Low-Level Laser Therapy and Cryotherapy in Tendinopathy Treatment

Clinical, biological, and biophysical effects of low-level laser therapy alone and in combination with cryotherapy

Sturla Haslerud

Thesis for the Degree of Philosophiae Doctor (PhD)
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Name: Sturla Haslerud

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Scientific environment

University of Bergen – Faculty of Medicine and Dentistry

- Physiotherapy Research Group
  Department of Public Health and Primary Care

Bergen University College – Faculty of Health and Social Science

- Centre for Evidence-Based Practice
- Department of Occupational Therapy, Physiotherapy and Radiography
- NorPhyPain Research Group

University of Mogi das Cruzes

- Department of Biomedical Engineering

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Abstract

Introduction

Low-level laser therapy (LLLT) and cryotherapy are applied to the human skin to trigger biological actions in the underlying tissue. LLLT modulates biological processes by emitting energy-charged photons to pathological tissue, whereas cryotherapy produces its effect on pathology through the reduction of tissue temperature. When a treatment leads to an unexpected clinical event, the underlying mechanisms involved are often uncertain. This thesis is based upon such a clinical observation, and a reversed translational research approach was used to further investigate the biophysical and biological effects of combining LLLT and cryotherapy in tendinopathy treatment.

Aim of Thesis

The overall purpose of this thesis is to investigate the clinical, biophysical, and biological effects of LLLT alone and in combination with cryotherapy for the treatment of tendinopathy.

Methods

This thesis consists of three studies. In Study I, a systematic review with meta-analysis was performed to determine the effectiveness of LLLT for shoulder tendinopathy. A structured search for relevant studies up to May 2013 was executed. Two independent assessors rated the included studies according to the Physiotherapy Evidence Database (PEDro) scale. Intervention quality assessments were performed according to World Association for Laser Therapy (WALT) guidelines. The included trials were sub-grouped by intervention quality and the use of other physiotherapy interventions. Study II was a basic in-situ research study of repeated measurements. The optical energy (from two different Class 3B lasers) penetrating the Achilles area of healthy adults was measured before and after 20 minutes of cryotherapy. In Study III, a blinded multiple-armed randomized controlled trial (RCT) design with a post

intervention test only was used to investigate the biological effects of LLLT and cryotherapy, both alone and in combination with each other. The study sample comprised in vivo rat Achilles tendons.

Results

Optimal LLLT can offer clinically relevant pain relief and initiate a more rapid course of improvement, both alone and in combination with physiotherapy interventions in patients suffering from shoulder tendinopathy. The systematic review identified parallel cryotherapy treatment as a possible confounder to LLLT, as it may induce inhibitory effects and negatively influence treatment outcomes. The penetration of laser energy increased significantly ($p<0.01$) through Achilles skin and tendons, for both lasers and at all time points, after 20 minutes of cryotherapy. Increased LLLT energy penetration occurred when mean skin temperature was 4.8˚C (SD±3.6), resulting in a significant reduction in the Achilles tendon ($p=0.03$) and skin-tendon-skin thickness ($p=0.05$). The biological effect of LLLT (3J) one hour after tendon trauma significantly ($p<0.05$) reduced pro-inflammatory interleukin (IL)-1ß expression in the presence of the highest median levels of IL-10 ($p=0.06$) across all treatment groups. Cryotherapy alone failed to reach statistical significance over no treatment for all the targeted cytokines. The parallel treatment of LLLT and cryotherapy produced an anti-inflammatory “add-on” effect and significantly reduced the expression of all targeted cytokines except IL-10. Biomechanical and histology results suggested that the order of therapy administration was essential, showing superior results when LLLT followed cryotherapy.

Conclusion

This thesis reveals that the parallel treatment of cryotherapy and LLLT can negatively influence the clinical effects of LLLT in shoulder tendinopathy treatment. The optical properties of healthy skin and tendons are altered by cryotherapy, which significantly increases the penetration of laser energy irradiation. The order of therapy administration determined if a positive or negative biological response in injured rat Achilles tendons occurred.

List of publications

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Abbreviations

A-P	anterior-posterior
Ad libitum	free access
ATP	adenosine triphosphate
°C	degrees Celsius
CAT	catalase
CG	cryotherapy group
CI	confidence interval
CINAHL	Cumulative Index to Nursing and Allied Health Literature
CLG	cryotherapy first/LLLT group
cm	centimeter
cm^2	square centimeter
CCO	cytochrome c oxidase
COX	cyclooxygenase
CW	continuous wave
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EPAs	electrophysical agents
FDA	American Food and Drug Administration
g	gram
GPx	glutathione peroxidase
HCG	healthy control group
Hz	hertz
ICC	intraclass correlation coefficient
IL	interleukin
ING	injured non-treated control group
J	joule
kg	kilogram
kHz	kilohertz
Laser	light amplification by stimulated emission of radiation
LET	lateral elbow tendinopathy
LCD	liquid-crystal display
LCG	LLLT first/cryotherapy group

LG	LLLT group
LLLT	low-level laser therapy
M	Molar
M-L	medial-lateral
MeSH	Medical Subject Headings
min	minute
mg	microgram
MHz	megahertz
mK	millikelvin
mm	millimeter
MMP	metalloproteinase
MOP	mean output power
mW	milliwatt
nm	nanometer
N	Newton
n	number in a sample
NAALT	North American Association for Photobiomodulation Therapy
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
nsec	nanosecond
OPM	optical power meter
PBMT	photobiomodulation therapy
PBST	phosphate-buffered saline with Tween 20
PEDro	Physiotherapy Evidence Database
pg	picogram
PGE_2	prostaglandin E_2
POLICE	protection, optimal loading, ice, compression, elevation
PRICE	protection, rest, ice, compression, elevation
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RCT	randomized controlled trial

REK	Regional Committees for Medical and Health Research Ethics
RevMan	Review Manager (software program)
RR	relative risk
RTUS	real-time ultrasonography
RICE	rest, ice, compression, elevation
ROS	reactive oxygen species
SAIS	subacromial impingement syndrome
sec	second
SD	standard deviation
SEM	standard error of mean
SMD	standardized mean difference
SOD	superoxide dismutase
SPW	super pulse wave
SRI-HD	high-definition speckle reduction imaging
TNF-α	tumor necrosis factor alpha
TTC	triphenyl tetrazolium chloride
μL	microliter
μm	micrometer
UV	ultraviolet
VAS	visual analogue scale
W	watt
WALT	World Association for Laser Therapy
WMD	weighted mean difference

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PAPER I, II & III

1. Introduction

Translational research is often associated with testing novel ideas in basic laboratory studies that aim to produce findings that can be turned into useful clinical interventions. However, the path between basic and clinical research is not unidirectional but runs both ways. Consequently, returning to the laboratory with observations made in human studies can lead to new discoveries, which may increase our understanding of human disorders and help us to optimize treatments (Mankoff et al., 2004; Littman et al., 2007). Indeed, the evidence for most physiotherapy interventions share this clinic-to-basic research history, in which observed clinical effects have been explained by plausible biological mechanisms of action in cell and animal trials. This thesis follows a similar research strategy, as an unexpected clinical observation intrigued and prompted us to gain more insight into the topic of low-level laser therapy (LLLT) in combination with cryotherapy for the treatment of tendinopathy.

Tendinopathy is a common and frequently disabling condition that is challenging to treat. Physiotherapy treatment options for tendinopathies are manifold, which reflects the partly unclear and multifactorial etiology behind the condition (Jarvinen et al., 2005; Seitz et al., 2011). The effectiveness of exercise-based physiotherapy treatment regimens has been extensively studied, and these are currently the cornerstone of treatment for tendinopathies. However, the ideal exercise program remains unclear, and many patients do not respond positively to exercise alone.

In physiotherapy, different interventions are often combined to best address the individual needs of each patient. However, research studies are often designed to measure the effect of these interventions as monotherapies. As most physiotherapy interventions have limited potential to cause harm, combining treatment modalities is usually a professional decision that is based on clinical reasoning and empiricism. Combining LLLT and cryotherapy to reduce pain and accelerate recovery in patients with tendinopathy is an example of a treatment combination lacking evidence of efficacy and knowledge about biological interactions.

Although the use of light and ice as therapeutic modalities share an ancient history, light amplification by stimulated emission of radiation (laser) devices first emerged as a commercially available option for treating painful musculoskeletal conditions in the 1960s (Mester et al., 1968a). Despite initial skepticism regarding its usefulness in mainstream medicine, laser therapy research has progressed over the years and consistently demonstrates its ability to produce beneficial photobiological effects in mammalian cells. However, the transfer of positive results from laboratory research to clinical trials on musculoskeletal conditions often produces mixed results. Indeed, the first American Food and Drug Administration (FDA) approval of a Class 3B laser device that could be marketed for treating musculoskeletal conditions was not issued until 2002 (FDA, 2002). In Norway, LLLT treatment was first included in the national tariff payment system for physiotherapists in 2001 (Bjordal et al., 2014).

There have been several studies investigating the efficacy of LLLT for tendinopathy, and reviewers were able to identify an optimal laser dose range for treating tendinopathy in 2001 (Bjordal et al., 2001). This discovery implies that LLLT trials should not be judged by methodological standards only but also by the validity of the dose and treatment procedure. Consequently, previously published randomized controlled trials (RCTs) and systematic reviews should be examined with new eyes if dose and procedural aspects have been left unaddressed. The efficacy of optimal LLLT has been synthesized in systematic reviews for some location-specific areas of tendinopathy. However, the evidence of effects related to the most prevalent site of tendinopathy in the general population, the shoulder, has not yet been reviewed.

This thesis investigates the clinical, biophysical, and biological effects of LLLT alone and in combination with cryotherapy for the treatment of tendinopathy. Chapter 1 is a review of relevant literature, which provides the context for the objectives of the thesis. The aim of the thesis and included studies are presented in chapter 2. Materials and methods are described in chapter 3. The systematic review and meta-analysis identifies cryotherapy as a possible confounder to LLLT in tendinopathy treatment, and two basic research studies was performed to follow up on this finding. The

results from these three studies are found in chapter 4. Finally, the last chapters (5–7) provide a discussion, a conclusion, and suggestions for future research.

1.1 Tendinopathy

Tendons play a critical role in body mechanics, predominantly by transferring force from muscle contraction to bone, thus allowing movement and joint stability. They consist of collagen fibrils (primary, secondary, and tertiary fibers), each sheathed by an endotenon, which in turn is wrapped in an epitenon. Enclosing the epitenon is a third sheath, the paratenon, forming the actual tendon. The microbiology of normal tendons is mainly composed of fibroblast-producing cells called tenocytes, which are surrounded by an extensive extracellular matrix (ECM). The ground substance of the ECM consists of proteoglycans, glycosaminoglycans, glycoproteins, and several other small molecules. These components are involved in the development, organization, and growth of the tendons. The water-binding proteoglycans enable cell migration and the diffusion of molecules. The glycoproteins are active in the repair and regeneration of tendon material, while other proteins are important for collagen fiber alignment and orientation. This hierarchical and morphological structure gives tendons the ability to withstand high unidirectional tensile loads (Sharma and Maffulli, 2006; Abate et al., 2009; Magnusson et al., 2010). The mechanical loading of tendons is known to have a major influence on ECM turnover, increasing both the collagen synthesis and the degrading metalloprotease enzymes (Kjaer, 2004).

Although tendons are metabolically active tissues dependent on blood supply, hypovascular areas have been identified in tendons such as the Achilles and supraspinatus (Åstroöm and Westlin, 1994; Mehta et al., 2003). The metabolic rate, oxygen consumption, and vascularization of tendons is lower, and the collagen turnover time higher, than those of skeletal muscles and other soft tissues (Vailas et al., 1978; Sharma and Maffulli, 2005). Consequently, the healing and regeneration processes of tendons are considerably slower than those of muscles.

Tendinopathy is a term used to describe the multifactorial pathology of non-ruptured tendon disorders characterized by localized pain and swelling, a decline in function, and a reduced tolerance for loading activities (Maffulli et al., 2003; Wang et al., 2006; Van Dijk et al., 2011; Magnan et al., 2014a). The origin of the term *tendinopathy* was much influenced by the complex and unclear etiological initiation of tendon pain (Maffulli et al., 2003; Rio et al., 2014). Prior to the 1990s, painful tendons were referred to as *tendinitis*, with the *-itis* ending implying that inflammation initiates and drives the condition. New perspectives on tendon disorders were published during the next decade, suggesting that the etiology of tendinitis was very different from the etiology of other inflammatory conditions.

The paper entitled "Time to Abandon the Tendinitis Myth," by Khan et al. (2002), had a great impact by strongly proposing that the condition of tendon pain originates from a non-inflammatory degenerative process. These non-inflammatory and degenerative models dominated thinking on tendinopathy during the first decade of the twenty-first century. However, the dogma of degeneration without inflammation in tendinopathy has been increasingly challenged in the past 10 years. Development in areas such as immunohistochemistry, molecular techniques, and gene expression analysis have identified inflammatory reactions in longstanding tendinopathy as well as in its early stages (Rees et al., 2013; Millar et al., 2017).

1.1.1 Risk factors of tendinopathy

The risk factors of tendinopathy are often characterized as both intrinsic and extrinsic, referring to internal tendon processes and external contributing factors. The most commonly reported extrinsic risk factor for developing tendinopathy is an increased overall volume of tendon loading, often referred to as *overuse tendinopathy*.

In terms of intrinsic risk factors, advancing age is demonstrated to be among the most significant (Maffulli et al., 2003; Seitz et al., 2011; Magnan et al., 2014a). As age progresses, the metabolic rate of the tendons decreases, which most likely influences reparative ability (Kannus, 2000). In addition, a decrease in the capillary blood

supply to the tendon and a degeneration of the tenocytes and collagen fibers are typical age-related tendon alterations (Kannus et al., 2005). The tendon loses tensile strength, stiffness, and rebound resilience, which may predispose it to injury. Degenerative tendon changes due to advancing age can be attenuated by physical activity (Narici et al., 2008). However, the optimal loading of the tendon is crucial, as the capacity to repair micro-trauma induced by mechanical loading may decrease with age (Cook and Purdam, 2009).

Overuse is not responsible for all tendon pathology. Tendinopathy may also occur following external trauma (Wedderkopp et al., 1997, Agel et al., 2007). In addition, there are several other factors associated with an increased risk for developing tendinopathy, such as anatomical anomalies, genetic factors, muscular insufficiency or imbalance, posture, soft-tissue inflexibility, drugs, and various environmental conditions (Maffulli et al., 2003; Seitz et al., 2011; Magnan et al., 2014a).

1.1.2 Tendon healing and regeneration

Tendons respond to acute injury by initiating several overlapping stages of repair. Immediate bleeding and the clotting of blood at the site of the injury characterize the hemorrhagic stage of tendon healing. Cytokines and growth factors are released by the infiltrated platelets and initiate the inflammatory tendon healing. During this stage neutrophils and macrophage phagocytose necrotic tissue, whereas a fibrin clot consisting of mainly collagen type 3 is formed by tenocytes to stabilize the injury. The increased synthesis of this immature granulation tissue represents the proliferative healing phase. The following process of remodeling and maturation is characterized by decreased inflammation and increased fibroblast activity, gradually replacing the mechanically weaker collagen 3 with the more resilient collagen 1. The tendon may remain in this state of fibroblast hypercellularity for up to a year (Wang et al., 2006; Voleti et al., 2012; Muller et al., 2013). Nevertheless, the tendon's structural and biomechanical properties may never be completely restored to pre-injury levels (Oliva et al., 2011; Wang et al., 2012), and many patients develop chronic symptoms.

It has been proposed that the typical micro-injuries associated with chronic overuse tendinopathy may fail to stimulate an adequate inflammatory response and that the consequence is a failed healing response by the immune system (Cook et al., 2002; Maffulli et al., 2010). Cytokines are frequently investigated inflammatory mediators in tendinopathy, much because of their immunoregulatory role and crucial interaction with resident tenocytes and ECM (Millar et al., 2017).

1.2 History of Electrophyscial Agents in Physiotherapy

"The ability of a clinician to reduce pain in a patient by exploiting the patient's own in-built neurophysiological mechanisms must surely rank as one of the greatest achievements of contemporary medical science."

(Woolf, 1984, as cited in Macdonald, 1993)

The first documentation of physiotherapy as a profession dates back to 1813, when the Swede Per Henrik Ling established the Royal Institute of Gymnastics for manipulation and exercise in Stockholm (Brodin, 2008). Other countries followed this initiative, and by 1920, the Chartered Society of Massage and Medical Gymnastics was granted its Royal Charter by King George V in the UK (Chartered Society of Massage and Medical Gymnastics, 1929). Physiotherapists were, at this point, educated in anatomy and biomechanics and given a license to interact with and treat patients using massage and manipulation. The adoption of a biomechanical framework and a growing attention to pathology were important for future advances in physiotherapy, ultimately leading to the assimilation of new treatment interventions in physiotherapy, such as electrotherapy (Nicholls and Cheek, 2006).

The therapeutic use of electrical stimulation for alleviating pain may originate from ancient Greece, where electrical impulses from fish or eels were applied to treat painful conditions such as gout and nuclei prolapse. The Greeks termed the electrical fish *narcs*, a precursor to the word *narcosis*, due to their numbing effect. The ability to stimulate or provide shock treatment using mechanically produced static electricity emerged during the eighteenth century. These electrical devices were used

therapeutically to treat numerous conditions ranging from painful musculoskeletal disorders to epilepsy and sterility (Macdonald, 1993). Although the first electrotherapy pioneers of this century were convinced of the effect, the medical establishment had so far responded with contempt and discredit. Treatment with EPAs was first made respectable after Dr. Golding Bird opened an electrical department at Guy's Hospital London in 1836 and gave a series of lectures on "Electricity and Galvanism in Relation to Physiology and Therapeutics" at the Royal College of Physicians (Selcon, 2001).

At the beginning of the twentieth century, the first investigative reports and clinical trials were published in recognized medical journals. These early papers suggested that EPAs should be used as an adjunct therapy to accelerate recovery in musculoskeletal conditions, such as peripheral nerve injuries, due to its ability to produce contractions in paralyzed muscles (Wolfson, 1931; Doupe et al., 1943). Following World War II, more research attention was focused on this phenomenon, referred to as *galvanic exercises* (Tiktinsky et al., 2010). However, the first major step forward for treatment with electrical currents was the introduction of the gate control theory in 1965, which provided clinicians and researchers with the first necessary theoretical framework to explain its pain-relieving effect (Melzack and Wall, 1965).

Over the years, technological improvements and innovations in the field of EPAs have been gradually adopted in physiotherapy. New electrophysical treatment interventions such as shockwave therapy and LLLT emerged, as well as devices suitable for diagnostics and tissue measurements.

The continued mapping of how EPAs interact with and modulate pathological processes in biological tissue, especially in the inflammatory process and tissue repair, provided physiotherapists with the necessary tool to possess a more autonomous role in the pain management of musculoskeletal disorders. From this perspective, new areas of research questions become clear: the effect of adding EPAs to other established interventions must be continuously updated; treatment

combinations that enhance or reduce the effect of EPAs must be identified; and the optimal EPA treatment parameters and timing in relation to different stages of musculoskeletal pathology must be determined.

1.3 Discovery of Low-Level Laser Therapy

The history of using light for therapeutic purposes goes back more than 3,000 years, when people suffering from depigmentation of the skin were exposed to sunlight (Fitzpatrick and Pathak, 1959). During the eighteenth century, medical reports appeared demonstrated that sunlight could improve and accelerate the healing of many different conditions, such as skin ulcers, wounds, and rickets (vitamin D deficiency causing bone fragility) (Palm, 1890; Rollier and Rosselet, 1923; Chesney, 2012; Hamblin and Huang, 2014). Therapeutic exposure to sunlight, known as *heliotherapy*, increased in popularity during the nineteenth century and was recommended for several different conditions including depression, rheumatic diseases, and scurvy (Cauvin, 1815).

The first researcher to successfully put artificial ultraviolet (UV) light to medical use was Nils Ryberg Finsen (1860–1904). He developed a carbon arc lamp to treat Lupus Vulgaris and was honored with the Nobel Prize in Physiology or Medicine in 1903 for his pioneering work (Grzybowski and Pietrzak, 2012). During the twentieth century, the use of both natural and artificial UV light in medicine rose, and consequently, much research into the physics of light followed. In the late 1950s, Basov and Prokhorov and Townes were able to produce the first laser (Karlsson, 2000). A decade later, Theodore Maiman developed the first ruby crystal laser, operating at a fixed wavelength (694 nanometers [nm]) in the visible red spectrum (Maiman, 1960).

