week study.[100] Conversely, knee flexion remained stable ($p=0.678$) in the group consuming 10 g CH (unknown animal source and containing other active ingredients), and the difference between groups was reported as approaching significance at the six week endpoint ($p=0.072$). Therefore, these two exploratory findings taken together may indicate at least six months are needed to study objective outcomes on structural and functional performance outcomes in future research.

8.3.2.2 Antioxidant

Oxidative stress is inevitable with intrinsic aging.[101] It is amplified in cases of KOA,[102,103] and augmented by photoaging of skin and excess body fat stores.[104,105] Moreover, oxidative stress upregulates a systemic response of proinflammatory mediators in circulation which exacerbates the progression of KOA and deterioration of skin condition.[39,65,106-108]

Proline is a free radical scavenger.[109] Tripeptides of Gly-Pro-Hyp have consistently demonstrated antioxidant properties of free radical scavenging (i.e. hydrogen atom transfer or single electron transfer), chelation (binding) of pro-oxidant metals for elimination (e.g. iron or copper ions), and inhibition of lipid peroxidation with *in vitro* research;[75,110-112] especially with formulations of CH at higher degrees of hydrolysis (i.e. lower MW) and higher concentrations of Pro-Hyp from terrestrial

mammal sources.[105,113] Therefore, the antioxidant potential of CH supplements (i.e. Pro-Hyp) in humans is a research opportunity to explore.[105,114,115] Yeum et al.[116] have assessed the antioxidant capacity of fat- and water-soluble vitamins (e.g. vitamins C and E) by measuring their rates of consumption within the aqueous and lipid components of human plasma incubated in free radical agents. A future study could adopt and adapt that design,[116] and measure the rate of Pro-Hyp consumption relative to the water and fat-soluble antioxidants to determine its antioxidant capacity. Furthermore, synergistic effects (interactions) between the vitamins and Pro-Hyp may also prove informative to inspect.[116]

There are several micronutrients with antioxidant properties which are reportedly effective for KOA symptom control (e.g. Vit C, E, zinc or selenium).[117,118] Edgar et al.[119] compared the effect of CH (derived from fish) with or without antioxidant ingredients on MMP activity of dermal fibroblasts *in vitro,* and they found CH alone was effective for down regulating MMP-1 and MMP-3 ($p<0.0001$ and $p<0.05$), but even more so in combination with the active antioxidant ingredients ($p<0.01$ and $p<0.05$). Moreover, Carrillo-Norte et al.[120] recently found in their study of 60 men and 60 women with confirmed KOA (KL Grade II-III), that 10 g/d CH (bovine origin) with 80 mg vitamin C consumed orally for six months versus placebo led to statistically significant improvements in biomarkers of inflammation (CRP and erythrocyte sedimentation rate), PROMs (VAS and the Lequesne Index) and self-reported analgesic use. Most participants in the CH group had mild pain at baseline whereas a majority in the placebo group were classified with moderate pain, but even so, the study generally provides support for evaluating the efficacy of CH from terrestrial mammals (with and without antioxidant ingredients versus isoenergetic and isoproteic controls) in a factorial study design to expand on the existing evidence on this potential mechanism of action for CH.[119,120]

Similarly, research on the synergistic effect of CH with or without glucosamine, chondroitin and hyaluronic acid (HA) is another future direction based on some exploratory studies completed after the CHAMP Study data were collected.[17,121,122] These proteoglycans (PGs) are fundamental for the optimal function of the articular cartilage ECM as explained in Chapter 2.2,[123-125] and their

depletion precedes collagen degradation in the early onset and progression of KOA.[126] Naumov et al.[122] reported supplements of CH with glucosamine, chondroitin and HA (doses unspecified) consumed orally for eight weeks significantly improved objective measures (analgesic use and functional performance tests) and PROMs (WOMAC and VAS) versus a comparator of the same ingredients but without CH in their study of 60 Russian patients (sex unspecified) with KOA (KL Grade II-III). A pilot study of 54 men and women with KOA (KL Grade II-III) allocated to treatment or placebo at a ratio of 2:1 by Fladerer-Grollitsch et al.[17] also found 120 mg CH (chicken cartilage) with glucosamine (700 mg), chondroitin (400 mg), HA (20 mg) and antioxidants (vitamins C, D, E) versus placebo for 12 weeks led to statistically significant improvements in the KOOS domains of symptoms ($p<0.05$), sport and recreation ($p<0.01$) and QoL ($p<0.001$). The pain domain significantly improved for the CH ($p<0.001$) and placebo groups ($p<0.01$) without a difference between groups ($p=0.6649$), as did the ADLs domain, and performances in a Chair Stand but not the Fast-Paced Walk or Stair Climb Tests.[17] Griffel et al.[121] demonstrated in their open, observational, single-arm, multicenter study of 186 Spanish participants with OA confirmed in the knee, hip or ankle or spine, that 3,000 mg CH (unspecified animal source) consumed daily for six months with 800 mg chondroitin, 700 mg glucosamine and antioxidant ingredients such as 70 mg zinc resulted in statistically significant reductions in PROMs (VAS and WOMAC scores) at three ($p<0.001$) and six months ($p<0.001$) compared to baseline, albeit without a placebo group for comparison. The doses of CH were less than the CHAMP study dose of 15 g, but these exploratory findings indicate the inclusion of CH with PGs (glucosamine, chondroitin and HA) in oral supplements is worthy of examining in a future RDBCT.