The discovery of LLLT is predominately associated with the pioneering work of Hungarian professor Endre Mester (1903–1984). In the 1960s, Mester implanted tumor cells beneath the skin in mice. In a failed attempt to destroy these malignant tumors with what he believed was a “high power” ruby laser, he instead discovered

that the skin incisions healed faster in treated mice. In fact, the custom-made laser used in the experiment was actually low powered, and the light accelerated tissue repair (Mester et al., 1968a). This observation formed the basis of a new experimental study, in which Mester successfully demonstrated faster wound healing in mice treated with LLLT (Mester et al., 1971). Inspired by these findings, Mester performed several clinical case studies on humans suffering from various chronic unhealed wounds and found that the wounds healed in 78% of the treated cases (Mester et al., 1985). Consequently, the effects of LLLT in biological tissue were referred to as *photobiostimulation*. However, the therapeutic application of LLLT extended beyond wound healing, and later research demonstrated that LLLT produced beneficial inhibitory effects in other conditions. It is now agreed that the term *photobiomodulation therapy* (PBMT) more accurately describes the mechanism by which low level lasers work in biological tissue.

1.3.1 Components and characteristics of LLLT irradiation

To produce laser light, three basic components are needed: a lasing medium, a power source, and a resonating cavity. Reflective mirrors, lenses, and other mechanical structures are added to manipulate the power output, irradiation mode (continuous or pulsed waves), and beam shape. The atoms or molecules from the lasing media are excited to higher energy levels by the power source, which generates photons of light (i.e., the emission of radiation). The lasing medium can be gaseous, liquid, solid crystal, or semiconductor. This component dictates the wavelength (nm) and the color of the light emitted from the machine (Baxter and Diamantopoulos, 1994).

Laser light is characterized by being monochromatic (single-colored) and of a defined wavelength. Because these waves of light travel in a synchronized phase, it is described as being highly coherent. The divergence of the laser beam is small and can be focused on a tiny area, which is referred to as *collimation*. Some devices also produce polarized light, meaning the waves of light are oriented in one plane only (Baxter and Diamantopoulos, 1994; de Freitas and Simoes, 2015). The biological and clinical relevance of coherence and polarization is not clear.

1.3.2 LLLT parameters

Lasers used for the treatment of musculoskeletal conditions are classified as 3B. These lasers typically have a wavelength ranging from 632 to 904 nm and a mean output power (MOP) between 5 and 500 milliwatts (mW). The energy absorption in water, cutaneous melanin, and hemoglobin differs with certain wavelengths but is lowest in the red and near-infrared spectrum (600–1000 nm) (Anderson and Parrish, 1981; Karu and Kolyakov, 2005). Infrared lasers (780–1000 nm) penetrate skin with less energy attenuation than red wavelength lasers do (600–700 nm) (Anderson and Parrish, 1981; Stolik et al., 2000). Thus, wavelength is an important parameter to consider if the targeted tissue is deeply situated.

Laser devices can deliver energy either continuously or in a pulsed mode. In continuous wave (CW) mode, the emitted energy (power output) over time is constant, whereas in pulsed mode, the energy may be delivered with high pulse peak powers and pauses. These pauses reduce the MOP of the laser, allowing it to still be classified as 3B even if the pulse peak power exceeds the 500 mW limit.

The therapeutic energy dose in LLLT is delivered in joules (J) and is calculated as the average power output emitted in watts (W) multiplied by seconds (s) of irradiation in a point. Energy dose is also reported as power density (mW/cm^2), which is defined by the power output divided by the laser beam spot size (cm^2) at the tissue surface. However, the validity of spot size measurements is debatable, as the distribution of power is not uniform across the laser beam (Baxter and Diamantopoulos, 1994; Nussbaum et al., 2003). Energy density is another parameter reported in LLLT literature and reflects the amount of energy (J/cm^2) received by the irradiated tissue. Energy density is calculated by adding time (s) to the equation of power density.

However, it has been argued that LLLT doses expressed in J/cm^2 are inadequate, as the calculations are based on the beam spot size, for which there is no agreement about how to define (Jenkins and Carroll, 2011). To overcome this shortcoming, Nussbaum et al. (2003) suggest that energy (J) per irradiated point should replace

power density and that the total energy of the treatment (J) should replace energy density.

1.3.3 LLLT penetration

The laser must penetrate the skin barrier with a sufficient amount of energy to modulate pathophysiological processes in musculoskeletal conditions. Wavelength is the main determiner regarding the penetration depth of a laser device. Lasers with longer wavelengths can penetrate tissue deeper than lasers with a shorter wavelength are able to. Furthermore, light energy can be "lost" before reaching deeper-situated pathology due to photon absorption and scattering in non-targeted tissue, and reflection of the skin surface (Bashkatov et al., 2011). The most commonly researched and clinically used wavelengths in LLLT are 632.8 nm helium-neon (HeNe), 810–830 nm gallium-aluminum-arsenide (GaAIAs), and 904 nm gallium arsenide (GaAs) (Enwemeka, 2000; Karu et al., 2001). The light penetration increases almost linearly with increasing wavelengths (450–1030 nm) in human skin samples (Ackermann et al., 2002). Red light lasers (600–700 nm) are easily absorbed by hemoglobin and melanin and penetrate approximately 4–5 mm into the skin (Ash et al., 2017). However, due to the circular-shaped scattering of red light, the indirect penetration depth should be deeper (de Freitas and Simoes, 2015). There is an "optical window" around 810 nm, where light can penetrate several centimeters (cm) into the tissue (Henderson and Morries, 2015; Hamblin, 2016). The elliptic-shaped scattering of infrared wavelengths can increase the indirect penetration even more (Mcleod, 2004; de Freitas and Simoes, 2015).

Although penetration depth in biological tissue is predominantly dependent upon wavelength, photons of light are more easily transmitted if the emitting diode is pressed firmly in contact with the skin. Conversely, the application of LLLT in non-contact mode will increase reflection and reduce the penetration of photons through the skin (de Freitas and Simoes, 2015).

1.3.4 Biophysical effects of LLLT treatment

The exact biochemical mechanisms responsible for the therapeutic effects of LLLT are not yet well established. It is suggested that the underlying mechanisms of action could be manifold, including molecular, cellular, and tissular responses. There is consensus that the effects of LLLT treatment occur according to the first law of photobiology, which states that for low-power light to have any effect on a living biological system, the photons must be absorbed by some molecular photoacceptors or chromophores. The construction of an action spectrum (i.e., a plot of biological effects against wavelength) supports the existence of cellular photoacceptors and signaling pathways stimulated by light (Huang et al., 2011; Chung et al., 2012).

The principal photoacceptors for monochromatic red and near-infrared light in mammalian cells have been attributed to the cellular respiratory chain, and the protein complex cytochrome c oxidase (CCO) located in the inner mitochondrial membrane (Karu, 1989; Karu and Afanas'eva, 1995). The application of LLLT to mitochondria increases the proton electrochemical potential, which results in increased adenosine triphosphate (ATP) production and electron transport. The activity of CCO is inhibited by nitric oxide (NO), which down-regulates cellular respiration (Fig. 1). It has been observed that LLLT releases NO from CCO, thereby preventing this process from occurring and promoting an increased cellular respiration rate (Karu et al., 2005; Moriyama et al., 2005).

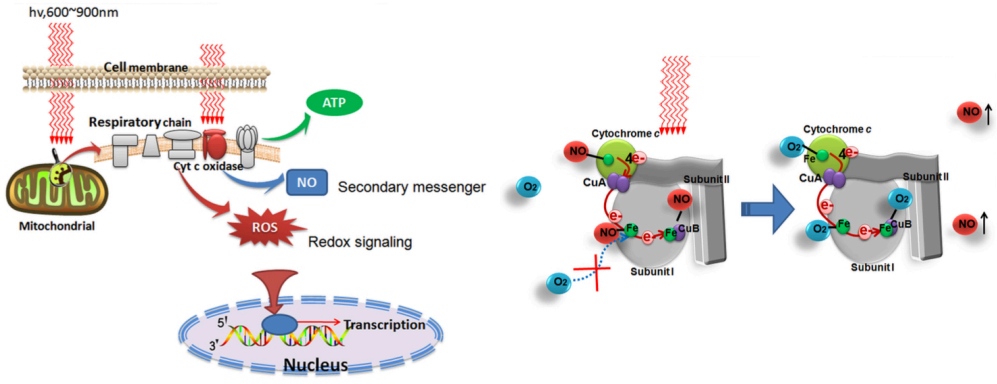

Figure 1. Schematic illustration of photon absorption by chromophores in the cell mitochondria and the release of NO, which is inhibiting CCO. Adapted from Huang et al. (2011).

Another possible mechanism of action for LLLT is an increased production of reactive oxygen species (ROS). ROS are natural by-products of cell oxidation and are involved in the signaling pathways from mitochondria to nuclei. Increased oxidation and expressions of ROS have been demonstrated after LLLT irradiation, which may influence the cellular redox state and induce several transcriptional changes. This cascade of cellular events triggers additional effects, such as increased cell proliferation and migration, a modulation of inflammatory mediators and growth factors, and increased tissue oxygenation (Huang et al., 2011; Chung et al., 2012; de Freitas and Hamblin, 2016).

An imbalance in the cellular redox state with high levels of ROS is seen in several soft tissue lesions; this is often referred to as *oxidative stress* (Ribeiro et al., 2016). The potential harmful effects of increased ROS on the healing process are controlled by cellular anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Barbieri and Sestili, 2012). LLLT has demonstrated a reduction of ROS in oxidatively stressed cells (Huang et al., 2013) and the attenuation of oxidative stress in several pathological lesions, such as arthritis, muscle injuries, and tendinopathy (Fillipin et al., 2005; dos Santos et al., 2017b; dos Santos et al., 2017a; De Marchi et al., 2017). Although the exact mechanisms are yet to be fully understood, LLLT seem to up-regulate the anti-oxidant defenses, reduce oxidative stress after injury, and increase ROS production in normal viable cells (Hamblin, 2017).

1.3.5 The effect of LLLT in tendinopathy treatment

When tendons are injured or traumatized, several biochemical mediators are released into the tissue. These include alterations in cytokine gene expression and metalloproteinase (MMP), increased levels of cyclooxygenase-1(COX-1) and cyclooxegynase-2 (COX-2), and, consequently, increased levels of inflammatory chemicals such as substance P and prostaglandins (Rees et al., 2013).

The anti-inflammatory effect of LLLT in tendinopathy has been extensively investigated over the years, and its ability to alter cytokine gene expression, COX-1,

COX-2, and the expression of inflammatory mediators have been demonstrated in several animal trials (Marcos et al., 2011; Marcos et al., 2012; Casalechi et al., 2013; de Jesus et al., 2015; Torres-Silva et al., 2015). There is some evidence that the anti-inflammatory effect of LLLT can be translated to humans, as reduced prostaglandin E_2 (PGE_2) levels have been measured by microdialysis in Achilles tendinopathy patients treated with LLLT (Bjordal et al., 2006b). LLLT has also demonstrated beneficial effects in the tendon repair process (Oliveira et al., 2009; Marcos et al., 2014), possibly by increasing collagen synthesis (Reddy et al., 1998), downregulating catabolic metalloproteinase enzymes (Marcos et al., 2014), regulating oxidative stress (Fillipin et al., 2005), and suppressing cell apoptosis (Sussai et al., 2010; Chen et al., 2011).

LLLT is used clinically to alleviate pain and accelerate recovery in tendinopathy. Although there is uncertainty about how to transfer consistent results from laboratory settings to clinical trials (Basford, 2005), the effect of LLLT appears to depend largely on the use of optimal laser doses. Systematic reviewers have identified such a dose-dependent effect of LLLT in lateral elbow (Bjordal et al., 2008) and generic tendinopathies (Tumilty et al., 2010), as well as in chronic joint disorders (Jang and Lee, 2012), neck pain (Chow et al., 2009), and osteoarthritis of the knee (Bjordal et al., 2007).

1.4 Cryotherapy

The history of medical cryotherapy dates back to 3000 BC, when ancient Egyptians used cold compresses to alleviate pain and reduce inflammation. The Greek physician Hippocrates advocated for the therapeutic use of cold to control hemorrhages and reduce the swelling of painful joints as early as 500 BC. Local application of cold for anesthetic purposes was described by monks in the mid-eleventh century (Grattan and Singer, 1952; Korpan, 2007). During the Napoleonic wars, Napoleon's surgeon, Dominique-Jean Larrey (1776–1842), used the vasoconstrictor and numbing effect of local cryotherapy to facilitate amputations (Larrey and Mercer, 1832). In the period of 1845 to 1851, Dr. James Arnott (1797–1883) focused much of his work on the use

of cold in anesthesia. He initially used cold to treat malignant disease and observed that even though this did not cure the patients, morbidity and pain were considerably reduced (Arnott, 1851; Bird, 1949; Korpan, 2007). The cooling of wounds to reduce local inflammation and pus formation was strongly recommended by German military surgeon Johan Friedrich August von Esmarch around the same time (Esmarch, 1865). His great passion for cryotherapy in emergency care led him to gain the nickname “Fritz the ice pack” (Beyer and Dick, 2001).

During the twentieth century, the application of ice packs and other local cryotherapy modalities became a widespread first aid treatment for acute soft-tissue injuries, in both domestic and sports medicine (Bierman, 1955; Blonstein, 1966). Physician Gabe Mirkin later suggested that first aid treatment for acute musculoskeletal injuries should be expanded to include four elements: rest, ice, compression, and elevation (resulting in the acronym RICE) (Mirkin, 1978). The RICE guidelines were quickly recognized by healthcare practitioners and implemented as the gold standard for the management of acute sports injuries (Wallace et al., 1979; Renström and Johnson, 1985), despite the discrepancy between the alleged therapeutic mechanisms and clinical effects (Swenson et al., 1996). The management of acute soft tissue injuries was later expanded to include the element of protection, and the acronym RICE was replaced by PRICE. Recently, Bleakley et al. (2011) suggested that the guidelines for the management of acute injuries should also reflect strategies for ensuring early optimal loading. Hence, a new acronym, POLICE (which represents protection, optimal loading, ice, compression, and elevation) was recommended to guide management.

1.4.1 Therapeutic mechanisms of cryotherapy

Cryotherapy can be defined as the application of any physical medium to the body that removes heat and decreases the temperature of the contact area and adjacent tissue (Nadler et al., 2004). The reduction in tissue temperature is regarded as the main trigger for any biophysical effect of cryotherapy.

These effects can be categorized as follows:

- Attenuation of inflammatory response (vascular changes)
- Effects on metabolism (reducing the sequela of injuries)
- Effects on nerve conduction (cold-induced neuropraxia)

Attenuation of inflammatory response (vascular changes)

The rationale for using cryotherapy in acute soft-tissue injuries is a general attenuation of the inflammatory process. Decreasing the temperature of the skin and underlying soft tissue reduces blood flow by causing a sympathetic vasoconstrictor reflex of the smooth muscle component on the vessel wall (Ho et al., 1994; Knobloch et al., 2007; Gregson et al., 2011). The cold-induced vasoconstrictor response and subsequent reduction in blood vessel diameter can help to reduce the amount of edema (Sloan et al., 1989; Deal et al., 2002; Schaser et al., 2007), which contains a large number of inflammatory cells.

However, we also suggest that the main possible mechanism to reduce edema is the decrease of biochemical activity at the inflammatory site, especially the enzymatic activity that cools down dramatically with the reduction of temperature. This hypothesis from our group make sense because increases in vascular permeability, which is the main phenomenon of edema, is not physiologically related to vasodilation (Claesson-Welsh, 2015). The leukocyte migration through the endothelial cells following soft-tissue injuries is allowed because of the increased vascular permeability (Menger et al., 1992; Vestweber et al., 2014). It has been demonstrated that cryotherapy reduces the amount of rolling and adhering leukocytes on the endothelium after muscle contusion injuries in rats (Menth-Chiari et al., 1999), which can also help to control the inflammatory process and edema. It is also argued that the increased viscosity of cooled blood increases blood flow resistance, which further contributes to reduced local circulation and decreased permeability of the blood vessels (McMaster, 1977; Swenson et al., 1996). A decrease in edema formation also puts the injured tissue under less mechanical tension, which together

with reduced stimulation of sensory nerve endings could have a pain-reliving effect (Hocutt Jr., 1981).

Although tendinopathy is a common musculoskeletal condition in both the general population and in sports medicine, the mechanism of effect and clinical benefit of cryotherapy for this condition are unclear. At present, very few studies have investigated the vascular and cellular effects of cryotherapy for tendinopathy. However, a significant decrease in capillary blood flow has been reported after local cryotherapy was performed on healthy human Achilles tendons (Knobloch et al., 2007). A reduced expression of COX-2 and the inflammatory mediator PGE_2 have also been demonstrated following local cryotherapy in acute mouse patellar and Achilles tendinopathy (Zhang et al., 2014). These findings have not been translated to human tendinopathy but could be explained by the temperature-sensitive expression of PGE_2. Synovial PGE_2 concentration decreased and correlated well with knee joint temperature in humans after postoperative cryotherapy (Stålman et al., 2011).

However, evidence for a beneficial anti-inflammatory effect of cryotherapy is somewhat conflicting. A single study using 20 minutes of local cryotherapy showed no effect on inflammatory cytokine expression in rats subjected to acute skeletal muscle injury (de Almeida et al., 2014). On the contrary, Schaser et al. (2007) demonstrated that six hours of percutaneous cooling following a crush injury to skeletal muscle in rats significantly reduced the number of leukocytes, granulocytes, and macrophages in the injured area. Another animal study suggested that local cryotherapy immediately after muscle injury could be harmful and significantly delay the regeneration process, despite decreasing inflammation. Twenty minutes of local cryotherapy following a crush injury to skeletal muscle in rats slowed the migration of macrophages and, thereby, the secretion of growth factors to the injured area (Takagi et al., 2011). In contrast, Ramos et al. (2016) found that intermittent cryotherapy (three 30-minute sessions, every two hours) during the first 48 hours after muscle injury in rats decreased macrophage invasion and inflammatory markers, without having a negative influence on the regeneration process.

Effects on metabolism

Cryotherapy is also used for reducing the metabolic rate of injured soft tissue. Following injury, cellular damage is directly caused by the trauma, but adjacent tissue areas may also suffer oxidative stress due to trauma-induced vascular disruption. A lack of oxygen hampers normal cellular processes and may lead to apoptosis and the release of more inflammatory mediators and edema (Rock and Kono, 2008). This process is referred to as *secondary hypoxic injury*. It is proposed that the slowing of cell metabolism by cryotherapy reduces the rate of oxygen consumption, and therefore has a protective effect on injured tissue. Evidence for such an effect on cell metabolism by cryotherapy has been demonstrated in rat muscle subjected to crush injuries and in humans suffering from acute traumatic injury to large joints (Ho et al., 1994; Merrick et al., 1999; Siqueira et al., 2016). Siqueira et al. (2016) found that intermittent cryotherapy (three 30-minute sessions of local cryotherapy) reduced ROS and oxidative stress in rat muscles subjected to crush injuries. Similar findings were reported after four hours of continuous icing, by Merrick et al. (1999). Ho et al. (1994) demonstrated that 20 minutes of local cryotherapy to human knees decreased arterial and soft-tissue blood flow as well as bone uptake, which can be explained by reductions in cell metabolism. It has also been demonstrated that cryotherapy reduces skeletal muscle damage in ischemic and reperfused muscle in rats, which could be attributed to a reduction in oxidative stress and inflammation (Mowlavi et al., 2003; Puntel et al., 2013).

Effects on nerves

The external cooling of tissue is well known for inducing an analgesic effect on peripheral nerves. The activation threshold of nociceptors situated in soft tissue decreases after cryotherapy, and the conduction velocity of pain signals slows down (Mc et al., 1984; Algafly and George, 2007; Herrera et al., 2010). The relationship between peripheral nerve conduction velocity and temperature was found to be roughly linear in a study of the saphenous nerve in cats (Franz and Iggo, 1968). Prolonged cooling and very low temperatures have been shown to cause transient

injury to peripheral nerves in humans (Bassett et al., 1992; Moeller et al., 1997). The threshold for inducing optimal cryoanalgesia in a clinical setting seems to occur at skin temperatures between 10 and 13˚C (Bugaj, 1975; Algafly and George, 2007).

1.4.2 Clinical effects of cryotherapy

To the best of our knowledge, no rigorous clinical trials investigating the effects of cryotherapy in human tendinopathy have been published. Only one basic research study has examined the potential anti-inflammatory effect of cryotherapy in tendinopathy (Zhang et al., 2014). There are numerous animal studies demonstrating beneficial biophysical effects of cryotherapy in other soft-tissue lesions such as muscle injuries (Hurme et al., 1993; Merrick et al., 1999; Menth-Chiari et al., 1999; Schaser et al., 2007; Puntel et al., 2013; Siqueira et al., 2016), but translational clinical studies demonstrating efficacy in humans are lacking (Bleakley et al., 2012).

In a systematic review including 22 RCTs, Bleakley et al. (2004) assessed the evidence in favor of using cryotherapy to manage a variety of acute soft-tissue injuries. The authors conclude that more high-quality trials are needed to provide evidence-based guidelines for the management of acute soft-tissue injuries. Similar conclusions were reported in a systematic review investigating the clinical effectiveness of RICE therapy in acute ankle sprains (van den Bekerom et al., 2012). The effect of cryotherapy in the acute phase post ACL surgery was assessed in another systematic review and demonstrated a significant effect on pain but no effects on functional outcomes (Raynor et al., 2005). In contrast, no effect on post-operative pain or function was found in a systematic review investigating the effect of cryotherapy after total knee arthroplasty (Adie et al., 2010).

There is a lack of scientific consensus regarding important treatment parameters such as the optimal application method, duration, frequency, and timing of cryotherapy (MacAuley, 2001). Consequently, there is considerable heterogeneity in treatment procedures across studies investigating the efficacy of cryotherapy, both in humans and animals. In addition, the majority of clinical cryotherapy trials investigating its

efficacy in soft-tissue lesions are of poor methodological quality (Bleakley et al., 2004; Hubbard and Denegar, 2004).

2. Objectives

The overall objective of this thesis was to investigate the clinical, biophysical, and biological effects of LLLT and cryotherapy for the treatment of tendinopathy.

Study I

The aim of Study I was to review literature for patients with shoulder tendinopathy to examine the effect of LLLT as monotherapy and the potential benefit of adding LLLT to exercises or a multimodal physiotherapy treatment regimen, including its effect magnitude compared with other electrophysical agents.

Study II

The aim of Study II was to investigate energy penetration from two therapeutic infrared lasers through skin and the Achilles tendon in healthy participants, before and after 20 minutes of cryotherapy.

H_0: Laser optical energy penetration through the Achilles tendon area does not change after 20 mins of cryotherapy.

H_1: There are changes in laser optical energy penetration through the Achilles tendon area after 20 mins of cryotherapy.

Study III

The aim of Study III was to investigate the anti-inflammatory and biomechanical effects of LLLT and cryotherapy as monotherapies and in combination with each other, one hour after acute Achilles tendon trauma in rats.

H_0: A single dose of cryotherapy followed by LLLT, or LLLT followed by cryotherapy, is no more effective on tensile strength and cytokine expression than no treatment, LLLT alone, or cryotherapy alone.

H_1: A single dose of cryotherapy followed by LLLT, or LLLT followed by cryotherapy, is more effective on tensile strength and cytokine expression than no treatment, LLLT alone, or cryotherapy alone.

3. Material and methods

3.1 Design

3.1.1 Study I

This study is a systematic review and meta-analyses of RCTs.

3.1.2 Study II

This study is a basic research study on in-situ human tissue. The study has a single factor experimental design and includes repeated measurements.

3.1.3 Study III

This study is a blinded RCT on LLLT and cryotherapy in in vivo rat Achilles tendons. The study has a post intervention test only, control group design.

3.2 Materials (subjects)

3.2.1 Study I

The systematic literature search identified a total of 395 potentially relevant trials. Two independent reviewers assessed these papers for suitability for inclusion. Only RCTs, controlled clinical trials, or trials with crossover design including human participants diagnosed with shoulder tendinopathy or subacromial impingement syndrome (SAIS) were eligible for inclusion. One group in the controlled trial had to be treated with LLLT (Class 3B) with reported outcome measures for pain or global improvement. Any disagreement regarding trial eligibility was resolved in consensus meetings between the reviewers. The final study population comprised 17 RCTs and 854 shoulder tendinopathy patients. The flowchart (Fig. 2) displays the results of the literature search and inclusion process.

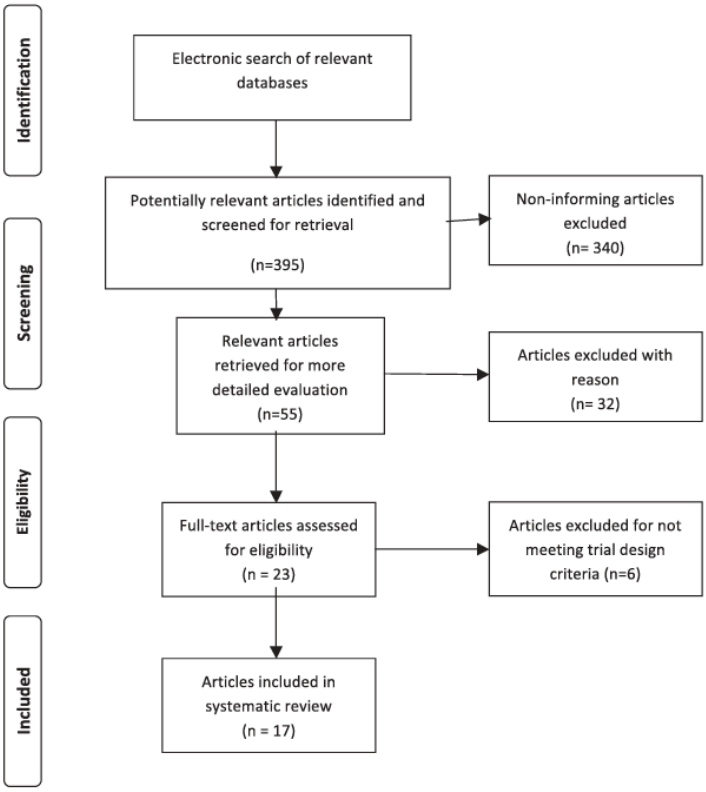

Figure 2. Flowchart illustrating the inclusion process

3.2.2 Study II

This study sample consisted of 54 healthy human Achilles tendons. Twenty-seven students from Bergen University College (20 women and 7 men), of light skin color and with ages ranging from 20 to 30 years, volunteered to participate in the study. Both the right and left Achilles tendons of the participants were included in the study sample.

3.2.3 Study III

This study was performed using 36 male Wistar rats weighing 200–250 g. The rats received food and water *ad libitum*. The rats were randomly divided into six groups, with six animals in each group:

1. Healthy control group (HCG)
2. Injured non-treated control group (ING)
3. LLLT group (LG)
4. Cryotherapy group (CG)
5. LLLT first/cryotherapy group (LCG)
6. Cryotherapy first/LLLT group (CLG)

3.3 Data collection (procedures)

3.3.1 Study I

A systematic literature search for clinical RCTs was performed on May 14, 2013 on Medline, PubMed, Embase, the Cumulative Index to Nursing and Allied Health Literature (CINAHL), the Physiotherapy Evidence Database (PEDro), and the Cochrane Controlled Trial Register Database. It is claimed that consulting Medline and Embase ensures a comprehensive literature search due to the minimal overlap between databases (Minozzi et al., 2000). However, we also applied the search strategy recommended by van Tulder et al. (2003). The keywords used were as follows: (low level laser therapy OR low intensity laser therapy OR low energy laser therapy OR phototherapy OR HeNe laser OR IR laser OR GaAIAs OR GaAs OR diode laser OR NdYag) AND (tendonitis OR tendinitis OR tendinopathy OR subacromial impingement OR impingement syndrome OR shoulder tendonitis OR shoulder tendinitis OR rotator cuff tendonitis OR rotator cuff tendinitis OR supraspinatus tendonitis OR supraspinatus tendinitis). Researchers in the field were contacted and contributed additional information. Article references were screened for potentially relevant trials. Unpublished material and abstracts were not included. No language restrictions were imposed.

Studies were subsequently categorized according to control group measures to answer these four core questions:

I. Does LLLT work in shoulder tendinopathy patients?
   - Control group receiving placebo LLLT or no therapy

II. Does LLLT work in combination with exercise?
   - Control group receiving exercise and placebo LLLT

III. Does LLLT work in combination with several physiotherapy interventions?
   - Control group receiving a combination of exercises and other modalities

IV. Does LLLT work better than other EPAs?
   - Control group receiving other EPAs

Two reviewers independently assessed the methodological quality of the included trials against the 10-point PEDro checklist (Maher et al., 2003), as exaggerated effect sizes have been reported for trials with weaker methodologies (Schulz et al., 1995). Disagreement regarding the rating of individual items between reviewers was resolved by consensus. Trials were labeled as "high," "moderate," or "poor," according to the total attainable sum score. The 17 included RCTs were also subjected to an in-depth assessment of possible confounders related to LLLT treatment parameters and procedures. Trials not adhering to the current treatment recommendations issued by the World Association for Laser Therapy (WALT) were classified as having "inadequate dosage."

3.3.2 Study II

The room temperature was recorded before each experiment began. The participants were placed in a prone position, lying on a bench, with both ankles placed off the end of the bench, leaving the ankle joint in a neutral position. The experiment was carried out in eight steps. The MOP of each laser device was recorded for 3 s before and after each irradiation. Lasers in the order of 810 nm and 904 nm wavelengths were applied, changing every second time. Following is a list of the eight steps:

1. A pen mark was drawn on both Achilles tendons, 2.5 cm proximal to the superior ridge of the Os calcaneus.

2. The Achilles area was ultrasonographically scanned in both the longitudinal and transverse planes, and one image in each plane was saved for further processing. The

longitudinal image covered the superior tip of the Os calcaneus and the distal third of the Achilles tendon. The transverse image was obtained with the probe covering the pre-drawn pen mark on the Achilles.

3. A thermographic camera was placed approximately 50 cm over the subject's ankles, covering both Achilles tendons, and one image was recorded.

4. Laser irradiation was performed with the subject lying on their side and the Os calcaneus placed on a mobilization wedge. The laser probe was placed in a tripod and pressed firmly to the medial side of the Achilles tendon (2.5 cm proximal to the Os calcaneus). A handheld optical power meter (OPM) was pressed firmly against the lateral side of the Achilles at the same level. The amount of energy penetrating through the skin and tendinous tissue was recorded after 1 s, 30 s, 60 s, 90 s, and 120 s irradiation by the 904 nm laser, and after 1 s, 30 s, and 60 s irradiation by the 810 nm laser.

5. The subject was lying prone during 20 mins of tissue cooling. An icepack containing 28 icecubes was applied directly over each Achilles area, covering the targeted distal third of the tendon. The subject was not allowed to change body position to ensure an equal and comparable cooling effect.

6. The icepacks were removed after 20 minutes, and a post-cooling thermographic image was recorded (per step 3).

7. The subject was again positioned on their side, and the post-cooling LLLT procedure was performed (per step 4). While measuring laser energy penetration on one Achilles, the other Achilles was kept cool.

8. The Achilles areas were scanned with real-time ultrasonography (RTUS) (per step 2). While scanning one Achilles, the other Achilles was kept cooled.

After all the participants had completed the procedures, the RTUS images were scored. The built-in caliper of the US device was used to measure the tissue size. In the longitudinal images, tendon thickness in an anterior-posterior (A-P) direction was

measured at 2.5 cm proximal from the tip of the Os calcaneus. In transversal images, tissue was measured 0.4 cm profound from the dorsal skin surface overlaying the Achilles. Tendon thickness was measured as the distance within the medial-lateral (M-L) border of the peritenon, and the total amount of tissue the laser irradiation should penetrate was measured as the skin-to-skin M-L distance.

3.3.3 Study III

The rats were anesthetized with ketamine/xylazine (100 and 20 mg kg^{-1}, respectively), and individually positioned with the right hind limb and knee extended and the ankle in 90° dorsiflexion. Then, a mini-guillotine consisting of a block weighing 200 grams (g), with a blunt edge 2 mm wide was dropped from 20 cm, with guided support, to induce injury to the Achilles tendon. Previous studies have shown that the mini-guillotine model produces acute inflammation and the degradation of tendon collagen (Oliveira et al., 2009; Joensen et al., 2012). After 24 hours, the animals were euthanized with an overdose of halothane for biomechanical, histological, and biochemical analyses. The skin and connective tissue was removed in order to harvest the Achilles tendons for further analyses. A sample of six tendons per group were available for analyses of cytokine expression. Four tendons were used for the biomechanical procedure, and the remaining two tendons were used for histology examinations. All procedures were performed by one observer. To ensure consistency in the analyses and the reproducibility of the histology and cytokine results, a second laboratory duplicated the blinded analyses, and any disagreement was resolved by consensus-based discussions.

Histology procedure

The tendon tissue samples were fixed in a 10% formalin solution (Formaldeído PA, Synth, Diadema, São Paulo, Brazil) for 72 hours. The samples were then dehydrated in a series of alcohol baths (Alcohol PA, Synth, Diadema, São Paulo, Brazil), beginning with 50% and progressing to 100%. The samples were cleared in xylol (Xilol PA, Synth, Diadema, São Paulo, Brazil) for four hours and embedded in Paraplast® (Tyco, Mansfield, MA, USA) for four hours for impregnation. Slices 5

micrometers (μm) in thickness were cut, stained with hematoxylin and eosin, and mounted on glass slides for histological evaluation.

Cytokine procedure

Interleukin (IL)-1β, IL-6, and IL-10, and tumor necrosis factor alpha (TNF-α), levels in the tendon samples were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. For this purpose, 96-well plates (R&D Systems, Minneapolis, MN, USA) were coated with 100 microliters (μL) of monoclonal antibody for each cytokine (anti-IL-1β, IL-6, and IL-10) and diluted in sodium carbonate buffer (Sigma, Aldrich, Brazil) (0.1 M, pH 9.6); anti-TNF-α was diluted in sodium phosphate buffer (0.2 M, pH 6.5). The plates were incubated (4°C) for 18 hours. For blocking, the plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) four times, and then filled with 300 L/well of blocking solution (3% gelatin in PBST; Sigma, St. Louis, MO, USA) at 37°C for three hours and subjected to a new cycle of washes. Next, 100 μL of properly diluted samples or standards of recombinant cytokines were added to the plate and left for 18 hours at 4°C. After washing, 100 μL of the respective biotinylated antibodies for the specific detection of each cytokine was added and left for one hour at room temperature. After the plates were washed, 100 μL of streptavidin-peroxidase was added and left for one hour at room temperature (22°C), followed by further washing. The reaction was revealed by adding 100 μL/well solution of 3,3', 5,5'-tetramethylbenzidine and stopped by adding 50 μL/well of sulfuric acid (2 Molar [M]).

Biomechanical procedure

Testing was performed immediately after tendon removal to avoid the influence of incubation (Screen et al., 2006). The mechanical characteristics were extracted from the force-displacement curves obtained from cyclic loading at a constant velocity of 1 millimeter per minute (mm/min) for each tendon (Marcos et al., 2014). At each cycle, tendon displacement was increased by 10% and force was released until 0.1 Newton

(N) was reached. The sequence was repeated until complete tendon failure was achieved.

3.4 Intervention (treatment)

3.4.1 Study I

Participants were treated with Class 3B lasers in the infrared spectrum with wavelengths ranging from 830 nm to 1064 nm. The mean treatment duration across the 17 included trials was three weeks. A total of 12 out of 17 trials used a direct laser application technique targeting the tendon pathology. In one study, a non-direct scan technique was used. The remaining four studies did not report the mode of LLLT application. Energy dose per irradiated point ranged from 0.72 to 4.5 J, and the total number of irradiated points ranged from 1 to 10 J.

3.4.2 Study II

Two commercially available infrared Class 3B lasers were used for irradiation of the Achilles tendons. The 810 nm-wavelength laser (Thor LX2, Thor UK) operates in CW mode with a 200mW MOP, a spot size of 0.0314 cm^2, and a power density of 6.37 W/cm^2 (manufacturer's specifications). The 904 nm-wavelength laser (MID-laser, Irradia Sweden) operates in a super pulse wave (SPW) mode with a peak power of 20W, pulse train frequency of 6 kilohertz (kHz), pulses at 100 nanoseconds (nsec) (10^{-9} sec), a width of 30.000 pulses per s, a 60 mW MOP, a spot size of 0.0364 cm^2, and a power density of 1.67 W/cm^2 (manufacturer's specifications).

The left and right Achilles tendon of each subject was irradiated before and after 20 minutes of ice application. Tendons were irradiated for a total of 60 s with the 810 nm laser and 120 s with the 904 nm laser (Table 1). Domestic ice cube bags containing 28 ice cubes per bag were produced in a freezer at -10°C and used for cooling the tendons.

Table 1. LLLT irradiation intervals

904 nm	810 nm
Seconds (Joule)	Seconds (Joule)
MOP	MOP
1 (0.06)	1 (0.2)
30 (1.8)	30 (6)
60 (3.6)	60 (12)
90 (5.4)	
120 (7.2)	

3.4.3 Study III

All treatments were performed one hour after administering the tendon trauma. The LG, LCG, and CLG were treated with a single LLLT application using an infrared laser unit (DMC®, São Carlos, Brazil). The laser unit emitted a continuous optical output of 100 mW with a wavelength of 810 nm to a spot size area of 0.028 cm^2, providing a power density of 3.57 W cm^{-2}. Laser irradiation was performed with skin contact in the middle portion of the Achilles tendon. The laser delivered energy of 3 J in one single point, corresponding to an irradiation time of 30 s. The laser treatment parameters were chosen according to previous studies performed by our research group (Marcos et al., 2011; Marcos et al., 2012).

The CTG, LCG, and CLG received cryotherapy to the injured Achilles tendons for 20 minutes. Small rubber bags containing 10 g of crushed ice were fixed to the region of the Achilles tendon with rubber bands, and the hind limb was kept elevated. The ice came from a freezer that held a stable temperature of -20 °C.

The HCG was not injured and not treated. The ING was injured but received no treatment.

3.5 Outcome measurement instruments

3.5.1 Study I

Methodological assessments of the included trials were scored using the 10-point PEDro scale. The intraclass correlation coefficient (ICC) for the total PEDro score is reported at 0.68 (CI=0.57–0.76) and interpreted to have “fair” to “good” reliability. Consensus scores are demonstrated to be in exact agreement with PEDro reviewers 46% of the time and differs by 2 points or less 99% of the time (Maher et al., 2003). We interpreted scores differing by one point or less to be in line with PEDro.

The WALT recommendations for treating tendinopathies with LLLT were used for examining the validity of the treatment procedure and the LLLT doses for each included trial. We used a calculation formula to compute the exact joules per point treated and total energy (J) per treatment session. LLLT treatment was ascribed as “inadequate” when LLLT doses ranged (± 50%) outside the stated therapeutic window regarding joules per point and/or total dose per treatment session.

3.5.2 Study II

Skin temperature was measured by a thermographic camera (Flir System, T640-25, USA) and ancillary software (FLIR Tools+). This software includes tools for quantifying the recorded temperature. The camera measures temperature with a precision of 50 millikelvin (mK) at 30°C and has an accuracy of ±2% (manufacturer’s specifications).

The laser MOP was measured with an OPM system (Thorlabs Instruments, NJ, USA). The OPM system consists of a PM100 display unit with a sample rate of 6 hertz (Hz), an accuracy of ±1%, and a S121B silicon sensor. The S121B sensor has an aperture diameter of Ø=9.5mm with an optical power range of 500 nW to 500 mW and an accuracy of ± 5% (manufacturer’s specifications).

The RTUS instrument was a Logiq-S8 (GE Healthcare, Minneapolis, USA). The unit has a 19” liquid-crystal display (LCD) screen and operates in B-mode with high-

definition speckle reduction imaging (SRI-HD), CrossXBeam resolution, and coded harmonic imaging. The linear matrix transducer (ML16-15-D) was tuned to a frequency of 12 megahertz (MHz). The RTUS instrument has a caliper (Somet INOX CHROM, Czech Republic) with measurement precision scaled in millimeters (mm).

3.5.3 Study III

The optical power output of the laser unit was measured before, halfway through, and after the experiment with a Thorlabs power meter (Thorlabs Instruments, NJ, USA).

Skin temperature over the Achilles tendon area was measured before cooling, after 3 mins, and after 10 mins of cryotherapy with a thermographic camera (Flir System, ThermaCAM S65HS, Boston, MA).

Histology samples were photographed using a microphotographic camera (Dino-Lite digital microscope®, Dino-Eye AM-423X model, Brazil) connected to a personal computer. Standardized photos were taken of all groups at a magnification of 100× at the specimen level.

A universal tensile test machine (zL2.5, Zwick, Roell, Germany) was used to perform mechanical testing of the tendons.

Cytokine readings were performed in a Spectrum Max Plus 384 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 450 nm, with correction at 570 nm. Sample concentrations were calculated from standard curves obtained from the recombinant cytokines.

3.6 Statistics

3.6.1 Study I

Two reviewers extracted data for analysis in the statistical software program Review Manager (RevMan) version 5.2. If insufficient data were reported in the original articles, authors were contacted to provide additional information. Testing for statistical heterogeneity was performed using the chi-square test, which determined

whether a random or fixed effects model was applied. Subgroup analyses were preplanned, as heterogeneity was expected in LLLT treatment parameters, composition of treatment interventions, and exercise designs. Sensitivity analysis was performed to reveal trials contributing to a statistical heterogeneity, and the meta-analysis was considered to be valid if the inclusion of these trials did not influence or inflate the overall effect size. End-of-treatment data were pooled as follows.

We extracted the means and standard deviation (SD) for comparisons using the outcome measures of pain, function, and active range of shoulder abduction. Pain intensity on a 100 mm visual analogue scale (VAS) and degrees of active abduction was defined as the pooled estimate of the difference in change between the means of the treatment and control groups, weighted by the inverse of the SD of change for each study (i.e., the weighted mean difference [WMD] of change between the groups). The variance was calculated from the trial data and presented as 95% confidence intervals in mm on VAS and degrees of abduction. Improved global health status was defined as any of the following categories: “improved,” “good,” “better,” “much improved,” “pain-free,” or “excellent.” The relative risk (RR) for change in health status was calculated by pooling the number of improved patients.