8.3.2.3 Prebiotic peptide

The prebiotic potential of Pro-Hyp within CH is also an emerging area of research.[127,128] Chronological (intrinsic) aging is associated with a decline in microbial diversity within the gastrointestinal tract,[128-131] but gut microbiota are integral to human health and disease as they moderate acute and chronic inflammatory responses.[127,131-133] In general, prebiotics are resistant to intestinal digestion, and they selectively promote the growth of 'good' (i.e. beneficial) bacteria which

produce short-chain fatty acids (SCFA) with anti-inflammatory and antioxidant properties.[82,127,130,132] Moreover, prebiotic peptides can alleviate oxidative stress and chronic inflammation associated with KOA progression,[82,127,128,132] but the effect from CH depends on the animal source of undenatured collagen (i.e. Pro-Hyp composition) and the degree of hydrolysis (i.e. MW) after extraction.[127,128]

Larder et al.[127] compared two commercially available formulations of CH from bovine hide at a dose of 1.2 g using a computer-controlled gastrointestinal model. They found the formulation of CH with a higher MW (i.e. longer peptide chains) significantly increased SCFA production ($p<0.05$) in the early (ascending) colon within 16 hours of ingestion. There was no effect observed further along the large intestine, nor was there anywhere along the colon for the formulation of CH with a lower MW (i.e. shorter peptide chains) which prompted the authors to surmise that CH has more than one potential mechanism of action.[127] For example, Gly-Pro-Hyp and Pro-Hyp are absorbed into the bloodstream for distribution to chondrocytes,[134-137] but longer peptides (i.e. higher MW and lower bioavailability) could escape absorption and exert their effect as a prebiotic in the large intestine.[127,138,139] This concept was outside the scope of the CHAMP study to investigate, but alongside recent work by Zhang et al.[139] it warrants more research with another computer-controlled gastrointestinal model for comparing formulations of CH (bovine or porcine hide) with several MWs and at higher doses than the 1.2 g/d studied by Larder et al.[127]

Zhang et al.[139] conducted an elaborate set of *in vivo* studies on rats and mice. Initially they demonstrated the superior bioavailability (absorption) of CH with a low MW (rich in Gly-Pro-Hyp tripeptides) compared to CH with a higher MW and fewer tripeptides in their rat study. They also found CH with a low MW (50% of its constituents were in the 0.2-0.5 KDa range, and 8% >2 kDa) consumed by mice on a low protein diet at 200 mg/kg (equivalent to 12 g for a 60 kg human) over eight weeks increased total SCFA production by the microbiota compared to lower doses of 50 mg/kg (3 g for the 60 kg human), 100 mg/kg (6 g for the 60 kg human) or 100 mg/kg of CH with a higher MW (11% of constituents were 0.2-0.5KDa, and 50% >2 KDa). The differences were not statistically significant, but the findings help with understanding a possible interrelationship of two different

proposed mechanisms of action for CH as a 'nutricosmetic' for skin condition through a gut-skin axis (i.e. absorption into the bloodstream, and as a prebiotic peptide escaping absorption).[139]

Zhang et al.[139] described the gut-skin axis as an interaction between gut microbiota, immune signaling and skin physiology. The microbiota can increase their production of SCFAs through CH supplements which escape digestion (i.e. Pro-Hyp), leading to the upregulation of immunoglobulin A (IgA) plasma cells in the bloodstream (which modulate the inflammatory response), and TGF-β which promotes collagen synthesis in the skin.[139] Simultaneously, Pro-Hyp are absorbed into the bloodstream for distribution to dermal fibroblasts for collagen synthesis. However, Pro-Hyp can also accumulate in articular cartilage,[95] and SCFAs produced by the microbiota can modulate the inflammatory response associated with KOA.[140] In other words, a gut-cartilage axis may exist alongside the gut-skin axis described by Zhang et al.[139] Albeit speculative, this highlights the need for a dose-response trial of CH from terrestrial mammals to reexamine the CHAMP study outcomes, and to explore the idea that two different postulated mechanisms of action may interrelate for improving articular cartilage and skin condition. Therefore, investigating the effect of CH at various MWs is needed considering the superiority of low MW formulations for absorption (bioavailability) and for a prebiotic effect as shown by Zhang et al.[139] Ultimately, a prudent next step of investigation is to identify a dose and MW where maximal absorption of Pro-Hyp is achieved, and enough escape absorption to reach the large intestine for potentially exerting a maximal prebiotic peptide effect too.

8.4 Conclusion

The CHAMP study was novel in its four-month (17 weeks) RDBCT design. It investigated the effect of 15 g/d protein as CH from bovine hide on joint pain and skin condition versus the comparators of either milk protein (MP), or maltodextrin (MDx) among women aged ≥50 years with knee pain (not necessarily due to KOA). Until now, placebos matched by weight rather than energy have been compared at lower doses, and without an isoenergetic, isoproteic comparator such as the MP supplement in this study. Furthermore, the CHAMP study examined subjective patient reported outcome measures (PROMs) alongside objective outcomes (i.e. functional tests, biomarkers and skin

parameters), so its results are relatively robust and make an important contribution to the existing evidence on the bioactive peptide (BAP) potential of CH as a nutraceutical for knee pain or deteriorating skin condition.

There was no evidence of a superior effect from the daily consumption of 15 g protein as CH in alleviating joint pain or improving functional performance versus MP or MDx. The three supplements performed similarly over time, without having any apparent impact on slowing an early stage of cartilage turnover dysregulation. There was some evidence that CH increased dermal thickness in skin regions partially protected from photoaging (i.e. ventral forearm) in contrast to the two comparators, but limitations associated with recruitment and data collection may restrict the generalizability of these findings. Even so, the CHAMP study has identified future research opportunities. For example, CH sourced from terrestrial mammals may or may not exert an independent effect as a nutraceutical promoting collagen synthesis (by articular chondrocytes and/or dermal fibroblasts) at a different dose (i.e. concentration of Pro-Hyp), and it may work in synergy with micronutrients to combat oxidative stress associated with aging (intrinsic and extrinsic), if not as a prebiotic with a similar effect on inflammation and oxidative stress. Therefore, the existing evidence base, now encompassing results from the CHAMP study, indicates a dose-response trial of CH from terrestrial mammals at various degrees of hydrolysis (i.e. MW) is needed to explore at least three potentially interrelated mechanisms of action with a factorial RDBCT design on women aged ≥50 years with early-stage KOA (i.e. KL Grade <II).

8.5 Chapter 8 references

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Chapter 9: Appendices

Appendix 1: The CHAMP study recruitment poster

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WHARENGA PUTAIAO

CHAMP STUDY

Do You Have a Stiff or Sore Knee?

Is your skin dry or showing signs of ageing?

You are invited to take part in the Collagen Hydrolysate and Milk Protein (CHAMP) study looking at the effects of collagen and milk protein on joint comfort and skin appearance

Participants will take 1 of 3 possible supplements (collagen, milk protein or placebo) each day for 4 months

All participants will get assessment of bone with a DXA bone scan

If you are a woman over the age of 50, who experiences regular knee pain in on or both knees, and are interested in joining this study, please contact,

CHAMP@massey.ac.nz | 021 764 868 | (06) 951 6321

Appendix 2: Metabolic syndrome definitions

Supplementary Table 9-1. Global definitions of metabolic syndrome (adapted with permission from Huang[1]).

	NCEP ATP III[A] (2005)	WHO[B] (1998)	EGIR[C] (1999)	IDF[D] (2005)
Absolute Requirement	None	Insulin Resistance	Hyperinsulinaemia	Central obesity (Waist circumference ≥80 cm[E])
Criteria	Any 3 of the 5 below	Insulin resistance or diabetes and any 2 from below	Hyperinsulinaemia and any 2 from below	Obesity and any 2 from below
Obesity	Waist circumference >89 cm[E]	Waist:Hip ratio (WHR) >0.85[E] or BMI >30 kg/m^2	Waist circumference ≥80 cm[E]	Central obesity already required
Hyperglycemia	Fasting glucose ≥5.55 mmol/L[F] or medically managed	Insulin resistance already required	Insulin resistance already required	Fasting glucose ≥5.55 mmol/L[F]
Dyslipidemia	TG ≥ 1.7 mmol/L[G] or medically managed	TG ≥ 1.7 mmol/L[G] or HDL < 1.0 mmol/L[E,H]	TG ≥ 2.0 mmol/L[G] or HDL <1.0 mmol/L[H]	TG ≥ 1.7 mmol/L[G] or medically managed
Dyslipidemia *(A second separate criteria)*	HDL cholesterol <1.3 mmol/L[E H] or medically managed			HDL <1.3 mmol/L[E H] or medically managed
Hypertension	>130 mmHg (SBP) or >85 mmHg (DBP) or medically managed	≥140/90 mmHg	≥140/90 mmHg or medically managed	>130 mmHg or >85 mmHg or medically managed
Other Criteria		Microalbuminuria[I]		