Shoulder function was measured by several different disability scales. If pooling of the data was justified, the standardized mean difference (SMD) of change between groups with a 95% confidence interval were calculated. SMD is a unitless pooled estimate of the difference between the mean of the treatment and control group, weighted by the inverse of the pooled SD of change between the groups.

3.6.2 Study II

The mean (± the standard error of mean [SEM]) amount of laser energy (mW) penetrating the Achilles area was calculated pre- and post-cooling. The difference in energy penetration pre- and post-cooling is illustrated as the mean change in mW and percentage of MOP when measured directly into the OPM. The mean (±SEM) tendon and skin thickness pre- and post-cooling is displayed in cm. Energy loss per cm of tissue was calculated as MOP (mW, no obstacle) – MOP (mW, through tissue) / skin-

to-skin tissue thickness in cm. Student's pairwise t-tests were used for pre- and post-cooling comparisons of energy penetration, tendon/skin thickness, and energy loss, with the statistical significance level set at $p<0.05$. Microsoft Excel (Microsoft Office Excel 2011) was used for statistical analysis and graphics.

3.6.3 Study III

The Kolmogorov–Smirnov test was applied to assess the normality of distribution of the dependent variables. The Kruskal–Wallis test, followed by pairwise comparisons using the Mann-Whitney U test, was used to test for differences between the ING and the four treatment groups (LG, CTG, LCG, and CLG). The HCG was compared to the ING to validate the trauma model. Biomechanical data and cytokine concentrations are expressed as medians and interquartile range. Differences were considered statistically significant at $p<0.05$. Tendon specimens were evaluated qualitatively for differences in collagen organization, tenocyte infiltration, and degree of eosin staining. To increase readability, the findings are presented quantitatively in a four-point histology score table, and the mean score for each group is reported.

3.7 Ethics

3.7.1 Study I

There is no universally accepted standard for ethical assessments in systematic reviews (Vergnes et al., 2010). Consequently, no systematic ethical assessments beyond the standards stated by the original authors were performed in this study.

3.7.2 Study II

LLLT is considered harmless when applied to healthy tissue. Due to the consistent absence of adverse events and the lack of treatment intentions in this study, no special ethical approval was necessary. This was confirmed orally by the Regional Committees for Medical and Research Ethics (REK) prior to the start of the study.

3.7.3 Study III

The protocol for Study III was approved by the University of Sao Paulo Animal Research and Care Committee (Appl. no 144/78-2).

4. Summary of results

4.1 Study I

All trials were of either moderate or high methodological quality (mean PEDro score of 7). While eleven studies (paper I, table 4) reported positive effects of LLLT, six studies reported no significant effects after LLLT treatment (paper I, table 5).

Four of the studies with non-significant trial results were performed with inadequate LLLT dosages (paper I, table 5). Three of these studies used the Roland (IR 904, Pagani) laser device, which has been revealed as faulty, with power outputs less than 1% of the stated output by the manufacturer (Bjordal, 2010). Although this is a potentially valid reason for excluding these papers from the review, these trials were subgrouped in the RevMan 5.2 analyses.

Eleven out of 15 trials (73%) that reported pain relief on VAS favored laser over placebo, no treatment, or other modalities. Statistically significant ($p<0.05$) effect sizes were found in 9 of these trials (53%), of which 7 trials exceeded the minimal important change of 14 mm on the VAS (Tashjian et al., 2009). When trials with inadequate laser dosage were excluded, 10 out of 11 trials (91%) displayed pain reductions exceeding 10 mm on the VAS. The 7 trials that provided data on global improvement were all in favor of LLLT, 4 of these trials with statistically significant effect sizes.

All included trials presented end-of-treatment data in a format that made it possible to use RevMan 5.2 for calculations of effect sizes in one or both of the primary outcome measures. Continuous data for pain relief on a 100 mm VAS was available from 13 trials in a format that made statistical pooling possible.

Following are answers to the four core questions posed at the beginning of Study I:

I. *Does LLLT work in shoulder tendinopathy patients? (Comparison with placebo LLLT and no therapy)*

LLLT was found to be significantly better ($p<0.0001$) than placebo LLLT or no therapy at the end of treatment, with a WMD of 20.41mm (95% CI: 12.38–28.44) on the VAS (paper I, fig. 2). Data from 5 trials found LLLT to be significantly better ($p=0.004$) than placebo or no therapy, with an overall RR of improvement at 1.96 (95% CI: 1.25–3.08) (paper I, fig. 4). Two trials comparing LLLT to placebo showed significantly better ($p<0.0001$) shoulder function with a SMD of 1.01 (95% CI: 0.53–1.50) at the end of treatment (paper I, fig. 5).

II. Does LLLT work in combination with exercise? (Comparison with exercise and placebo LLLT)

Data from two trials showed that LLLT and exercise were significantly better ($p<0.0001$) than exercise and placebo laser, with a WMD of 16.00 mm (95% CI: 11.88–20.12) on the VAS (paper I, fig. 2).

III. Does LLLT work in combination with several physiotherapy interventions? (Comparison with exercise and other modalities)

When used as an adjunct to exercise and other therapies, LLLT was significantly ($p=0.02$) better than other therapies, with a pain reduction of 12.80 mm (95%CI: 1.67–23.94) on the VAS (paper I, fig. 2). Two trials made statistical pooling on global improvement possible for LLLT as an adjunct to other interventions. The RR for improvement was significantly higher ($p=0.006$) at 1.51 (95%CI: 1.12–2.03), in favor of LLLT (paper I, fig 4.). When LLLT was used as an adjunct to other interventions, improvement in shoulder function was not significantly different ($p=0.27$) from placebo laser, with an SMD of 0.33 (95% CI: -0.26–0.91) (paper I, fig. 5). We were able to pool data for shoulder function expressed as improvement in active shoulder abduction for two trials investigating the effect of LLLT as an adjunct to other interventions. Although the overall effect was negligibly in favor of laser therapy, the effect did not reach statistical significance ($p=0.09$), with a WMD of 8.08 degrees (95% CI: -1.27–17.43) (paper I, fig. 6).

IV. Does LLLT work better than other EPAs? (Comparison with other EPAs)

Two trials compared laser therapy to ultrasound and both trials favored LLLT, with a significant effect size on the VAS. However, significant heterogeneity in treatment procedures and a lack of trial data did not justify statistical pooling (paper I, fig. 3).

V. Trials subgrouped for not adhering to WALT treatment recommendations

In trials performed with inadequate laser dosages, LLLT was not significantly better (p=0.38) than controls, with a WMD of 2.77mm on the VAS (95% CI: -3.46–8.99) (paper I, fig. 3). Shoulder function was not significantly different (p=0.26) from controls, with an SMD of -0.17 (95% CI: -0.48–0.13) (paper I, fig. 5). Data from three of the four trials with inadequate laser dosages showed no difference in active abduction with the application of LLLT, with a WMD of -0.08 degrees (95% confidence interval [CI] -0.81–0.65) (paper I, fig. 6).

4.2 Study II

The H_1 hypothesis, "Laser optical energy penetration through the Achilles tendon area changes after 20 mins of cooling," was confirmed, and the H_0 hypothesis, "There are no changes in energy penetration through the Achilles tendon area after 20 mins of cooling," was rejected.

All study subjects (n=54) completed the experimental procedure according to protocol (paper II, fig. 1). The baseline room temperature was 21.9°C (SD ±0.7). Thermography recordings before cooling showed the Achilles mean skin temperature to be 28.2°C (SD ±1.8). Skin temperature dropped to a mean value of 4.8°C (SD ±3.6) after 20 mins of cooling.

MOP (no obstacle)

The 904 nm laser was stable and not significantly different (p=0.22) during the 120 s pre-cooled and post-cooled irradiations at 54.5 mW (SD ± 2.41) and 55.2 mW (SD ±

3.05) ($p=0.22$), respectively. The 810 nm laser was stable during the 60 s laser exposure period, at 202.7 mW (SD 3.75) before ice and 204.3 mW (SD 2.99) after ice. The MOP increased by 1.6 mW in the post-ice measurement, which was found to be a statistically significant increase ($p<0.05$).

904 nm laser energy penetration before and after ice (per steps 4 and 7)

Irradiation with the 904 nm laser showed a linear increase in tissue penetration with time (30 s–120 s), and during this laser exposure period, penetration increased by 0.03 mW (19%) before cooling and 0.09 mW (45%) after cooling. The energy penetration after cooling was significantly increased ($p<0.01$) at all time intervals (30 s, 90 s, and 120 s) compared to the before-cooling measurements (paper II, fig. 3a, table 3).

The percentage of energy penetrating the Achilles area during the exposure period was 0.34–0.39% of the MOP before cooling (i.e., a relative increase of 15%). The percentage of energy penetrating the same area was 0.43–0.52% of the MOP after cooling (i.e., a relative increase of 21% during the laser exposure period) (paper II, fig. 3b). Laser energy loss per cm of tissue was significantly ($p<0.05$) higher at all time intervals after cooling (paper II, fig. 4a).

810 nm laser energy penetration before and after ice (per steps 4 and 7)

Irradiation with the 810 nm laser showed a stable energy penetration during the 60 s exposure. Laser energy penetration at the interval 30 s–60 s increased 0.012 mW (2%) in non-cooled Achilles and 0.02 mW (3%) in cooled Achilles tendons. The amount of energy penetrating the tissue in the Achilles area after cooling was significantly increased ($p<0.01$) at all time intervals compared to the before-cooling measurements (paper II, fig. 5a, table 3).

The percentage of energy penetrating the Achilles area was 0.24–0.25% of the MOP before cooling and 0.30–0.31% after cooling (paper II, fig. 5b), meaning there was a relative increase of 4% before and 3% after cooling during the laser exposure period.

Laser energy loss per cm of tissue was significantly ($p<0.05$) higher at all time intervals after cooling (paper II, fig. 4b).

RTUS measurements before and after ice (per steps 2 and 8)

The mean thicknesses of the Achilles tendons before cooling were 0.51 cm (SD ±0.07) in the longitudinal (A-P size) images and 1.83 cm (SD ±0.40) in the transversal (M-L size) images. The total amount of tissue the laser should penetrate before cooling (i.e., the M-L size skin-to-skin distance) was 2.20 cm (SD ±0.30).

After 20 mins of cryotherapy, the mean A-P thickness of the Achilles tendons were not significantly different ($p=0.49$) in longitudinal images compared to the before-cooling measurements, at 0.51 cm (SD ±0.07). The mean transversal M-L Achilles tendon thickness after cooling was 1.77 cm (SD ±0.38) and was significantly reduced ($p=0.03$) compared to before-ice measurements. The M-L skin-to-skin distance after cooling was significantly reduced ($p=0.05$) compared to the before-cooling measurements, at 2.14 cm (SD ±0.34) (paper II, fig. 6).

4.3 Study III

The H_1 hypothesis, "A single dose of cryotherapy followed by LLLT, or LLLT followed by cryotherapy, has an effect on inflammation and tendon biomechanics," was confirmed. The H_0 hypothesis, "A single dose of cryotherapy followed by LLLT, or LLLT followed by cryotherapy, has no effect on inflammation and tendon biomechanics," was rejected.

The biomechanical properties measured as force (N) and displacement (mm) at the rupture point were statistically different across the ING and the four treatment groups (X^2 (4)=5.3, $p=0.02$ and X^2 (4)=5.3, $p=0.02$, respectively). The expression of inflammatory cytokine IL-1β, TNF-α, and IL-6 were significantly different across the ING and the four different treatment groups (at X^2 (4)=6, $p=0.00$; X^2 (4)=18.5, $p=0.00$; and X^2 (4)=5.9, $p=0.00$, respectively). No statistically significant differences across the groups for the expression of IL-10 was found at X^2 (4)=2.5, $p=0.64$.

Combination of LLLT and cryotherapy (comparison against ING)

The pairwise comparisons against the ING showed improved force (p=0.02) and displacement rates (p=0.04) for the CLG. The LCG showed significantly lower displacement (p=0.04), but there was no difference in force at the rupture point (p=0.15) compared with the ING (paper III, fig. 1, table 2).

Both groups combining LLLT and cryotherapy showed significantly reduced levels of IL-1β (p=0.00, p=0.01) and TNF-α (p=000, p=0.02). The expression of IL-6 increased significantly (p=0.00) in both groups, while IL-10 remained at levels (p=0.75, p=0.63) similar to those of the ING (paper III, fig. 2a–d, table 3).

The histology analyses of the CLG showed a near-normal appearance, with no sign of tendon rupture. As seen in the healthy controls, the collagen bundles were lightly stained and largely uninterrupted. The distribution of tenocytes was situated regularly between the collagen bundles (paper III, fig. 3). As such, this group achieved a histology score of three, similar to that of healthy control tendons (paper III, table 4). The LCG showed consistent signs of moderate tendon rupture. There were larger regions of irregularly shaped collagen bundles and heterogeneous staining compared to the other treatment groups. Tenocytes were scattered irregularly around the lesion (paper III, fig. 3). This treatment group displayed morphological features that most closely resembled those of the ING, resulting in a total histology score of eight out of twelve in our analyses (paper III, table 4).

Monotherapy groups (comparison against ING)

Tendons treated with LLLT tolerated higher forces (p=0.04), but they were not significantly less displaced than the non-treated tendons (p=0.19). Tendons treated with cryotherapy were significantly less displaced (p=0.02), but they did not tolerate higher forces at the rupture point than the non-treated tendons (p=0.56).

The LLLT group showed significantly decreased levels of IL-1β (p=0.00) and bordered the threshold for statistical significance (p=0.06) for IL-10 cytokine expression (paper III, fig. 2a/d). TNF-α expression was non-significantly (p=0.15)

reduced in the LLLT group (paper III, fig. 2b), whereas IL-6 expression remained equal ($p=0.42$) to that of the ING (paper III, fig. 2c). The cryotherapy group displayed cytokine expression in favor of an anti-inflammatory response, but no significant differences were found between the groups (paper III, fig. 2a–d).

The histology analyses of the groups treated with LLLT or cryotherapy alone had a general appearance indicating slight tendon rupture. Small areas of irregularly shaped collagen bundles and disruption were evident, with somewhat larger regions of dark staining in the cryotherapy group compared with the LLLT group. The distribution of tenocytes was slightly disorganized, with small cluster formations around the lesion (paper III, fig. 3). LLLT and cryotherapy treatment resulted in total histology scores of six and seven, respectively, out of twelve (paper III, table 4).

5. Discussion

This thesis investigated the clinical, biological, and biophysical effects of LLLT alone and in combination with cryotherapy for the treatment of tendinopathy. To explore these issues, a systematic review, a basic research study on in-situ human tissue, and an RCT were the methodologies used. All these approaches are robust and well-established evidence-based research designs. Regarding the main purpose of the thesis, there is evidence from the systematic review that LLLT is effective if guidelines regarding dosage and application are followed. The systematic review identified cryotherapy as a possible confounder of LLLT, with the potential to induce inhibitory effects and negatively influence treatment outcomes for LLLT.

Interestingly, our basic research study demonstrated that the penetration of laser light through healthy Achilles tendons increased significantly after 20 min of cryotherapy. Finally, the results of our RCT animal trial were polarized and showed both positive and negative biological effects using the combination of ice and LLLT, depending on the order of therapy administration. The rule of thumb from this study was as follows: ice followed by LLLT seems like a good idea, but LLLT followed by ice does not seem wise. These novel findings illuminate a new area in LLLT research and raise several questions that require further exploration and verification. The main points and issues raised in this thesis will be discussed in conjunction with current knowledge regarding LLLT and cryotherapy for the treatment of tendinopathy. The results in each of the studies have been discussed in the respective papers. In the following chapter, the general aspects, including the main findings in this thesis and methodological considerations across the studies, will be discussed.

5.1 General discussion

The history of physiotherapy is a tale of relatively young profession. The Chartered Society of Massage and Medical Gymnastics was established in the early 1920s with support from medical community (Chartered Society of Massage and Medical Gymnastics, 1929). Physiotherapy quickly became a profession that developed within

the framework of biomedicine, with a subordinate and assistive role to medical doctors. Physiotherapists have traditionally been dedicated the role of treatment executioners, leaving the defining powers of diagnostics to doctors. Since diagnostics guides therapy, much research into treatment methods for musculoskeletal diseases have been initiated and defined by others than physiotherapists. This can be easily illustrated by conducting a simple database search in PubMed. While a search combining the terms “low level laser therapy” or “cryotherapy” and “musculoskeletal” results in a little more than 200 papers, “non-steroidal anti-inflammatory drugs” and “musculoskeletal” increase the amount of published papers fourfold. From this perspective, LLLT may still be regarded a novel treatment in musculoskeletal diseases compared to non-steroidal anti-inflammatory drugs (NSAIDs), even though both interventions arose as potential treatment options around 50 years ago (Adams and Cobb, 1967; Mester et al., 1967; Rainsford, 2007). Furthermore, early studies stating that low-level lasers could produce beneficial biological effects and act at a molecular level were met with skepticism and discredit by a majority of scientists. Consequently, the method of LLLT was established, and still remains, outside of mainstream medicine (Hamblin and Huang, 2014).

The term *evidence-based medicine* (EBM) was introduced in the 1990s and marks a transition in patient decision-making processes. It is emphasized that clinical decision making should be based on the best level of evidence from clinical studies rather than subjective expert opinions and clinical expertise (Guyatt et al., 1992). Extending this idea, a hierarchical model for grading levels of evidence was suggested, placing a systematic review with meta-analysis at the highest level of evidence (Evans, 2003). Although the main purpose of systematic reviews is to summarize existing research on a topic and make judgements regarding evidence, an equally important task is to identify gaps in the existing literature and direct future research. The requirement to bring the best evidence into clinical decision making highlights the need for more research in physiotherapy-related interventions. The number of RCTs and systematic reviews published per year in the PEDro database increased from less than 500 in the early 1980s to more than 2,000 in the late 1990s, and along with this, there was also a

substantial improvement in the methodological quality of the studies (Moseley et al., 2002). The cumulative number of physiotherapy papers in the PEDro database has now exceeded 37,000 (PEDro, 2017).

Another factor that has led to confusion in scientific and clinical laser therapy literature is the lack of consistency and consensus regarding terminology. The great heterogeneity in nomenclature has most likely contributed to a loss of momentum and increasing skepticism in the emerging field of LLLT. In addition to the term *laser biostimulation*, introduced by Mester et al. (1968b), other names such as *cold laser*, *soft laser*, *low-power laser therapy*, *low-intensity laser therapy*, and *low-level laser* (or *light*) *therapy* were used by the industry and researchers. Although LLLT is a well-established, searchable Medical Subject Headings (MeSH) term and extensively used by researchers, patients, and clinicians, it has been criticized for not accurately reflecting the variety of light sources used or the mechanisms of actions at play. In a nomenclature consensus meeting organized by WALT and the North American Association for Photobiomodulation Therapy (NAALT) in 2014, a majority of participants opted for *photobiomodulation therapy* as a more accurate and specific description of the therapeutic application of light (Anders et al., 2015). However, this term was not MeSH indexed at the time and was first included in the MeSH database as an entry term for "laser therapy, low level" in 2016.

One major consequence of lacking adherence to a universal term is a neglect of the literature. Researchers may not be able to perform a comprehensive systematic search for literature if the many synonyms to LLLT and PBMT are not included. This limitation is not unique to the field of LLLT. A conglomeration of different terms has also been used to describe tendinopathy, which mirrors the uncertain pathophysiology driving the condition (Van Dijk et al., 2011). In literature, the move away from the term *inflammatory tendinitis/tendinosis* to *degenerative non-inflammatory model of tendinopathy* is evident (Rees et al., 2013). Consequently, studies containing both *LLLT* and *tendinopathy* are at risk of falling between the cracks and going unnoticed. This can be demonstrated by investigating the number of LLLT trials included in published systematic reviews of several shoulder tendinopathy interventions over the

past decade. We identified six systematic reviews that made conclusions regarding the effectiveness of LLLT for shoulder tendinopathy and were based on only a handful of studies, ranging from 1-4 RCTs (Green et al., 2003; Faber et al., 2006; Kromer et al., 2009; Valen and Foxworth, 2010; Gebremariam et al., 2013). In contrast, our systematic review and meta-analysis (Study I) includes a total of 17 LLLT trials.

The LLLT modality struggles to gain general acceptance in medical communities and remains controversial, much due to the uncertainties and confusion related to treatment parameters. A large number of parameters (e.g., wavelength, output power, irradiation mode, irradiation time, treatment timing, and repetition) can be chosen, showing the complexity of designing LLLT protocols (Nussbaum et al., 2003). Furthermore, the first law of photobiology states that for LLLT to have any effect on a biological system, a sufficient amount of energy from the irradiated light must be absorbed by photoacceptors in the targeted tissue. Consequently, if the amount of light energy delivered to the target tissue is too low, a response in the tissue will not occur. Conversely, if the amount of light energy is too high, inhibitory effects may occur. The existence of this biphasic dose-response phenomenon in LLLT has been frequently observed. Interestingly, this phenomenon contradicts our natural assumption that if a small dose of LLLT produces a significant therapeutic effect, a larger dose should produce an even greater effect (Hashmi et al., 2010; Huang et al., 2011; Chung et al., 2012; Cotler et al., 2015). By acknowledging these challenges related to LLLT treatment dosages, a more refined interpretation of the many negative studies that have been published is appropriate. Rather than making firm conclusions that LLLT in general does not work, the key message should be that the laser parameters used in those particular studies were ineffective.