[A] National Cholesterol Education Program Adult Treatment Panel III, [B] World Health Organization, [C] European Group for the Study of Insulin Resistance, [D] International Diabetes Foundation, [E] Females, [F] mg/dl x 0.0555, [G] mg/dL x 0.01129, [H] mg/dL x 0.02586, [I] urinary albumin ≥20 µg/min or albumin-to-creatinine ratio ≥30 mg/g.

Appendix 2 References

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Appendix 3: Supplementary file of the CHAMP study

Supplementary Table 9-2. Eligibility criteria of the collagen hydrolysate and milk protein (CHAMP) study.

INCLUSION: Total 'Pain' raw score ≥8 / 36 OR (*from August until October, 2023*)[A] Total 'Pain' raw score of 6-7 *AND* Quality of Life (QoL) raw score ≥7 / 16
EXCLUSION[B]
Male
Total 'Pain' raw score <8 OR (*from August until October, 2023*)[A]: Total 'Pain' raw score of 6-7 *and* QoL raw score <7
BMI > 40
Age <50
Medically diagnosed or self-reporting autoimmune conditions that can influence biomarkers of inflammation independent to KOA (i.e. Rheumatoid arthritis, Coeliac Disease[C], fibromyalgia).
Conditions causing knee pain independently from KOA (i.e. knee surgery or acute injury) within the past 6 months[D]
Conditions influenced by, or sensitive to, total daily protein intake. (i.e. all stages of chronic kidney disease)
Conditions with altered ability to digest and absorb dietary protein. (i.e. all forms of bariatric surgery)
Already consuming (or consumed within 6 months) oral nutrition supplements containing any amount of chondroitin, glucosamine, or collagen (undenatured or hydrolyzed)[E,F]
Anyone having started hormone replacement therapy (HRT) within 6 months
Heavy smoker (≥21 per day)[1]
Heavy alcohol consumer (>2 units of alcohol / day)
Lactose intolerance
Vegetarian

[A] The eligibility criteria were revised after several respondents with a pain score <8 reported to have changed their living circumstances to avoid evoking further pain each day (i.e. avoiding stairs). Consequently, all future respondents with a pain score of 6-7 were provided with the Quality of Life (QoL) domain questions adopted from the KOOS.[2]

[B] Respondents with pre-existing medical conditions using prescribed medications that can affect the biomarkers of cartilage turnover and or inflammation (i.e. statins or NSAIDs) were instructed to continue with their medical management supervised by a registered health care professional for the duration of the study

[C] Well-controlled Coeliac Disease (CD) of at least two years since diagnosis was not excluded.

[D] Assuming older trauma, thus predisposing to early-onset OA, should not be associated with acute pain.

[E] Respondents having started collagen (UC or CH) within two weeks of the screening survey were allowed to cease treatment and undergo a washout of three months before participating.

[F] Respondents using supplements with anti-inflammatory properties (i.e., omega 3 or 6), were instructed to continue their treatment throughout the intervention.

Supplementary Table 9-3. Nutrition information panel for Blackmores Multivitamin for 50+.

Nutrient	Per Tablet (daily dose)
Vitamin A	750 µg Retinol Equivalents
Vitamin B2 (Riboflavin)	1.6 mg
Vitamin B5	6 mg
Vitamin B6	1.7 mg
Vitamin B9 (folic acid)	200 µg
Vitamin B12	2.4 µg
Vitamin C	45 mg
Vitamin D3	600 IU
Vitamin E	14.9 IU
Vitamin K	35 µg
Biotin	30 µg
Iron	5 mg
Calcium	325 mg
Iodine	150 µg
Magnesium	105 mg
Manganese	3.5 mg
Zinc	10 mg
Chromium	35 µg
Copper	600 µg
Selenium	50 µg
Colloidal anhydrous silica	20 mg
Thiamine	1.2 mg

https://www.blackmoresnz.co.nz/products/multivitamins-for-50

Supplementary Table 9-4. Protocol for inspection of normal distribution assumptions (Chapters 4 and 5).[3,4]

1. Generate descriptive statistics
2. Inspect skewness via histogram and Q-Q plot
   - *Aim for a value ≤±1.0*
3. Inspect kurtosis via histogram and Q-Q plot
   - *Aim for a value ≤±2.0*
4. Perform and assess Shapiro-Wilk test
5. Perform and assess Levene's test
6. Identify extreme outlier(s) for inclusion and exclusion during per protocol analysis
7. Log10 transform data and repeat steps 1-6,
8. Remove any persistent outliers starting with the most extreme and repeat steps 1-7.

Supplementary Table 9-5. Adverse events by supplement group.

Event	Supplement Group		
	Collagen Hydrolysate	Milk Protein	Maltodextrin
Gastrointestinal (GI) discomfort	1	2	7
Heartburn	2	0	2
Weight Gain	0	2	2
Unrelated			
- *Cholecystitis*	*0*	*0*	*1*
- *GI attributed to other foods, medications, preexisting conditions*	*4*	*2*	*3*
- *Itchy eyelid*	*0*	*1*	*0*
- *Post-Covid cough*	*1*	*0*	*0*
- *Vertigo*	*1*	*0*	*0*

Supplementary Table 9-6. Minimal detectable change (MDC) values for the functional tests.

Functional Test	Author(s)	MDC	Study Characteristics
Timed Up and Go (TUG)	Alghadir et al.[5]	1.10 – 1.14s	*65 participants (25 male, 40 female) aged 45-70 years with KOA graded I-III by radiography*
	Dobson et al.[6]	1.21s	*51 participants (47% female, mean age: 64.5±6.2 years)-with KOA graded ≥II by radiography*
30-second Chair Stand (30s-CST)	Dobson et al.[6]	n=2	*51 participants (47% female, mean age: 64.5±6.2 years)-with KOA graded ≥II by radiography*
	Holm et al.[7]	n=2.4	*40 participants (55% female, mean age: 68.8±8.3 years) with KOA graded ≥II by radiography or clinical assessment*
Stair Climb Test (SCT)	Dobson et al.[6]	2.33s	*11-step protocol, 51 participants (47% female, mean age: 64.5±6.2 years)-with KOA graded ≥II by radiography*
	Holm et al.[7]	2s	*9-step protocol, 40 participants (55% female, mean age: 68.8±8.3 years) with KOA graded ≥II by radiography or clinical assessment*
	Iijima et al.[8]	0.102s	*11-step protocol, 59 participants (72.9% female) aged 50-69 years with a history of self-reported knee pain without radiographic evidence of KOA*

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Appendix 4: Chapter 5 supplementary data

Supplementary Table 9-7. Blood sample collection/processing protocol.

Laboratory analysis was done by Canterbury Health Laboratories (CHL). They are accredited against the ISO 15189:(2012), ISO 22870:(2006), and AS/NZS 4308:2008 standards in biosecurity and safety procedures. After the collection and preparation described below for each biomarker, all blood samples were sent to CHL on February 14, 2024.

Serum PIIANP

Whole blood was drawn into 3.5 mL 13x75 mm BD Vacutainer SST Advance tubes (gold) and inverted 5 times before storage at room temperature for two hours. They were centrifuged for 15 minutes at 1100 g at 4°C in a bench top centrifuge (Thermo Scientific, Multifuge X1R230V 50/60Hz). Duplicate aliquots of 500 µL were stored upright in cryo-tubes at -80°C until analyses by CHL on November 19 and 26 (baseline samples), and November 20-21, 2024 for endpoint (Catalog # EZPIIANP-53K, EMD Millipore, Burlington, MA, USA}.

Plasma COMP

Whole blood was drawn into 4 mL 13x75 mm K2E Vacutainers (lavender) and inverted 10 times before storage at room temperature for one hour. They were centrifuged for 10 minutes at 2200 g at 4°C in a bench top centrifuge (Thermo Scientific, Multifuge X1R230V 50/60Hz). Duplicate aliquots of 500 µL were stored upright in cryo-tubes at -80°C until analyses by CHL on September 27 and October 04, 2024 for baseline, and October 04, 2024 for endpoint (Invitrogen Human Thrombospondin-5/COMP ELISA Kit, Catalog # EH453RB / EH453RBX10; Thermo Fisher Scientific, Waltham, MA, USA).