To minimize dose issues related to the clinical application of LLLT, the WALT constructed guidelines for treating common conditions, such as tendinopathy and arthritis, based on the current best evidence from systematic reviews and meta-analyses (WALT, 2006). These guidelines were made in an attempt to reveal optimal dose ranges (e.g., the biphasic dose-response window). To overcome the inadequacy

of reporting doses calculated from the laser spot size (energy density in J/cm^2), WALT implemented the idea from Nussbaum et al. (2003) and suggested that the area size in cm^2 should be replaced with energy (J) per point irradiated. The explanation for this decision can be illustrated by the following equation: two LLLT probes of different output power (500 mW, 1 mW) and different spot sizes (0.001 cm^2, 1 cm^2) were used for 8 s and 4 s, respectively. Dosage reported as energy density would be identical at 4 J/cm^2 for both lasers, whereas the energy delivered to the tissue (in joules per point treated) is in fact completely different, at 4 J and 0.004 J, respectively (Carroll, 2017). Consequently, one should not expect similar clinical results. Although the consensus agreement issued by WALT (2006) regarding reporting of LLLT parameters in clinical trials is comprehensive (including wavelength, power density, irradiation time, joules per point treated, number of points treated, and total dose in joules), the published guidelines only state recommendations for minimum total dose in joules, minimum joules per point treated, and number of points to treat (WALT, 2005). The guidelines contain no recommendations regarding irradiation time (i.e., a typical dose of 4 J can be delivered in 8 s with a 500 mW laser and 80 s with a 50 mW laser). Thus, more studies are needed to refine these guidelines and the optimal dose ranges for LLLT. Nevertheless, the validity of the guidelines has increased over the past ten years as systematic reviewers consistently report positive results across trials adhering to the recommendations, as opposed to trials using unsupported doses (Bjordal et al., 2008; Tumilty et al., 2010; Jang and Lee, 2012). The results from our systematic review (Study I) were not different.

The identification of an optimal dose-response relationship is even more limited for the therapeutic use of cryotherapy in musculoskeletal conditions. To date, optimal recommendations regarding the mode, duration, and frequency of cryotherapy treatment for tendinopathy or other soft-tissue lesions do not exist. The basic rationale for applying cooling agents after soft-tissue injury is related to van ’t Hoff’s law, which states that for every 10°C reduction in tissue temperature, a two- or threefold decrease in the rate of chemical reactions will occur. The current best evidence for reducing metabolism and enzymatic activity in the early stages of injury

is limited to animal trials, which demonstrate optimal effects when the targeted tissue is cooled to temperatures between 5 and 15°C (Bleakley and Hopkins, 2010; Bleakley et al., 2012). Similar to treatment success in LLLT, the treatment success of cryotherapy depends on penetrating the skin barrier to reach deeper-situated pathology. Moreover, a sufficient amount of temperature reduction is necessary to modulate pathophysiological processes. At present, the transfer of basic scientific theory and results from animal trials to clinical cryotherapy trials on soft-tissue lesions is frequently unsuccessful (Bleakley et al., 2004). As no study has demonstrated temperatures below 20°C in human muscle tissue following cryotherapy, its potential to reduce metabolism in deeper-situated soft tissue has been questioned (Bleakley and Hopkins, 2010). Skin and adipose tissue have an insulating effect, and individual variations in skinfold thickness require different cooling durations to produce an identical temperature reduction (Otte et al., 2002). The target area for reducing temperature in human muscle tissue is often 2 cm beneath the subcutaneous tissue (Merrick et al., 2003), and the amount of adipose distribution varies within and between subjects.

To our knowledge, no studies have investigated intra-tendinous temperature reductions following cryotherapy. Superficial tendons are usually covered by a thin layer of subcutaneous tissue and located at anatomical body parts that are not associated with great inter-subject variability in adipose distribution. The Achilles, lateral elbow extensors, and rotator cuff tendons have all been measured and located less than 1 cm from the skin surface in an RTUS study (Bjordal et al., 2003). Hence, a greater reduction in intra-tendinous temperature in superficially located tendons should be expected when compared with most muscles.

Research studies may be biased and produce erroneous results due to the influence of both known and unknown variables. An adequately powered and well-designed RCT will, on average, limit the influence of many potential cofounders (Polit and Beck, 2008). Concomitant therapy is a well-known confounder and, therefore, often imposed as an exclusion criterion in RCTs. This is especially true for pharmaceutical studies, where polypharmacy may induce harmful drug interactions (Bjerrum et al.,

2003; Rothwell, 2005). In contrast, most physiotherapy interventions, including modalities used for treating tendinopathy, display limited potential to cause harm. Moreover, different interventions are often combined into a physiotherapy treatment regimen, targeting individual clinical findings and etiological characteristics of the specific tendon disorder at hand (Cook and Purdam, 2009; Kuhn, 2009; Seitz et al., 2011; Magnan et al., 2014a). Although these multimodal treatment regimens are designed to optimize effect, the evidence for such an effect is unclear for many treatment combinations (Green et al., 2003; Rees et al., 2009; Hanratty et al., 2012).

In our systematic review and meta-analysis (Study I), we identified cryotherapy as a possible negative confounding variable if administered parallel to LLLT in patients suffering from shoulder tendinopathy. This hypothesis emerged as a trial with high methodological and procedural quality but which displayed ineffective results across all reported outcome measures (Dogan et al., 2010). Indeed, we expected this trial to be in favor of LLLT, as the remaining 10 trials performed with adequate LLLT doses reported reductions in pain and/or accelerated improvement. Unique to this study was the use of cryotherapy as a co-intervention. Consequently, a reversed translational research strategy was used to explore the potential biophysical and biological effects of the treatment combination of LLLT and cryotherapy in tendinopathy treatment.

5.1.1 Clinical effects of LLLT and cryotherapy

Studies on animal and cell models show that LLLT can modulate pathophysiological processes in tendinopathy, predominantly by modulating inflammatory components and stimulating the tendon repair process (Bjordal et al., 2006a; Cotler et al., 2015). Similarly, cryotherapy may induce beneficial anti-inflammatory effects by reducing microcirculation and cell metabolism, in addition to its neuro-analgesic potential (Malanga et al., 2015). Consequently, both therapies display a mechanism of action in which pain relief seems to be the most important outcome measure. A more rapid course of improvement may also be expected if pain subsides and the tendon healing process is either stimulated by LLLT or the sequelae of a tendon injury is limited by cryotherapy. However, none of these therapies target other crucial etiological factors

in human tendinopathy, such as soft-tissue inflexibilities, impaired muscular activation pattern, loss of muscular strength or stability, or any environmental risk factor (Jarvinen et al., 2005; Seitz et al., 2011; Magnan et al., 2014a). Therefore, LLLT and cryotherapy should be considered as possible pain-modifying treatments and their usefulness in the clinical management of tendinopathy should be evaluated based on these premises.

The current best evidence suggests that both LLLT and cryotherapy produce clinical effects in a dose-dependent manner. Evidence from animal studies show that a sufficient level of temperature reduction in the skin (<12°C) and soft tissue (5–15°C) is necessary to produce optimal clinical effects (i.e., pain relief and lower cell metabolism) (Bleakley et al., 2012; Bleakley and Hopkins, 2010). The critical threshold for skin cooling deemed necessary to produce cryo-analgesia can be easily achieved in humans using ice-based cryotherapy modalities (Merrick et al., 2003), as demonstrated in Study II, in which domestic ice-cube application to the Achilles area produced a mean skin temperature value of 4.8°C in healthy adults. However, skin temperature is a weak predictor of deeper-situated tissue temperature (Jutte et al., 2001), and the ability of cryotherapy to lower temperature to the threshold for metabolic reduction seen in animal studies (5–15°C) may not be possible in most human soft-tissue disorders (Bleakley et al., 2012). Furthermore, these emerging recommendations for cryotherapy must be validated in rigorous clinical trials, across different soft-tissue injuries and pathological stages, before any firm conclusions regarding dose-response relationship can be established. As yet, no clinical trials have attempted to investigate the effects of cryotherapy alone or as an adjunct to LLLT in human tendinopathy.

The effect of cryotherapy as a supplement to therapeutic exercises have been investigated in two controlled clinical pilot trials, which included patients suffering from lateral elbow and rotator cuff tendinopathy. Parle et al. (2016) found significant improvements in pain and shoulder function in the presence of reduced bursal thickness following one week of cryotherapy or isometric exercises alone. There were no significant differences between the groups and there was no evidence for any

"add-on" effect when these two therapies were combined in a third treatment arm. The authors submitted no information regarding cryotherapy treatment parameters. Manias and Stasinopoulos (2006) investigated the potential "add-on" effect of 10 min of ice-bag application (five times a week for four weeks) post eccentric exercises in lateral elbow tendinopathy (LET), compared to exercise only. The authors concluded that the magnitude of pain relief did not differ between the two groups at the end of treatment. However, the validity of these preliminary results is very limited, as they arise from two small pilot trials of poor methodological quality. Hence, it is not reasonable to make any conclusions regarding the clinical benefit of using cryotherapy as a supplement to exercise in human tendinopathy.

There is evidence from previous systematic reviews that LLLT is effective for treating Achilles tendinopathy and LET if the currently recommended dosage guidelines issued by WALT are followed (Bjordal et al., 2008; Tumilty et al., 2010). Results from Study I supported the existing evidence and demonstrated that LLLT can produce beneficial clinical effects alone, as an adjunct to exercise or a multimodal physiotherapy regimen in shoulder tendinopathy patients. Our results demonstrated significant pain relief and accelerated recovery rates if LLLT was included across these intervention strategies.

Interestingly, the Dogan et al. (2010) trial, which combined LLLT, exercise, and cryotherapy, displayed a WMD for pain relief and an SMD for shoulder function at 0.4 cm and -0.33 over placebo, respectively. These effect sizes are almost identical to the trials subgrouped for using non-valid laser treatment doses. Although this high-quality study (10/10 PEDro score) adhered to the treatment recommendations issued by WALT, no measures of laser output testing were reported. Consequently, laser device failure or errors in actual irradiation dose cannot be excluded as a possible confounding factor.

In Study II, we discovered that 20 min of cryotherapy significantly decreased skin-to-skin and Achilles tendon M-L thickness. Furthermore, we found that penetration of laser light increased significantly through healthy Achilles tendons subjected to the

cryotherapy treatment protocol. Dogan et al. (2010) treated all patients with 10 min cold pack application. It is possible that the duration of cryotherapy treatment and choice of modality (cold packs) used in this study predominantly produced temperature reductions in the skin. Merrick et al. (2003) reported an overall temperature reduction of only 5°C 1 cm sub-adipose tissue and a superficial skin temperature of 12°C after 10 min of cold pack application to the thigh of healthy adults. Levy et al. (1997) investigated subacromial space temperature following 90 minutes of cryotherapy (Cryo/Cuff) in post-operative shoulder arthroscopy patients and found that the subacromial temperature was similar to controls. It can be argued that the cryotherapy treatment protocol used by Dogan et al. (2010) targeted the skin and did not penetrate the rotator cuff tendons situated in the subacromial space. With regard to the results in Study II, this would have allowed LLLT to penetrate the skin with less energy attenuation and perhaps "over-dose" the tendon tissue. Indeed, Dogan et al. (2010) treated patients with an 850 nm infrared laser (100 mW MOP) and irradiated each point with 6 J, which is an energy dose in the absolute upper margin according to WALT recommendations. However, the authors did not report the order of therapy administration, and cryotherapy may have been applied after LLLT treatment.

In Study III, we found that LLLT followed by cryotherapy produced counter-productive biomechanical and histological effects in acute Achilles tendinopathy of rats. The immediate application of a cooling agent on injured LLLT stimulated tendons and seemed to interfere with some cellular responses in a non-beneficial fashion. Conversely, superior effects were observed in the group treated with cryotherapy followed by LLLT.

To the best of our knowledge, no other clinical trials have examined the effect of LLLT in combination with cryotherapy in tendinopathy treatment. It should be mentioned that the trial by Yeldan et al. (2009), which is included in Study I, also combined LLLT, cryotherapy, and exercise. However, the findings of this trial were deemed invalid because the participants were treated with a discredited laser device, which had a MOP output of less than 1% of the displayed dose (Bjordal, 2010).

5.1.2 Biophysical and biological effects of LLLT and cryotherapy

Physiotherapy interventions such as LLLT and cryotherapy are applied to the human skin for the purpose of triggering biological actions in underlying tissue. The penetrative ability of both therapies depends primarily on mode of delivery (i.e., wavelength for lasers and the thermodynamic properties of the cooling agent for cryotherapy) (Merrick et al., 2003; Bashkatov et al., 2011). The amount of dermal and adipose tissue between the skin surface and the underlying target is obviously of great importance. Although the depth to tissue target should affect both cryotherapy and LLLT treatment parameters, it has not been a prioritized area of research and is seldom reflected in clinical treatment protocols. The WALT consensus agreement on the design and clinical conduct of LLLT studies has not been revised since its release in 2006 (WALT, 2006). An updated version should perhaps encourage researchers to include ultrasonography measurements of dermal tissue thickness in LLLT tendinopathy trials. This would allow for future assessments of correlation between inter-subject variability in "depth to target tissue" and magnitude of effect. Indeed, such evidence is important to refine, optimize, and individualize the treatment parameters of both LLLT and cryotherapy.

It has also been demonstrated that the biophysical penetration of laser light is influenced by skin color. LLLT doses within WALT recommendations increased skin temperature beyond the threshold for painful thermal stimuli in people with dark skin, whereas the skin temperature in those with light/medium skin tones remained negligible (Joensen et al., 2011). The melanosomes of dark skin are heavily pigmented, which increases the ability to absorb ultraviolet light (Yamaguchi et al., 2007). Hence, dark skin most likely acts as a more efficient photon barrier than lighter skin tones can during LLLT irradiation. Skin color in relation to biophysical penetration of cryotherapy and thermodynamics has not been investigated. However, skin structure and function are known to vary across different ethnic skin types (Rawlings, 2006), and their response to heat or cold application may also differ (Taylor, 2006; Lee et al., 2010).

How the biophysical penetration of LLLT and cryotherapy is influenced by variables such as gender, age, disease, activity level, and various drugs are also unknown. Interestingly, the co-contaminant use of the cortisol antagonist mifepristone completely blocked the anti-inflammatory effect of LLLT in the carrageenan-induced pleurisy of mice (Lopes-Martins et al., 2006). The authors explained this negative event as resulting from a steroid-induced downregulation of cortisol receptors, suggesting that the effect of LLLT may depend on the cortisol/adrenal gland pathway. Notably, when the adrenal glands responsible for endogenous cortisol secretion were excised in rats, an inhibition or even a blockade of LLLT effects occurred (Albertini et al., 2004). It is also well known that corticosteroid treatment is able to produce a downregulation of its receptors (Silva et al., 1994; Felszeghy et al., 1996; Zovato et al., 1996; Sanden et al., 2000), which in clinical situations may be responsible for the poor effects of LLLT in patients submitted to corticosteroid therapy. However, skin atrophy and hypopigmentation are well-known side effects of both local and systemic steroid use (Schäcke et al., 2002; Liang and McElroy, 2013; Coondoo et al., 2014). Consequently, the biophysical penetration of LLLT may increase through skin and possibly "over-dose" the underlying target tissue if administered parallel to steroid treatment. With regard to Study II, careful consideration should be given before generalizing the results to a heterogeneous general population of varying ages, activity levels, skin colors, pathologies, and pharmaceutical histories.

We have shown that skin and tendon thickness is significantly reduced after 20 min of ice pack application to the Achilles area of healthy adults with light skin color. The total amount of tissue reduction (skin-to-skin distance) following cryotherapy was small (0.6 mm/2.7%) but may have contributed to the increased LLLT energy penetration observed in Study II. Optical energy penetration increased significantly for both lasers and at all measured time points after cryotherapy application. Laser energy may penetrate more easily in cooled tissue due to reduced skin and tendinous microcirculation and the amount of laser energy–absorbing hemoglobin (Knobloch et al., 2007; Yanagisawa et al., 2007). It is also possible that the reduced attenuation of

laser energy reflects the slowing of cell metabolism in cooled tissue. Conversely, heat therapy is frequently used by physiotherapists to increase blood flow, cell metabolism, and the elasticity of collagenous tissue (Malanga et al., 2015). Future studies should investigate whether heat induces the opposite effect on the optical properties of skin and tendon tissue (i.e., increases the attenuation of LLLT).

The combination of cryotherapy and LLLT also produced different biological effects in acute Achilles tendinopathy of rats when compared to the effects of LLLT or cryotherapy alone (Study III). This finding suggests that some cellular or tissular interaction occurred. LLLT induces photochemical actions on mammalian cells by increasing the activity of the enzyme CCO in mitochondria (Hamblin, 2016), which in turn modulates cellular responses responsible for limiting inflammation and stimulating tendon repair. Merrick et al. (1999) investigated whether five hours of continuous local cryotherapy reduced secondary injury after crush injury to skeletal muscle in rats. In this study, triphenyl tetrazolium chloride (TTC) reduction rates in assayed muscle tissue served as a measurement of CCO activity and was found to significantly increase in injured rats treated with cryotherapy, thus limiting the magnitude of the secondary injury. It has also been demonstrated that acclimation to low temperatures provokes a compensatory increase of CCO activity in fish and in the adipose tissue of hamsters (Klingenspor et al., 1996; Hardewig et al., 1999).

Our study on Achilles tendinopathy of rats demonstrated that cryotherapy in combination with LLLT was the only intervention that significantly reduced the expression of all pro-inflammatory cytokines, thus displaying evidence for an anti-inflammatory add-on effect, despite which therapy was administered first or last. This may reflect a reinforced upregulation of CCO activity when these therapies are combined. However, the effect on tendon biomechanics and histology was only positively influenced if LLLT was applied after cryotherapy. In fact, tendons treated with LLLT before cryotherapy negatively influenced these outcomes, which suggests that the order of therapy administration is essential to achieving a beneficial effect.

The photons emitted from LLLT irradiation is suggested to increase CCO activity in damaged cells by liberating it from the inhibitory actions of nitric oxide (Hamblin, 2016), but the exact mechanisms of action for cryotherapy remain unknown. However, a decreased formation of NO has been demonstrated in heart muscles of hypothermic rats subjected to myocardial inflammation (Scumpia et al., 2004). NO is a potent signaling molecule that can activate both beneficial and harmful immune responses (Bogdan, 2001). The NO released by LLLT may induce different intra- and inter-cellular signaling pathways in cooled tissues compared to those of tissues at a normal temperature, producing a beneficial biomechanical and histological outcome in the CLG and a negative response in the LCG, as observed in Study III.

Another aspect to consider regarding Study III is timing of LLLT intervention. It is well established that LLLT limits inflammation and promotes tendon healing in a laboratory setting (Oliveira et al., 2009; Marcos et al., 2011; Marcos et al., 2012; Laraia et al., 2012; Tsai et al., 2012; Casalechi et al., 2013; Torres-Silva et al., 2015). Indeed, LLLT alone significantly reduced pro-inflammatory cytokine IL1 expression in the presence of increased levels of IL-10 in Study III. However, we were surprised that the anti-inflammatory response was not more profound and included a significant reduction of the pro-inflammatory cytokine TNF-α. The optimal time frame for initiating LLLT treatment after injury is still an open question in the literature. In Study III, rat Achilles tendons were irradiated with 3 J one hour after crush injury, which is a recommended irradiation dose for human inflammatory conditions according to WALT treatment guidelines. It is possible that an irradiation dose of 3 J was a case of “too much, too soon” to achieve an optimal anti-inflammatory effect in small rodents. Indeed, increased Achilles tendon edema has been demonstrated in rats treated with a similar dose (3 J) 30 min after blunt injury (Joensen et al., 2012).

However, the optimal LLLT dose may also differ depending on which trauma model is used to inflict tendon injury in animals. It is likely that the course of inflammation and the cytokine expression profile varies between different trauma methods. COX-2 gene expression was found to peak 2 hours after injury in collagenase-induced Achilles tendinopathy of rats, and a single LLLT dose of 3 J was more effective than

1 J in reducing inflammation one hour after injection (Marcos et al., 2011). Another study with LLLT treatment one hour after collagenase-induced tendinopathy in rats demonstrated significant reductions in COX-2 and pro-inflammatory markers IL-6 and TNF-α after 3 J but not after 1 J (Torres-Silva et al., 2015). Conversely, de Almeida et al. (2014) treated rats with LLLT one hour after blunt muscle injury and found significantly reduced TNF-α levels after 1 J but not after 3 J. Both de Almeida et al. (2014) and Joensen et al. (2012) used a similar injury model as did Study III, and in retrospect, these findings suggest that the early initiation of LLLT treatment after blunt injury to tendons should be performed with a lower dose than 3 J.