Serum IL-1β

Whole blood was drawn into 4 mL 13x75 mm Plain CAT Vacutainers (red) and inverted 5 times before storage at room temperature for one hour. They were centrifuged for 10 minutes at 2200 g at 4°C in a bench top centrifuge (Thermo Scientific, Multifuge X1R230V 50/60Hz). Aliquots of 500 µL serum were stored upright in cryo-tubes at -80°C until analyses by CHL on October 17, 2024 (baseline), and October 23 and November 14, 2024 for endpoint 1β (Invitrogen Human IL-β ELISA Kit, Catalog # BMS224-2 / BMS224-2TEN; Thermo Fisher Scientific).

Serum TNF-α

Whole blood was drawn into 4 mL 13x75 mm Plain CAT Vacutainers (red) and inverted 5 times before storage at room temperature for one hour. They were centrifuged for 10 minutes at 2200 g at 4°C in a bench top centrifuge (Thermo Scientific, Multifuge X1R230V 50/60Hz). Aliquots of 500 µL serum were stored upright in cryo-tubes at -80°C until analyses by CHL on November 14, 2024 for baseline, and October 23 and November 14, 2024 for endpoint (Invitrogen Human TNF-α ELISA Kit, Catalog # BMS223-4 / BMS223-4TEN; Thermo Fisher Scientific).

Supplementary Table 9-8. Published coefficient of variation (CV) values and limits of detection for soluble biomarker assays.

Biomarker	Coefficient of Variation		Limit of Detection
	Intra-assay	Inter-assay	
Serum PIIANP	<10%	<10%	39 ng/mL
Plasma COMP	<10%	<12%	0.085 ng/mL
Serum IL-1β	5.1%	8.6%	0.3 pg/mL
Serum TNF-α	6.0%	7.4%	2.3 pg/mL
uCTX-II	≤7.8%	≤12.2%	0.20 µg/L

Supplementary Table 9-9. CTX-II at baseline (BL) & endpoint (EP) by supplement group (without data causing an elevated intra-assay CV).

	Supplement Group											
	Collagen Hydrolysate (CH)				Milk Protein (MP)				Maltodextrin (MDx)			
	n=	Mean	SE	95% CI	n=	Mean	SE	95% CI	n=	Mean	SE	95% CI
CTX-II (ng/mmol Cr) *Intra-assay: 10.3%* *Inter-assay: 8.9%*	20				14				18			
Time (EP vs. BL)	$F(1.000, 49.000) = 1.964$, $p=0.167$, $\eta_p^2 = 0.039$											
Time*Group	$F(2.000, 49.000) = 1.776$, $p=0.180$, $\eta_p^2 = 0.068$											
Baseline		512.7	61.2	399.7 635.7		595.5	73.2	448.4 742.5		546.1	64.5	415.4 674.7
Endpoint		550.2	57.3	435.1 665.4		510.0	68.5	372.3 647.6		455.5	60.4	334.1 576.9
Within-group p value[A,B]		*p=0.475*				*p=0.177*				*p=0.110*		

[A] EP versus BL

Appendix 5: Chapter 7 supplementary data

Supplementary Table 9-10. Outlier identification and management protocol.

Part I: Visual Inspection / Outlier Identification

1. Generate descriptive statistics
   - *Raw (untransformed) data*
   - *Common (log10) transformation of data*
2. Inspect skewness via histogram and Q-Q plot
   - *Aim: ≤±1.0*
3. Inspect kurtosis via histogram and Q-Q plot
   - *Aim: ≤±2.0*
4. Perform and assess Shapiro-Wilk test
5. Perform and assess Levene's test

Part II: Outlier Management

6. Identify any extreme outlier(s) from box-and-whisker plots and check for extreme values (high & low) in each group
   - *Drop data outside an evidence-informed reference range (Supplementary Table 9-11) and assume a measurement error has occurred*
7. Drop any data identified as an outlier if:
   a. *The case at BL or EP remains an outlier after log10 transformation*
   b. *The case at BL or EP is resolved with log10 transformation BUT falls outside the evidence-informed reference range (Supplementary Table 9-11)*
8. Retain any data identified as an outlier if:
   a. *The case at BL or EP is resolved by Log10 AND falls within the evidence-informed reference range (Supplementary Table 9-11)*
9. Repeat Steps 1 to 6
10. Execute split-plot ANOVA of:
    a. untransformed data without outliers to report *F statistics, p value and partial eta*
    b. log10 data without outliers for sensitivity analysis comparisons with reported data

Supplementary Table 9-11. Evidence-informed reference ranges for identifying outliers.