5.2 Methodological discussion

The research question for this thesis was to investigate the clinical, biophysical, and biological effects of LLLT alone and in combination with cryotherapy for the treatment of tendinopathy. Hence, the target tissue in all the three included studies are tendons. This section addresses the methodological aspects of the thesis with an emphasis on research design, subjects, and internal and external validity. Specific methodological limitations of the individual studies are presented separately in subsections.

5.2.1 Study design and study population

Systematic review and meta-analysis (Study I) are powerful tools for collecting and summarizing existing knowledge in a research field and for combining the results of individual studies in one paper (Laake et al., 2007). The aim of Study I was to investigate the clinical effectiveness of LLLT for shoulder tendinopathy by synthesizing evidence from already-published RCTs. Heterogeneity across trials related to procedural aspects of LLLT treatment and different co-interventions to LLLT was expected and formed the basis of our a priori subgrouping (Chapter 3). The review question was formulated using the population, intervention, comparison, outcome (PICO) model (Table 2, below), and these parameters constituted the basis of our systematic literature search. The subjects in Study I originate from RCTs of

patients diagnosed with shoulder tendinopathy or SAIS. The diagnostic accuracy of the clinical tests used to confirm SAIS and shoulder tendinopathy are known to be limited (Hegedus et al., 2008). However, rotator cuff disorders are by far the most common associated source of shoulder pain, accounting for more than two thirds of all cases (Murphy and Carr, 2010). These limitations in clinical diagnostics may cause some subject heterogeneity across trials, which could arguably impose a threat to the internal validity of Study I. Perhaps more importantly, the subjects in Study I most likely mimic the diversity of shoulder tendinopathy patients treated by clinicians, which is a crucial element for the generalization of the findings to clinical practice.

In Study II, the biophysical ability of LLLT energy (from two different Class 3B lasers) to penetrate healthy human Achilles tendons was measured before and after cryotherapy, and at several time points during exposure (Table 2). The amount of optical LLLT energy penetration before cryotherapy was compared—using a similar part of the body, the Achilles—to that after cryotherapy (i.e., the subjects were their own control). This basic in-situ research study follows a repeated measurements design, which is sensitive in detecting differences within the same subject. The healthy subjects in Study II were recruited by nonprobability convenience sampling. This non-random method of recruiting participants limits the ability to generalize findings (Polit and Beck, 2008), but a conservative interpretation of the results can still yield important and clinically relevant knowledge.

In Study III, a blinded multiple-armed RCT design with post intervention test only was used to investigate the biological effects of LLLT and cryotherapy as monotherapies, as well as in combination with each other (Table 2). The study population comprised in vivo rat Achilles tendons. Animals included in this study were (outbred) male Wistar rats of the same age and weight, produced at the Biotery of Sao Paulo University, comprising a homogenous study sample. The rats were randomly divided into six experimental groups. The probability sampling using random assignment increases internal validity by reducing bias related to pre-existing differences between the groups before the experiment begins (Polit and Beck, 2008).

Table 2. PICO key components of individual studies

Study no.	*Population*	*Intervention*	*Comparison*	*Outcome*
I	Humans diagnosed with shoulder tendinopathy or SAIS	LLLT (Class 3B) administered to one group in the controlled trial	Placebo LLLT or no therapy groups EPA groups Exercise and placebo LLLT Physiotherapy regimen and placebo LLLT or another EPA	Pain Global improvement Shoulder function
II	Achilles tendons of healthy young adults	LLLT (Class 3B) Cryotherapy	Subjects are their own control (repeated measurements)	Optical energy penetration before and after cryotherapy RTUS tendon thickness measurements Skin temperature
III	Achilles tendons of male Wistar rats	LLLT alone Cryotherapy alone LLLT & cryotherapy Cryotherapy & LLLT	No treatment	Inflammatory mediators Tendon biomechanics Tendon histology

5.2.2 Internal validity

All research studies should be evaluated based on their internal validity, and if it is found to be adequate, they should then be evaluated based on their results and external validity (Biondi-Zoccai et al., 2011). The level of internal validity reflects how confident one may be that the dependent variable (outcome) was caused by the independent variable (intervention) rather than some other variable (Polit and Beck, 2008). All three studies in this thesis were performed with detailed predefined protocols and standardized procedures to minimize biases, and they were controlled for the influence of possible extraneous variables.

Study I

The systematic review and meta-analyses (Study I) included as part of this thesis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement and was conducted with an a priori methodological approach (Moher et al., 2009). With PRISMA, several measures are taken to ensure that the review process is clearly defined, thereby establishing a framework for reproducibility of the results by other researchers and limiting potential sources of bias. However, the protocol for Study I was not registered in the PROSPERO database, which reduces the transparency of this a priori process.

A comprehensive literature search strategy, adequate coverage of relevant databases, article reference screening, and personal contact with experts in the field were conducted to identify relevant trials for inclusion. No language restrictions or exclusions of "old" studies were imposed, to limit selection bias. Grey literature (e.g., unpublished trials) was not included in Study I, and their exclusion can lead to exaggerated estimates of intervention effectiveness (McAuley et al., 2000). However, as negative publication bias has been reported in LLLT literature, a devalued estimate of effect sizes seems like an equally likely outcome (Bjordal et al., 2008).

The internal validity of LLLT trials depends on both methodological and procedural quality. There is no universal agreement or gold standard regarding which checklist

to use for methodological assessments of RCTs, and different quality scores for the same study may be reported across different systematic reviews (Olivo et al., 2008). The PEDro scale used for assessing trials in Study I was developed for use in physiotherapy and is a reliable and valid instrument (Maher et al., 2003; Macedo et al., 2010; Sherrington et al., 2010). According to the study by Maher, consensus scores were in exact agreement 46% of the time and differed by two points or less 99% of the time. The ICC for the total PEDro score has been reported to range from 0.68 to 0.91 (i.e., acceptably good reliability) (Maher et al., 2003; Foley et al., 2006). However, some disagreement between independent reviewers should be expected. For Study I, any disagreement in the rating of individual items between the two independent reviewers was solved by consensus, and scores differing by one point or less were interpreted to be in line with PEDro reviewers. For only one trial were reviewers in significant disagreement with the PEDro reviewers, and the results outlining this discrepancy were made available in the appendix of the published paper.

The assessment of possible confounders related to LLLT treatment parameters and procedures were handled according to the standards issued by the WALT musculoskeletal advisory board, and trials using non-optimal treatment doses were subgrouped (WALT, 2006). The justification and validity of applying these standards in systematic reviews and meta-analysis of LLLT trials have been discussed in the general section.

Two reviewers extracted data from the primary studies for the purpose of statistical pooling. There is an ongoing debate regarding how to handle cases of significant statistical heterogeneity ($I^2 > 0\%$, $p < 0.05$), and whether reviewers should avoid performing meta-analysis in such cases (Biondi-Zoccai et al., 2011). Higgins et al. (2003) investigated 509 meta-analyses of dichotomous outcomes in the Cochrane Database of Systematic Reviews and found that a quarter displayed I^2 values over 50%. Moreover, adjectives were suggested to interpret the degree of heterogeneity as follows: low (25%), moderate (50%), and high (75%). Although no attempt was made to categorize the meta-analysis of our study, sensitivity analyses were

performed to reveal trials contributing to statistical heterogeneity. If the inclusion of these trials did not increase the overall effect size and the random or fixed effect model produced similar effect estimates, the meta-analysis was considered valid.

Study II

The within-subject repeated measurement design was used to investigate the penetration of laser energy through healthy Achilles tendons under two different conditions: before and after cryotherapy. An a priori power analysis was not possible to perform, as no similar studies containing expected means and variance data had been published (D'Amico et al., 2001). However, our study sample consisted of 54 healthy tendons, which decreased the probability of beta errors (i.e., the probability of not finding a difference when one truly exists). The repeated measurement design also reduces error variance associated with individual differences between subjects, as they are their own control. In RCTs, important differences may exist between the two groups, which effects the dependent variable (Bordens and Abbott, 2002). While threats to internal validity such as history, maturation, and "carryover effects" are well-known disadvantages of this design, they do not apply in this study as the time interval between measurements (before and after cryotherapy) is limited to 20 min (Kirk, 1982, Polit and Beck, 2008). The internal validity of this study is largely dependent on the reduction of extraneous variables in the experimental set up, as well as the use of reliable measurement instruments and procedures.

Two Class 3B lasers (an 810 nm 200 mW CW-mode laser and a 904 nm 60 mW SPW-mode laser) were used for irradiation of the Achilles tendons. Both lasers displayed a stable MOP during irradiation with no obstacle measured before and after cryotherapy. However, a small but significant increase in the 810 nm laser MOP (1.6 mW) was observed in the after-cryotherapy measurement. Although we handled this inconsistency in our analysis by reporting the percentage of MOP penetrating the Achilles before and after cryotherapy, randomization of the intervention order could have eliminated this weakness. To obtain reliable measurements of non-cooled tissue, the tissue temperature must return to baseline levels after cryotherapy. The restoration

of skin temperature to baseline levels following cold-air exposure was found to exceed two hours (Kim et al., 2002), and the relationship between skin surface temperature and underlying tissue temperature is poorly understood (Hardaker et al., 2007). The experiment could have been performed on consecutive days, but this approach was not taken because of the increased risk of influence from extraneous variables within subjects and "loss to follow-up."

Skin temperature was measured by thermographic imaging (Flir System, T640-25). Modern thermographic cameras have a small error rate of only ±2% and can produce high-definition infrared images with an accuracy of 0.1°C in skin temperature measurements (Jiang et al., 2005; Villaseñor-Mora et al., 2009). Abnormal body temperature is a natural indicator of illness, and thermographic imaging has been successfully used in the diagnoses of several medical conditions, including breast cancer, neuropathy, vascular disorders, and chronic joint and tendon disorders (Ring and Ammer, 2012). This imaging technique is based on the phenomenon that different materials emit infrared thermal radiation, which can be used for calculating the temperature of the emitting object and visualizing it in real-time images. The emissivity of objects range from 0 (no emission) to 1 (complete emission), and the emissivity of human skin has been found to be almost constant, at 0.96 to 0.98 in both white and dark skin colors (Lahiri et al., 2012; Ring and Ammer, 2012). The reliability of thermographic temperature measurements can be affected by environmental changes in temperature, airflow, and moisture (Ring et al., 2004). The mean room temperature in our laboratory was 21.9°C and varied less than 1°C throughout the experiment; air filtered through a balanced ventilation system and the windows were double-glazed.

Another factor to consider is the distance from the camera to the target measurement area (the Achilles), as this parameter can influence the pixel resolution. In Study II, a standardized distance of 50 cm was used for all measurements, as recommended by Ring and Ammer (2000). The skin surface temperature can also be affected by topical substances such as ultrasound gel or water from the ice packs (Bernard et al., 2013). Although measures were taken to ensure that the skin surface was dry before images

were captured, the skin surface temperature at a baseline would perhaps have been more reliable if the images were captured before the ultrasound examination. In a review by Costello et al. (2012), the use of thermal imaging to assess skin temperature following cryotherapy was investigated, and the authors concluded that thermal imaging appeared to be a safe, accurate, and reliable method of collecting such data.

The Achilles area (skin and tendon) was scanned in the longitudinal and transverse plane using an ultrasonography instrument (Logiq-S8, GE Healthcare, Minneapolis, USA). A built-in caliper was used to measure the amount of tissue. Images were obtained in a standardized manner at 2.5 cm from the tip of the OS calcaneus bone. The inter- and intra-observer reliability of Achilles tendon thickness measurement has been reported as high (Bjordal et al., 2003; Ying et al., 2003; Brushøj et al., 2006).

The stability of the laser MOP and the relative loss of energy after penetrating through skin-tendon-skin was measured with an OPM (ThorLabs model PM100 with a S121B optical sensor). This instrument measures power output at five specific wavelengths, including 810 nm and 904 nm, which corresponds to those of the two LLLT devices used in Study II. OPM systems are widely used to measure and control laser parameters and are associated with a high degree of stability and low occurrence of measurement errors (Chen et al., 2016).

Study III

Evidence hierarchies often rank well-designed individual RCT studies on the second rung in evaluating healthcare interventions, below the strongest evidence, which comes from the systematic review of multiple RCT studies (Polit and Beck, 2008).

The aim of Study III was to investigate the biological effects of LLLT and cryotherapy as mono- and combined therapies on the tendon mechanical properties, inflammation and histology. Even though the rats form a homogenous group, the induction of injury to the tendon may still produce some variation between the study subjects. Hence, manually assigning animals to specific groups may cause selection

bias and ultimately influence the results (Van der Worp et al., 2010). The failure to randomize animals and employ blinded outcome assessors are associated with exaggerated effect sizes across several different disease areas (Hirst et al., 2014). In this study, the animals were randomly divided into six different cages by one person, and group allocation was decided by another blinded researcher. However, randomization does not ensure an unbiased comparison of the treatment groups. In clinical trials, the most robust method of limiting systematic bias is to employ a double-blind, placebo-controlled procedure, meaning neither the patient, researcher, nor caregiver know the details of the treatment given (Laake et al., 2007). As animals are not likely to experience any placebo effect, double-blinding procedures are not necessary (Van der Worp et al., 2010). Given the nature of the treatment intervention in our study, blinding the caregiver was not possible. However, we ensured that the outcome assessors were unaware of the group allocation. Tendon biomechanical tests can only be performed once, as the tendon ultimately ruptures. Two independent blinded assessors performed the histology and cytokine analysis to ensure the consistency of the analyses.

ELISA is the most widely used and best-validated method for measuring individual inflammatory cytokine expression. The high sensitivity ELISA from R&D Systems, as used in Study III, has been reported as having a sensitivity of less than 1 picogram per milliliter (pg/ml) for IL-1, IL-6, and IL-10 measurements (Leng et al., 2008).

The evaluation of biomechanical properties using a tensiometer has been widely used in animal research (Ferry et al., 2007; Joensen et al., 2012; Marcos et al., 2012; Marcos et al., 2014). In Study III, the tendons were attached to a universal tensile test machine (zL2.5, Zwick, Roell, Germany), and several bouts of loading and unloading sequences with increasing strength were performed until the tendon ruptured. A major prerequisite for obtaining reliable measurements is to avoid slipping of the tendon grip as tension increases. The musculotendinous junction was firmly fixed at the bottom, whereas the osteotendinous junction was fixed at the top of the tensiometer grip. This procedure reliably measured the force and displacement needed to rupture the healthy control tendons of the rats.

An a priori power analysis was not possible to perform, as no studies containing expected means and variance data for the treatment combination of LLLT and cryotherapy had been published. We requested a total of 36 rats (i.e., six rats per group), based on experience from previous animal studies conducted by our research group (Marcos et al., 2011; Torres-Silva et al., 2015). Also, six animals should be sufficient according to the following power calculation and our estimate of cytokine expression due to LLLT treatment. A previous study by our group investigated the effect of LLLT on expression of inflammatory mediators in rat Achilles tendinopathy (Torres-Silva et al., 2015). Using the means for IL-10 (15 SD±2 for the injured controls, 19 SD±0.5 for the LLLT group) and TNF-α (35 SD±1 for the injured controls, 18 SD±3 for the LLLT group), we calculated an effect size of 2.7 and 7.6, respectively. Hence, conducting a power analysis (using G*Power software) for the difference between independent means (two-tailed t-test) with these effect sizes revealed a sample size of two to four rats per experimental group.

5.2.3 External validity

External validity refers to the generalization of results from tightly controlled research settings to real-world clinical practice settings (Polit and Beck, 2008). From this perspective, some shortcomings across the included studies of this thesis require that the results should be interpreted with caution.

In Study I, the included subjects most likely represent a subsample of the target population. In line with previous reviews, our study supports the validity of the WALT guidelines, which dictate that LLLT acts in a dose-dependent manner in tendinopathy. The external validity of Study I is primarily limited by the internal validity of the results, which originates from four different comparison groups, ranging between two and five studies for each comparison. A common critique of systematic reviews and meta-analysis is that they are not original research, as the data used for analyses originates from primary research studies. However, the originality of the research should be based on its novelty and usefulness rather than what appears as original or secondary research (Biondi-Zoccai et al., 2011).

In Study II, the subjects consist of 27 healthy young adults (22.5 years SD±2), comprising 54 healthy Achilles tendons. Skin and tendons degenerate with increasing age, and tendon pathology can produce changes in both morphology and biology. Hence, the generalization of the results from Study II to a heterogeneous population with tendon pathology has its limits.

The use of animal models as surrogates for researching human health conditions has several shortcomings. The most obvious threat to external validity is the anatomical and physiological differences between species. There is no animal model that mimics human tendinopathy perfectly. The etiology and pathophysiology of tendinopathy is incompletely understood, which limits all animal models used for investigating aspects of human tendinopathy (Lui et al., 2011). Hence, the results of Study III should not be used to impose firm conclusions on human tendinopathy. One major advantage of using animal models is that the effect of an intervention can be measured at the tissue level as signs of accelerated healing or pathology improvement. The disadvantage is that pathology improvement cannot accurately be correlated with subjective outcomes such as pain relief, improved function, or quality of life. However, Study III provides evidence that cryotherapy can negatively influence the effect of LLLT in tendinopathy treatment, which can explain the lack of improvement in the study by Dogan et al. (2010).

5.2.4 Statistics

The meta-analysis in Study I was performed with RevMan (version 5.2), which is the software used in the Cochrane Database of Systematic Reviews. The validity of RevMan meta-analysis has been compared to analyses performed with several different commercial software programs, and no discrepancies in the results were reported (Bax et al., 2007). In Study II, Microsoft Excel 2011 was used for statistical analysis because of this software's advantage in creating visualizations of the data sets. Some of the statistical functions of Microsoft Excel versions until 2007 have been criticized by statisticians, but these functions have been improved in later versions (Mélard, 2014). The p-values calculated from Microsoft Excel using

Student's t-tests have been compared to SPSS analyses on similar data sets previously and found to be almost identical (Joensen, 2013). All statistical analyses of Study III were calculated on SPSS version 22. Non-parametric statistical methods were applied, as the data did not follow normal distribution. The Kruskal–Wallis analysis is adequate for detecting differences between several groups, but it will not indicate which groups are significantly different from each other. Consequently, multiple pairwise comparisons (Mann–Whitney U tests) had to be performed in Study III. We decided to compare the different treatment groups to one common reference group (ING), as the combination of cryotherapy and LLLT was a novel experimental treatment approach, and to limit the number of pairwise comparisons.

5.2.5 Limitations of Studies I–III

Study I

Even though the overall effect for LLLT on pain and global improvement is encouraging in this review, the result should be interpreted with caution. The included trials were subjected to analysis in one out of four different comparison groups, depending on control group measures. Hence, the result for each comparison and outcome arises from a handful of studies. It should also be noted that our outcome measures are based solely on end-of-treatment data (2–12 weeks) and display the potential short-term effects of LLLT for shoulder tendinopathy. Only three trials (Al-Shenqiti and Oldham, 2003; Bal et al., 2009; Otadi et al., 2012) provided post-treatment follow-up data, and no robust conclusions can be drawn regarding the long-term effects. Synthesizing evidence was challenging for numerous reasons. In LLLT research, the validity of a study is based on both methodological quality and the validity of the intervention procedure. Clinical application procedures and laser parameters were poorly or inaccurately described in some studies (England et al., 1989; Logdberg-Andersson et al., 1997; Santamato et al., 2009; Abrisham et al., 2011). In addition, there were large variations in laser wavelength (nm), number of points treated, composition of co-interventions, and exercise design across the included studies. A lack of therapist blinding and an intention to treat analysis

were the two most frequent methodological shortcomings, consequently increasing the potential bias of this review. Although we thoroughly searched databases for available literature, hand-searches of the grey literature were not performed. As negative publication bias has been reported for the LLLT literature (Bjordal et al., 2008), we cannot exclude the possibility that published trials may have been overlooked.

Study II

The use of a laser tripod and a hand-held OPM has its limitations. The exact amount of pressure exerted on the Achilles tendon from these devices was not measured. Although our experimental set up was identical for each measurement, we cannot exclude that small variations in Achilles squeezing occurred and influenced the outcome. The operator holding the OPM was blinded to all measurement recordings but could not be blinded to whether the tendon was cooled or not. We also observed a difference in MOP (no obstacle measurements) before and after ice for the 810 nm CW laser. Although this increase in MOP after ice was very small (1.6%), it was still significantly ($p<0.01$) higher than the before-ice measurements. However, a higher percentage of MOP-penetrating cooled Achilles were also found for the 810 nm CW laser, indicating that the increased penetration after ice cannot be explained by a higher MOP from the laser device alone. It should also be emphasized that our results originate from a homogenous population consisting of healthy young adults with a light skin tone. Dermal and tendinous tissue degenerates with increasing age (Lephart, 2016; Svensson et al., 2016) and can be influenced by both disease and activity levels (Wang, 2005; Kongsgaard et al., 2010; Babalola et al., 2014; Kjaer and Heinemeier, 2014). Careful consideration should be given before extrapolating the results of this study to a heterogeneous general population with pathology.

Study III

The experimental trauma model produced increased expression levels of pro- and anti-inflammatory cytokines, consistent with reactions known to occur in local inflammatory tendon disease (Kindt et al., 2007). It also caused a weakening of

tendon material, loss of stiffness, and loss of ability to withstand force before rupturing. These findings correlated well with the histopathological appearance of the tendons. Nevertheless, the use of animal models in tendinopathy research has several shortcomings. At present, there is no ideal model to induce tendinopathy in animals (Lui et al., 2011), and all of the models share the common limitation of an unclear pathophysiology in human tendinopathy (Rees et al., 2006). We decided to use the mini-guillotine model to mimic a tendon disorder with inflammatory components, which is comparable to early or acute tendinopathy (Millar et al., 2010). Achilles tendinopathy in humans is often associated with overuse, and it can develop over months or even years (Paavola et al., 2002). In vivo animal models, such as repeated uphill treadmill running, would better mimic the etiology of chronic human Achilles tendinopathy. However, it is also a more stressful procedure for the animals than our mini-guillotine model. We also found no evidence of its superiority in causing inflammatory reactions. Perhaps the most important limitation of this study is the absence of a functional outcome, as activity-related pain is a common clinical feature in human tendon disorders (Rio et al., 2014). This point should be emphasized, as histopathological changes and pain intensity do not necessarily correlate in humans (Magnan et al., 2014b). Consequently, the validity of our findings would be strengthened by indirect measures of pain such as the analysis of gait pattern (Lui et al., 2011). Our cytokine analysis also showed considerable variance, indicating that the sample size should be increased in future replica studies. In view of these shortcomings, careful consideration should be given before extrapolating the findings of this study to clinical practice.

6. Conclusion

The main purpose of this thesis was to investigate the clinical, biophysical, and biological effects of LLLT alone and in combination with cryotherapy for the treatment of tendinopathy. The hypothesis arose from our systematic review and meta-analysis (Study I), in which cryotherapy was identified as a possible negative confounding variable if administered parallel to LLLT in shoulder tendinopathy patients. This clinical finding revealed a gap in knowledge, stimulated our curiosity, and brought us to explore the potential biophysical and biological effects of combining LLLT and cryotherapy. The main insights from this thesis can be summarized as follows.

In Study I, we demonstrated that LLLT acts in a dose-dependent manner in shoulder tendinopathy. Optimal LLLT can offer clinically relevant pain relief and initiate a more rapid course of improvement, both alone, in combination with exercises, and in combination with several physiotherapy interventions. However, the effects of optimal LLLT seemed to be inhibited if administered in combination with cryotherapy.

In Study II, it was hypothesized that cryotherapy alters the biophysical penetration of LLLT. Consequently, we investigated the penetration of a CW 810 nm laser and an SPW 904 nm laser through skin and Achilles tendon in healthy humans, before and after 20 min of cryotherapy. LLLT penetration increased significantly through the Achilles area (skin-tendon-skin) for both lasers and at all measured time points, when skin surface temperature was lowered from 28.2˚C to 4.8˚C. This may indicate that cryotherapy can be used prior to LLLT to reach more deeply situated pathology but also that “over-dosing” the tendon with LLLT is possible if the biophysical effects of cryotherapy are limited to the skin.

Finally, it was hypothesized that the combination of LLLT and cryotherapy would produce different biological responses in injured tendons when compared with no treatment or with LLLT or cryotherapy alone. Although we found that cryotherapy in combination with LLLT can produce an anti-inflammatory “add-on” effect in

Achilles tendinopathy of rats, other outcomes suggested that the order of therapy administration is essential to produce a beneficial biological effect in injured tendon tissue. Superior biomechanical and histology results were demonstrated if LLLT treatment followed cryotherapy. Conversely, cryotherapy after LLLT treatment produced the poorest biomechanical behavior and histological score of all treatment groups.

7. Perspectives

This thesis has drawn attention to some interesting aspects regarding LLLT and cryotherapy in tendinopathy treatment. There are, however, several new questions to be answered:

- To what extent do different cryotherapy modalities produce intra-tendinous temperature reductions in humans?
- How is the infrared Class 3B laser's ability to penetrate human skin and tendons after heat therapy?
- What is the ideal timeframe for the initiation of LLLT and cryotherapy after tendon trauma in animals, and what are the optimal treatment parameters?
- Is the ideal timeframe for the initiation of LLLT and cryotherapy different across different trauma models used to inflict tendon injury in animals?

Most importantly, our findings clearly elucidate the need for future clinical RCTs that investigate whether cryotherapy in combination with LLLT can produce beneficial "add-on" effects in tendinopathy patients. As an extension of Study III, our research group is now recruiting Achilles tendinopathy patients to participate in a double-blind placebo-controlled RCT in which the effects of cryotherapy, LLLT, and exercise are compared to cryotherapy, placebo LLLT, and exercise. This ongoing clinical trial will also provide ultrasonography measurements of subcutaneous dermal tissue thickness and allow initial assessments of correlation between the depth to tissue target and magnitude of effect.

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PAPER I

RESEARCH ARTICLE

The Efficacy of Low-Level Laser Therapy for Shoulder Tendinopathy: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

Sturla Haslerud[1,2]*, Liv Heide Magnussen[2,4], Jon Joensen[2,4], Rodrigo Alvaro Brandao Lopes-Martins[3] & Jan Magnus Bjordal[1,2]

[1]Centre for Evidence Based Practice, Faculty of Health and Social Sciences, NorPhyPain Research Group, Bergen University College, Bergen, Norway

[2]Department of Global Public Health and Primary Care, Physiotherapy Research Group, University of Bergen, Bergen, Norway

[3]Department of Biomedical Engineering, University of Mogi das Cruzes, SP, Brazil

[4]Department of Occupational Therapy, Physiotherapy and Radiography, Faculty of Health and Social Sciences, Bergen University College, Bergen, Norway

Abstract

Background and Purpose. Low-level laser therapy (LLLT) is proposed as a treatment for tendinopathies. This is the first systematic review focusing solely on LLLT treatment effects in shoulder tendinopathy. **Methods.** A systematic review with meta-analysis and primary outcome measures pain relief on 100-mm visual analogue scale (VAS) and relative risk for global improvement. Two independent assessors rated the included studies according to the PEDro scale. Intervention quality assessments were performed of LLLT dosage and treatment procedures according to World Association for Laser Therapy guidelines. The included trials were sub-grouped by intervention quality and use of other physiotherapy interventions. **Results.** Seventeen randomized controlled trials (RCTs) met the inclusion criteria, and 13 RCTs were of high and 4 RCTs of moderate methodological quality. Significant and clinically important pain relief was found with weighted mean differences (WMD) over placebo, for LLLT as monotherapy at 20.41 mm (95% CI: 12.38 to 28.44) and as adjunct to exercise therapy at 16.00 mm (95% CI: 11.88 to 20.12). The WMD when LLLT was used in a multimodal physiotherapy treatment regime reached statistical significance over placebo at 12.80 (95% CI: 1.67–23.94) mm pain reduction on VAS. Relative risks for global improvement were statistically significant at 1.96 (95% CI: 1.25–3.08) and 1.51 (95% CI: 1.12–2.03), for laser as monotherapy or adjunctive in a physiotherapy regime, respectively. Secondary outcome measures of shoulder function were only significantly in favour of LLLT when used as monotherapy. Trials performed with inadequate laser doses were ineffective across all outcome measures. **Conclusion.** This review shows that optimal LLLT can offer clinically relevant pain relief and initiate a more rapid course of improvement, both alone and in combination with physiotherapy interventions. Our findings challenge the conclusions in previous multimodal shoulder reviews of physiotherapy and their lack of intervention quality assessments.

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Keywords

low-level laser therapy; shoulder; subacromial impingement; supraspinatus; tendinopathy

***Correspondence**

Sturla Haslerud, PT, MSc, PhD student. NorPhyPain Research Group, Centre for Evidence Based Practice, Faculty of Health and Social Sciences, Bergen University College, Inndalsveien 28, 5020 Bergen, Norway.

E-mail: sturla.haslerud@hib.no

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Introduction

Rotator cuff disorders are by far the most common problem affecting the shoulder complex (Van Der Windt *et al.,* 1995) and account for more than two-thirds of all shoulder cases in primary care (Vecchio *et al.,* 1995). Unfavourable outcome, with a subsequent increased risk for chronicity, is reported for many of these patients (Kuijpers *et al.,* 2004). Only 50% of all patients diagnosed with rotator cuff tendinopathy seem to be fully recovered within the first 12 months (Van Der Windt *et al.,* 1996), which indicates the absence of a self-limiting disease course and points to the necessity of developing effective treatment programmes. Conservative treatment options for shoulder tendinopathy is manifold, including non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticosteroid injections and a broad spectrum of physiotherapy treatment regimens (Green *et al.,* 2003). The effectiveness of physiotherapy encompassing exercise therapy is extensively studied across several systematic reviews and has exhibited beneficial effects for this condition (Green *et al.,* 2003; Faber *et al.,* 2006; Kromer *et al.,* 2009; Kuhn, 2009; Kelly *et al.,* 2010, Littlewood *et al.,* 2011; Hanratty *et al.,* 2012). The value of adding other therapeutic modalities to exercise is largely unknown, even though combining interventions to optimize effect most likely reflects clinical practice (Green *et al.,* 2003). Corticosteroid injections (CSI) are commonly administered to treat shoulder pain (Gruson *et al.,* 2008), and despite the controversy regarding their effectiveness for rotator cuff pathology (Alvarez-Nemegyei *et al.,* 2008; Van Der Sande *et al.,* 2012), they seem to have acquired recognition for being an efficacious treatment among clinicians (Johansson *et al.,* 2002).

Low-level laser therapy (LLLT) has been used as a non-pharmacological alternative to treat painful musculoskeletal conditions for three decades (Chow *et al.,* 2009). While laboratory research consistently shows that low energy irradiation from lasers alters cellular processes, producing among others anti-inflammatory effects and increased collagen turnover (Lopes-Martins *et al.,* 2007). The transfer of these superior and consistent results to clinical LLLT trials on tendinopathy is frequently unsuccessful (Basford, 2005). Scattered positive results seem to be almost equally countered by negative trial results.

Several systematic reviews have identified a dose-dependent effect of LLLT, both in elbow and generic tendinopathy (Bjordal *et al.,* 2008; Tumilty *et al.,* 2010), but also for chronic joint disorders (Jang and Lee, 2012). These reviews show consistently better effect of LLLT across trials using the recommended LLLT dosages suggested by Bjordal *et al.* (2001) and Lopes-Martins *et al.* (2007). The growing body of evidence for an 'effective dosage-window' in LLLT cannot be ignored and is currently the most important construct in the World Association For Laser Therapy (WALT) guidelines.

At present, no systematic review focusing solely on the treatment effect of LLLT for shoulder tendinopathy has been published. Previous reviews covering the effect of physiotherapy interventions in general have not advocated LLLT as a potential option for treating shoulder tendinopathy (Green *et al.,* 2003; Kromer *et al.,* 2009), primarily because of conflicting findings across an insufficient number of published trials. However, none of these reviews assessed the validity of the LLLT treatment variables and procedures. Nor were the literary search strings tailored to explore the largest possible number of clinical LLLT trials. For this reason, we undertook a new systematic review and meta-analysis of LLLT in shoulder tendinopathy, where laser treatment protocols were systematically assessed in the process of establishing whether LLLT treatment relieves shoulder pain and triggers a more rapid course of improvement for patients with this condition. Objectives were set to evaluate the effect of laser as monotherapy, the potential benefit of adding LLLT to exercise or a multimodal physiotherapy treatment regimen, including its effect magnitude compared with other electrophysical agents.

Method

Search strategy

A literature search for randomized controlled clinical trials was performed on Medline, PubMed, Embase, CINAHL, PEDro and the Cochrane Controlled Trial Register databases. Key words used were as follows: low-level laser therapy OR low intensity laser therapy OR low energy laser therapy OR phototherapy OR HeNe laser OR IR laser OR GaAIAs OR GaAs OR diode laser OR NdYag, AND tendonitis OR tendinitis OR tendinopathy OR subacromial impingement OR impingement syndrome OR shoulder tendonitis OR shoulder tendinitis OR rotator cuff tendonitis OR rotator cuff tendinitis OR supraspinatus tendonitis OR

supraspinatus tendinitis. Researchers in the field were contacted and contributed additional information. Article references were screened for potentially relevant trials. Unpublished material and abstracts were not included. No language restrictions were imposed.

Inclusion criteria

Randomized controlled trials (RCTs), controlled clinical trials or trials with crossover design were included if the following criteria were fulfilled:

- *Study population:* the trials included human participants diagnosed with shoulder tendinopathy or subacromial impingement syndrome, experiencing pain and functional disability.
- *Type of intervention:* treatment with LLLT at wavelengths in the range of 632–1064 nm, targeting the tendon pathology, acupuncture or trigger points, and administered to one group in the controlled trial.
- *Type of outcome:* trials reporting endpoints for pain intensity or global improvement of health were considered for inclusion.

Classification of trials

Potentially relevant trials were independently assessed by two reviewers (SH and JMB), and any disagreement regarding trial eligibility was resolved in consensus meetings. Studies were subsequently categorized according to control group measures.

Comparisons were made regarding the following:

- Placebo or no therapy control groups
- Electro Physical Agents (EPA) control groups
- Control groups receiving exercise therapy and placebo LLLT
- Control groups receiving a combination of exercise therapy and other modalities

Quality assessment

Two reviewers (RLM and JMB) independently assessed the methodological quality of the included trials against the 10-point PEDro checklist (Maher *et al.,* 2003), as exaggerated effect sizes have been reported for trials with weaker methodology (Schulz *et al.,* 1995). Any disagreement in the rating of individual items between reviewers was resolved by consensus. Trials were labelled as 'high', 'moderate' or 'poor' according to the total attainable sum score. The label 'high' was given to trials attaining a PEDro score of seven or more, whereas five or six out of 10 were rated as 'moderate', and trials with a score of 4 or less were ascribed a label of 'poor'.

The included trials were subjected to an in-depth assessment of possible confounders related to the LLLT treatment parameters and procedures. WALT guidelines for Laser class 3B (780–904 nm) were used when applicable. Trials exhibiting an LLLT treatment regimen that was not in line with the current recommendations by WALT were classified as having 'inadequate dosage'. Any adverse effects reported in the included trials were registered with comment on reason. Trials explicitly stating no adverse events or withdrawals were classified as safe.

Statistical analysis

Two reviewers (SH and JJ) extracted data for analysis in the statistical software program Reviewmanager (RevMan) (2013). If insufficient data were reported in the original articles, authors were contacted to provide additional information. Testing for statistical heterogeneity was performed using the chi-square test, which determined whether a random or fixed effect model was applied. Sub-group analyses were preplanned, as heterogeneity in LLLT treatment parameters, composition of treatment interventions and exercise designs were expected. Sensitivity analysis was performed to reveal trials contributing to a statistical heterogeneity, and the meta-analysis was considered to be valid if the inclusion of these trials did not influence, or inflate, the overall effect size. End of treatment data were pooled as follows.

Primary outcome measures

a) Pain intensity on a 100-mm visual analogue scale (VAS) was defined as the pooled estimate of the difference in change between the means of the treatment and the control groups, weighted by the inverse of the standard deviation of change for each study, that is, WMD of change between groups. The variance was calculated from the trial data and presented as 95% confidence intervals in millimetre on VAS.
b) Improved global health status was defined as any of the following categories: 'improved', 'good', 'better', 'much improved', 'pain-free' or 'excellent'. The relative risk for change in health status was calculated by pooling the number of improved patients.

Secondary outcome measures

c) Improved shoulder function defined as the mean change between groups in Shoulder Pain and Disability Index, Constant Murley Shoulder Score, Shoulder Disability Questionnaire or VAS for function.
d) Improved shoulder function defined as the mean change between groups in active abduction.

If pooling of data was justified, results were expressed as the standardized mean difference (SMD) of change between groups with 95% confidence interval. For shoulder function expressed as the mean change between groups in active abduction, WMD was calculated.

Results

The flow diagram (Figure 1) displays the results of the literature search conducted on 14 May 2013. A total of 395 potentially relevant articles were identified and assessed by their title and abstract. Of these, 340 articles were excluded as non-informing based on their abstracts. The remaining 55 trial reports were retrieved for more detailed evaluation, of which another 32 trials were excluded because of a variety of reasons. These included systematic reviews, duplicated reports, trials not addressing shoulder tendinopathy or impingement syndrome, or trials that did not include an active LLLT intervention. Of the 23 potentially conforming trials for inclusion to the systematic review, a further six trials were excluded for inappropriate trial designs such as lack of an inert placebo control group, lack of randomization or lack of a full trial report (Table 1). Finally, 17 RCTs were included in the review (Table 2).

Included trials in the review were subjected to a qualitative level of evidence assessment against the PEDro checklist (Table 3). All trials were of either moderate or high quality (mean PEDro score of 7). While 11

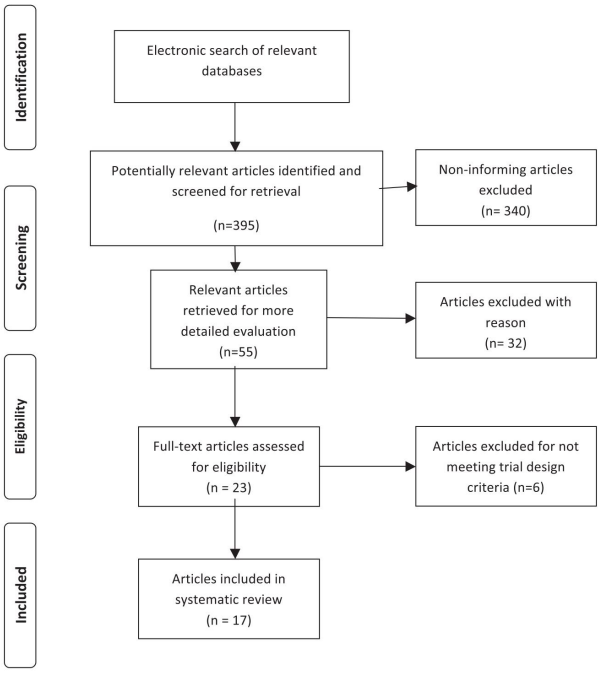


Figure 1. Flowchart

Table 1. Trials excluded for not meeting design criteria for systematic review on low-level laser therapy (LLLT)

Study by first author	Year	Design	Laser wavelength	Application technique	Result	Reason
Montes-Molina	2012	Randomized controlled trial (RCT)	810 nm	Site of maximal pain and four adjacent points	No significant differences between interferential and single diode LLLT.	Excluded because of lack of placebo control group. Both groups received active laser according to World Association For Laser Therapy recommendations.
Montes-Molina	2012	Randomized controlled pilot study	660 nm 905 nm	Not stated	Significant differences in favour of interferential LLLT compared with conventional LLLT.	Excluded because of lack of placebo control group.
Aydeniz	2011	RCT	905 nm	Tendon	No significant differences between LLLT and ultrasound.	Excluded for not being a full report. Only abstract available.
Tascioglu	2003	RCT	780 nm	Tendon	No significant between-group differences	Excluded for not being a full trial report.
Bringmann	1998	Double blind crossover	785 nm	Acupuncture points Duche	Between-group effects not stated.	Excluded because of lack of randomization.
England	1985					Duplicate

studies (Table 4) reported positive effects of LLLT, six studies reported no significant effects after LLLT treatment (Table 5).

Assessment of low-level laser therapy treatment procedures

Four of the studies with non-significant trial results were performed with inadequate LLLT dosages (Table 5). Three of these studies used the Roland (IR 904, Pagani, Milano, Italy) laser device, which has been revealed as faulty with power outputs less than 1% of the stated output by the manufacturer (Bjordal, 2010). Although a potentially valid reason for excluding these papers from the review, these trials were sub-grouped in the RevMan 5.2 analyses.

Trials with positive effect

Eleven out of 15 trials (73%) that reported pain relief on VAS favoured laser over placebo, no treatment or other modalities. Statistically significant ($p < 0.05$) effect sizes were found in nine of these trials (53%), of which seven trials exceeded the minimal important change of 14 mm on VAS (Tashjian *et al.,* 2009). When trials with inadequate laser dosage were excluded, 10 out of 11 trials (90%) displayed pain reductions exceeding 10 mm on VAS. The seven trials, which provided data on global improvement, were all in favour of LLLT, four of these trials were with statistically significant effect sizes.

Meta-analysis of effects

All included trials presented end of treatment data in a format that made it possible to use RevMan 5.2 for calculations of effect sizes in one or both of the primary outcome measures. Sixteen trials reported end of treatment data at 2–4 weeks after randomization. Mean treatment duration was 3 weeks. The trial by Vecchio *et al.* (1993) reported end of treatment data at 8 weeks. Bal *et al.* (2009) presented end of treatment scores at 12 weeks, but LLLT was only administered during the first 2 weeks. Continuous data for pain relief on a 100-mm VAS were available from 13 trials in a format that made statistical pooling possible.