Parameter	Inclusion range	Reference
Dermal Thickness (DT)	Arm 700-2,000 µm	Arm Peperkamp et al.[1] Mean±SD: 1308±254 µm Range: (705-2120)
	Cheek 500 -2,000 µm	Cheek Chopra et al.[2] Mean±SD: 1291±427 µm Žmitek et al.[3] Mean±: 972±154 µm
Viscoelasticity (VE)	Arm <0.2 and >16 MPa	Arm Peperkamp et al.[1] Mean±SD: 2.9±2.0 MPa Range: (0.2-15.1 MPa)
	Cheek <0.2 and >15 MPa[A]	Cheek Žmitek et al.[3] Mean±SD: 4.5±1.6 MPa
Epidermal Hydration (EH)	Arm >300 µS	Arm Bogdan et al.[4] Mean±SD: 251±29 µS i.e. 2sd (z scores for 95%)
	Cheek >300 µS	Cheek Voegeli et al.[5] Range (middle cheek): ≈25-300 µS

[A] Values >15 removed because of the anticipated lower VE in cheek vs arm due to sun exposure damage and aging (i.e. unlikely that higher values are accurate) [6].

Appendix 5 references

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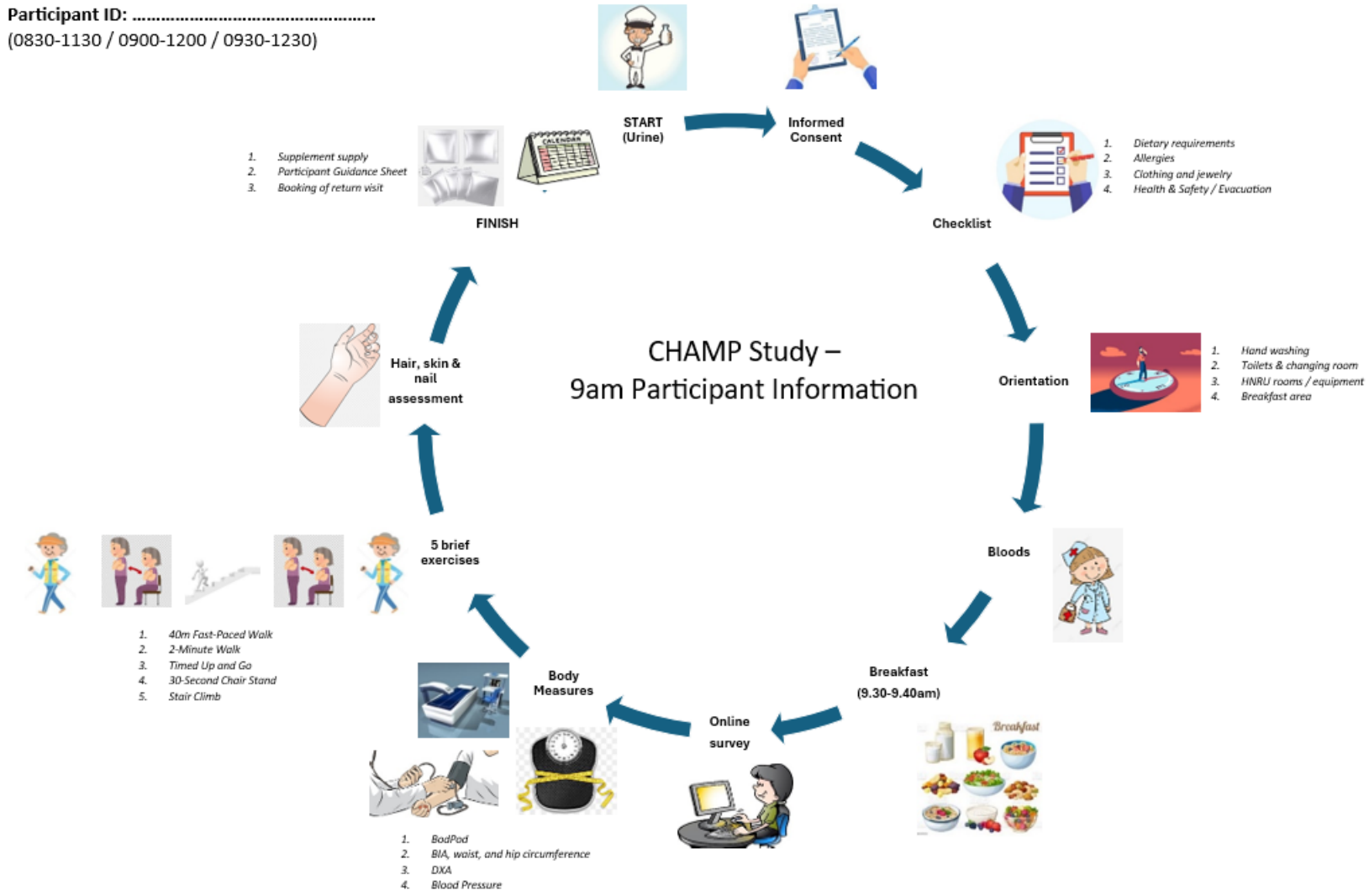
Appendix 6: Flow diagram of the CHAMP study activities at baseline and endpoint

Participant ID: .....
(0830-1130 / 0900-1200 / 0930-1230)



Appendix 7: Facial cleaner ingredients

Product name: Johnson's Daily Essentials Refreshing Facial Cleansing Wipes

(Buy Johnson's Daily Essentials Refreshing Facial Cleansing Wipes Normal 25 Online at Chemist Warehouse®)

Ingredients: Aqua, Isostearyl Palmitate, Cyclopentasiloxane, Pentaerythrityl Tetraethylhexanoate, Isononyl Isononanoate, Cetyl Ethylhexanoate, Hexylene Glycol, Stearyl Ethylhexanoate, PEG-6 Caprylic/Capric Glycerides, Sucrose Cocoate, Panthenol, Magnesium Aspartate, Zinc Gluconate, Copper Gluconate, Ethylhexylglycerin, Glyceryl Oleate, Hydrogenated Palm Glycerides Citrate, PEG-4 Laurate, Coco-Glucoside, Carbomer, Citric Acid, Sodium Hydroxide, Ascorbyl Glucoside, Benzoic Acid, Dehydoracetic Acid, Iodopropynyl Butylcarbamate, Phenoxyethanol, Parfum

Appendix 8: Bioavailability of the CHAMP study supplement

An absorption study was conducted in 2024 to supplement data analyses in the CHAMP study. It examined if prolyl-hydroxyproline (Pro-Hyp) dipeptides from the CH supplement were absorbed after ingestion by a comparable cohort of participants. Six free-living healthy women aged ≥50 years were recruited from advertising on social media. Exclusion criteria were BMI >26 kg/m^2, vegan/vegetarian unwilling to consume gelatin or collagen, and any known inflammatory condition such as medically diagnosed osteoarthritis (any joint) or a connective tissue disorder (e.g. rheumatoid arthritis). The study was approved by the NZ Health and Disability Ethics Committee (2024 exp 19781).

Participants attended the Human Nutrition Research Unit (HNRU) in Palmerston North after an overnight fast ≥12 hours. With written informed consent, whole blood venipuncture samples were collected from each participant before they consumed the 15 g CH supplement in 200 mL water. A second blood sample was taken two hours later, and all samples were analyzed for the presence of Pro-Hyp with tandem liquid chromatography-mass spectrometry (LC-MS/MS) in accordance with the methodology of Ichikawa et al.[1] and Shigemura et al.[2]

The results are presented as descriptive statistics for each participant (mean±SD) in Table 9-12. It shows free Pro and Hyp concentrations in baseline plasma samples were minimal, albeit the levels of Pro were higher than Hyp. This was expected considering proline is absorbed from other foods (e.g. milk protein), and it is endogenously synthesised in the human body while its hydroxylated residues only appear after collagen polypeptides are synthesised (i.e. post-translational modification).[3,4] After 2h, free Pro and Hyp were markedly increased in all participants compared to their baseline levels, indicating Pro-Hyp dipeptides were absorbed intact (i.e. before the fragmentation from liquid chromatography) for presumed distribution to potential target cells (i.e. articular cartilage chondrocytes and dermal layer fibroblasts).[5] This was consistent with Aito-Inoue et al,[6] so the CH supplement was verified as a bioavailable source of Pro-Hyp to examine its proposed bioactive peptide (BAP) potential for joint pain and skin condition in the existing CHAMP study project.

Supplementary Table 9-12. Absorption study data (Mean±SD) for the CHAMP study supplement.

Participant	Pro (ng/mL)		Hyp (ng/mL)	
	0h	2h	0h	2h
1	13.02±0.99	88.89±2.72	2.23±0.61	82.26±3.02
2	11.84±0.19	89.53±0.82	4.68±0.41	91.78±4.46
3	8.41±0.29	80.49±3.64	0.89±0.09	77.89±4.98
4	8.69±0.45	100.21±3.44	0.38±0.13	97.26±2.26
5	9.97±0.37	126.60±7.76	1.02±0.52	127.49±10.64
6	9.07±0.36	83.62±2.45	0.16±0.06	78.75±4.76

Acknowledgements

This study was designed and executed by Dr Katie Schraders. The methodology for the analysis was designed by Trevor Loo and the late David Lun, who also carried out the LC-MS/MS analyses.

Appendix 8 references

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