Results for primary outcomes

LLLT was found to be significantly better ($p < 0.0001$) than placebo LLLT or no therapy at end of treatment, with a WMD of 20.41 mm (95% CI: 12.38–28.44) on VAS (Figure 2). Data from two trials showed that LLLT and exercise were significantly better ($p < 0.0001$) than exercise and placebo laser, with a WMD of 16.00 mm (95% CI: 11.88–20.12) on VAS (Figure 2). When used

Table 2. Characteristics of included randomized low-level laser therapy (LLLT) trials

Study	Year	Diagnosis	Control	Outcomes
Eslamian	2012	Rotator cuff tendinitis	Exercise and other modalities	VAS for pain Shoulder ROM Shoulder disability questionnaire
Otadi	2012	Shoulder tendonitis	Ultrasound and exercise	VAS for patient perception of change CMS for shoulder function Muscle force Tenderness Severity Scale
Abrisham	2011	Shoulder tendonitis	Exercise therapy and placebo LLLT	VAS for pain Shoulder ROM
Calis	2011	Subacromial impingement	1) Hot pack, ultrasound and exercise 2) Hot pack and exercise	VAS for pain VAS for movement Shoulder ROM CMS for shoulder function
Dogan	2010	Subacromial impingement	Cold pack, exercise and placebo LLLT	VAS for pain Shoulder ROM SPADI
Bal	2009	Subacromial impingement	Home exercises	VAS for pain SPADI for shoulder function UCLA for improvement
Yeldan	2009	Subacromial impingement	Placebo laser, cold pack and progressive exercises	VAS for pain VAS for activity CMS for shoulder function DASH questionnaire Shoulder disability questionnaire Muscle strength Shoulder ROM
Santamato	2009	Subacromial impingement	Ultrasound	VAS for pain CMS for shoulder function Simple shoulder test (SST)
Bingol	2005	Unilateral shoulder pain	Placebo LLLT and exercise	VAS for pain Palpation sensitivity Algometric sensitivity Shoulder ROM
Saunders	2003	Supraspinatus tendinoses	1) Ultrasound 2) No therapy	Muscle force VAS for pain VAS on activities Tendon tenderness
Al-Shenqiti	2002	Rotator cuff tendonitis	Placebo LLLT	VAS for pain SPADI for shoulder function Algometry over trigger points Shoulder ROM
Lodgberg-A	1997	Tendinitis or myofascial pain (majority shoulder tendonitis)	Placebo LLLT	VAS for pain Algometry Satisfaction with treatment Consumption of analgesics Patient perception of improvement
Saunders	1995	Supraspinatus tendinitis	Placebo LLLT	Huskisson's analogue pain scale Pain diary Muscle strength in empty can position Algometry of Tendon
Vecchio	1993	Rotator cuff tendinitis	Placebo LLLT and Exercise	VAS for pain VAS for movement VAS for activity Shoulder ROM Painful arc score
Taverna	1990	Shoulder periarthritis and cervical Osteoarthritis (OA)	Placebo LLLT	VAS for pain VAS for function
England	1989	Supraspinatus or biceptial tendonitis	Placebo LLLT	VAS for pain VAS for stiffness VAS for restriction VAS for function Shoulder ROM
Gudmundsen	1987	Rotator cuff syndrome	Placebo LLLT	Patient perception of improvement Sick leave

Studies listed by first author, year of publication, diagnosis of included patients, control group and outcome measures. VAS = visual analogue scale; ROM = range of movement; CMS = Constant Murley Shoulder score; SPADI = Shoulder Pain and Disability Index; UCLA = University of California-Los Angeles end-result score; DASH = Disabilities of the Arm, Shoulder and Hand questionnaire.

as an adjunct to exercise and other therapies, LLLT was significantly ($p = 0.02$) better than other therapies with a pain reduction of 12.80 mm (95% CI: 1.67–23.94) on VAS (Figure 2). A further two trials compared laser therapy to ultrasound, and both trials favoured LLLT with significant effect size. However, significant heterogeneity in treatment procedures and lack of trial data did not justify statistical pooling. In trials performed with inadequate laser dosages, LLLT was not significantly better ($p = 0.38$) than controls, with a WMD of 2.77 mm on VAS (95% CI: −3.46–8.99) (Figure 3). A total of seven trials, distributed in two different comparison groups, provided details allowing us to pool categorical data on global improvement. Data from five

Table 3. Methodology

Study	Year	1	2	3	4	5	6	7	8	9	10	11	Score	PEDro
Eslamian	2012	+	+	+	+	−	−	+	+	−	+	+	8	7
Otadi	2012	+	+	+	+	+	−	+	+	−	+	+	8	8
Abrisham	2011	+	+	+	+	+	−	+	+	+	+	+	9	9
Calis	2011	+	+	+	+	−	−	−	−	−	+	+	5	5
Dogan	2010	−	+	+	+	+	−	+	+	+	+	+	9	9
Bal	2009	+	+	+	+	−	−	+	+	+	+	+	8	7
Yeldan	2009	+	+	+	+	+	−	+	−	−	+	+	7	7
Santamato	2009	+	+	+	+	−	−	+	+	+	+	+	8	8
Bingol	2005	+	+	+	+	+	−	+	+	+	+	+	9	8
Saunders	2003	+	+	+	+	−	−	+	+	+	+	−	6	2
Al-Shenqiti	2002	+	+	+	+	+	−	+	+	−	+	+	8	*
Lodgberg-A	1997	+	+	−	+	+	+	+	−	−	+	−	6	5
Saunders	1995	+	+	−	+	+	+	+	+	+	+	+	9	9
Vecchio	1993	+	+	−	+	+	+	+	+	−	+	+	8	8
Taverna	1990	−	+	−	−	+	−	+	+	+	+	−	6	4
England	1989	+	+	−	−	+	−	+	+	+	+	+	7	6
Gudmundsen	1987	−	−	−	−	+	−	+	+	+	+	+	6	5

Studies listed by first author. Total method scores compared with PEDro reviewers in right column.

PEDro criteria:

1 — Eligibility criteria

2 — Random allocation

3 — Concealed allocation

4 — Baseline comparability

5 — Blind subjects

6 — Blind therapist

7 — Blind assessors

8 — Adequate follow-up

9 — Intention to treat analysis

10 — Between-group comparisons

11 — Point estimates and variability

+ = criterion met.

− = criterion not met (eligibility criteria do not contribute to total score).

*Trial not assessed by PEDro.

trials found LLLT to be significantly better ($p = 0.004$) than placebo or no therapy, with an overall relative risk of improvement at 1.96 (95% CI: 1.25–3.08). Two trials made statistical pooling on global improvement possible for LLLT as an adjunct to other interventions. The relative risk for improvement was significantly higher ($p = 0.006$) at 1.51 (95% CI: 1.12–2.03) in favour of LLLT. These results are displayed in Figure 4.

Results for secondary outcomes

Two trials comparing LLLT to placebo showed significantly better ($p < 0.0001$) shoulder function with an SMD of 1.01 (95% CI: 0.53–1.50) at end of treatment. When LLLT was used as an adjunct to other interventions, improvement in shoulder function was not significantly different ($p = 0.27$) from placebo laser, with an SMD of 0.33 (95% CI: −0.26–0.91). For trials performed with inadequate laser dosages, shoulder function was not significantly different ($p = 0.26$) from controls with an SMD of −0.17 (95% CI: −0.48–0.13). Results are displayed in Figure 5. We were able to pool data for shoulder function expressed as improvement in active shoulder abduction for two trials investigating the effect of LLLT as an adjunct to other interventions. Although the overall effect was negligibly in favour of laser therapy, the effect did not reach statistical significance ($p = 0.09$) with a WMD of 8.08 degrees (95% CI: −1.27–17.43). Data from three of the four trials with inadequate laser dosages showed no

Table 4. Trials reporting positive effects of low-level laser therapy

Study	Diagnosis	Application technique	Energy (J)	Power output (mW)	Wavelength (nm)	PEDro
Eslamian	Rotator cuff tendinitis	Tendon	2	100	830	8
Otadi	Shoulder tendonitis	Tendon	1.2	30	830	8
Abrisham	Shoulder tendonitis	*	*	*	890	9
Santamato	Subacromial impingement	Scan	*	*	1064	8
Saunders	Supraspinatus tendinitis	Tendon	4.5	50	820	6
Al-Shenqiti	Rotator cuff tendonitis	Tendon and trigger points	4	100	820	8
Lodgberg-A	Majority shoulder tendonitis	Tendon	*	8	904	6
Saunders	Supraspinatus tendonitis	Tendon	3.6	40	820	9
Taverna	Shoulder periarthritis	*	7.5	25	904	6
England	Shoulder tendonitis	*	0.9	3	904	7
Gudmundsen	Rotator cuff syndrome	Tendon	1.92	4	904	6
Total (mean)						7.5

Trial characteristics by first author, diagnosis, laser application technique, laser energy (J) per point, power output in milliwatts, laser type by wavelength in nanometers and PEDro method score.

*Insufficient information provided, or treatment parameters missing to calculate laser dosage.

Table 5. Trials reporting no effect of low-level laser therapy

Study	Diagnosis	Application technique	Energy (J)	Power output (mW)	Wavelength (nm)	PEDro
Calis	Subacromial impingement	*	0.72	6	904	5
Dogan	Subacromial impingement	Tendon	6	100	850	9
Bal −	Subacromial impingement	Tendon	1.6	13.2	904	8
Yeldan −	Subacromial impingement	Tendon	3	33.3	904	7
Bingol −	Unilateral shoulder pain	Tendon	*	*	904	9
Vecchio	Rotator cuff tendinitis	Tendon	3.6	40	830	8
Total (mean)						7.6

Trial characteristics by first author, diagnosis, laser application technique, laser energy (J) per point, power output in milliwatts, laser type by wavelength in nanometers and PEDro method score.

*Insufficient information provided, or treatment parameters missing to calculate laser dosage. Trials conducted with the malfunctioning Roland Pagani laser manufactured before 2011 when the emitted output was <1% than the output power stated by the manufacturer are given the abbreviation (−).

difference of LLLT in active abduction, with a WMD of −0.08 degrees (95% CI: −0.81–0.65). Results are displayed in Figure 6.

Treatment was well tolerated, and a general absence of side effects was reported, except for one trial (Otadi *et al.,* 2012), in which two diabetic patients experienced transient increased pain in the intervention group. No other trial reports on adverse treatment reactions were registered. Eight trials (Taverna *et al.*, 1990; Vecchio *et al.*,1993; Al-Shenqiti and Oldham, 2003; Bingöl *et al.,* 2005; Bal *et al.,* 2009; Yeldan *et al.,* 2009; Dogan *et al.,* 2010; Abrisham *et al.,* 2011) explicitly stated that there were no adverse events. Seven trials (Gudmundsen and Vikne, 1987; England *et al.,* 1989; Saunders, 1995; Saunders, 2003; Santamato *et al.,* 2009; Calis *et al.,* 2011; Eslamian *et al.,* 2012) failed to report such information; however, no participants withdrew from these studies. In another trial (Logdberg-Andersson *et al.,* 1997), the dropout rate was 12%, reportedly because of the lack of compliance to the inclusion criteria drug restrictions.

Discussion

Our results show that LLLT reduces pain and accelerates improvement when used as monotherapy, add-on therapy to exercise or in a physiotherapy treatment regimen. Except for LLLT alone, no strong evidence for an effect on shoulder function was found.

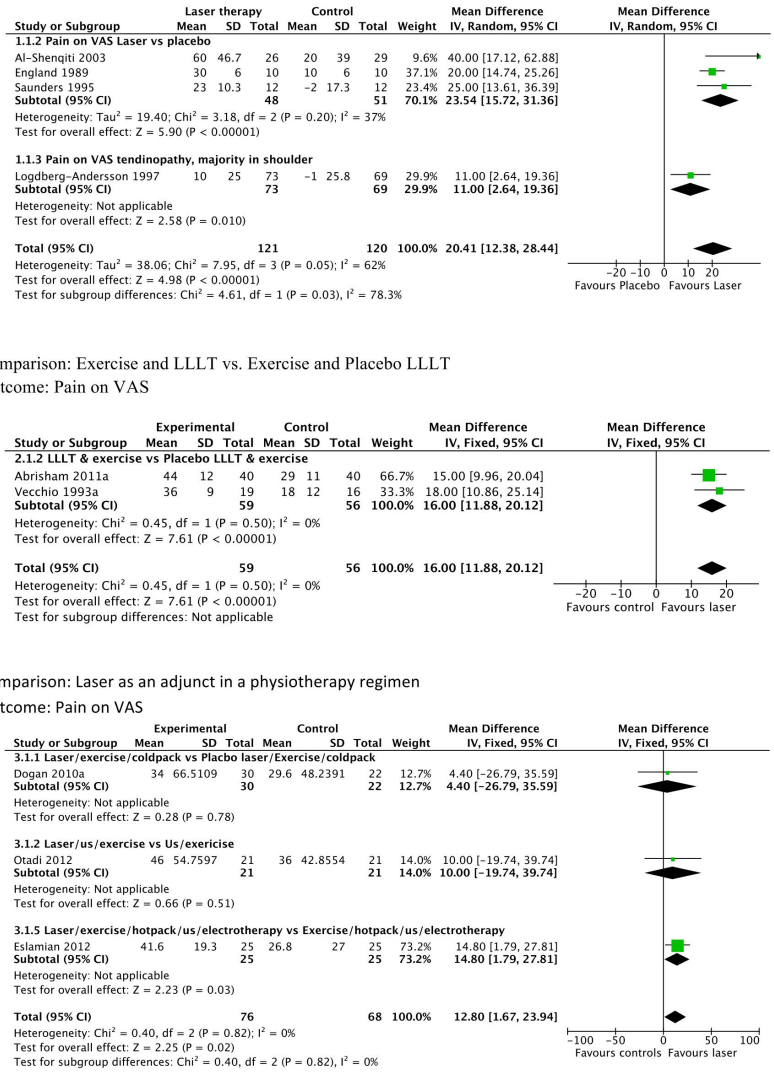


Figure 2. End of treatment results for low-level laser therapy (LLLT) measured as the weighted mean difference pain reduction on 100-mm visual analogue scale (VAS). Trials were sub-grouped by control treatment, and the overall effect is shown at the bottom of the table. Plots on the right hand side of the middle line indicate that the effect of LLLT is superior to the control treatment

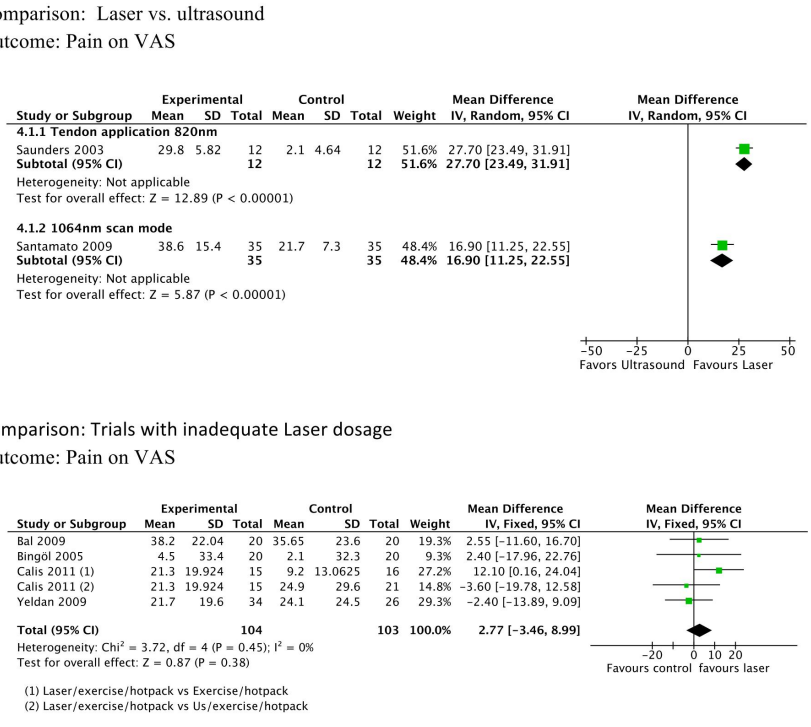
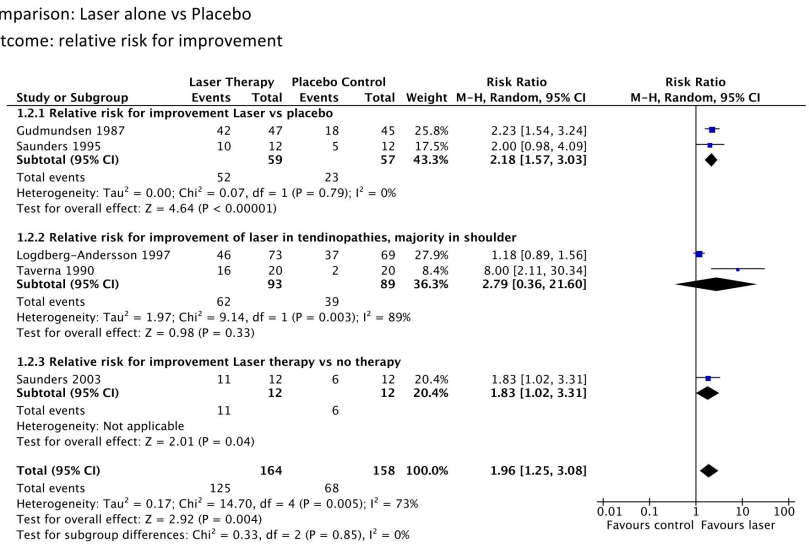


Figure 3. End of treatment results for low-level laser therapy (LLLT) measured as the weighted mean difference pain reduction on 100-mm visual analogue scale (VAS). Trials were sub-grouped by control treatment, and the overall effect is shown at the bottom of the table. Plots on the right hand side of the middle line indicate that the effect of LLLT is superior to the control treatment

The comparison of LLLT to placebo or no therapy may be of less interest for clinicians, as the complex aetiology of shoulder tendinopathy arguably makes LLLT unsuitable as a stand-alone therapy (Seitz *et al.*, 2011). However, pain relief exceeded the minimal value for clinical importance by a substantial margin (Tashjian *et al.*, 2009). Similar effect size for LLLT as monotherapy has been reported for lateral elbow tendinopathy (Bjordal *et al.*, 2008), which suggests that LLLT is a viable pain-modifying treatment alternative for physiotherapists. Considering the well-established benefits of treating these patients with exercise therapy (Green, *et al.* 2003; Kelly *et al.*, 2010; Littlewood *et al.*, 2011; Hanratty *et al.*, 2012), deflated effects were expected for any trial using this as a co-intervention to LLLT. Perhaps, the most important finding of this review was that statistically significant and clinically important pain relief was found in patients treated with LLLT as an additive to exercise. Even though smaller effect sizes were found for LLLT as a co-intervention in a physiotherapy treatment regimen, pain relief was still significant with reductions bordering the clinically important threshold on VAS. Consequently, adding LLLT to an exercise-based treatment programme may accelerate improvement of physical function, possibly by keeping the inflammation under control or stimulating tendon repair, with the end result being reduced pain and more rapid improvement.

An interesting perspective to arise from this analysis is whether outcome measures of pain are clinically valid if pain relief is not accompanied by improvements in measures of disability (Kromer *et al.*, 2010). We



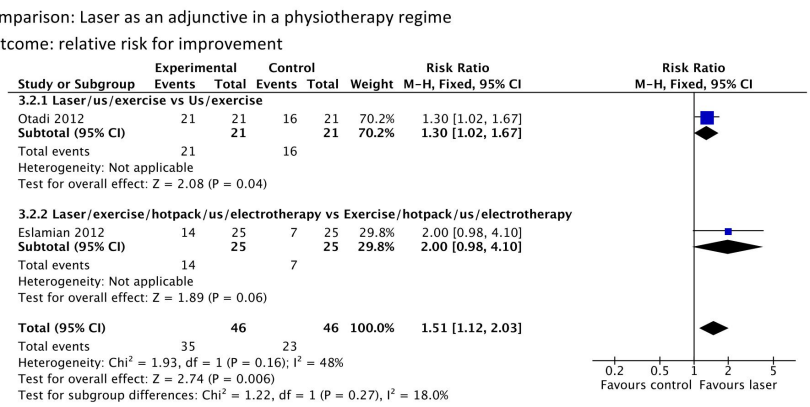
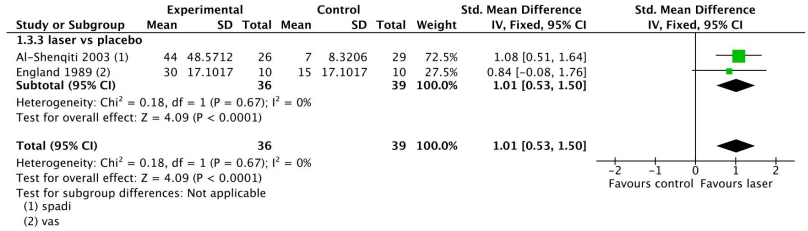


Figure 4. End of treatment results for low-level laser therapy (LLLT) measured as relative risk for global improvement. Trials were subgrouped by control treatment, and the overall effect is shown at the bottom of the table. Plots on the right hand side of the middle line indicate that the effect of LLLT is superior to the control treatment

found no strong evidence for such relationship in laser treatment, as effects on physical function were only significant for LLLT as monotherapy. However, pain is often evaluated separately from disability in clinical practice, and pain intensity levels control exercise progression in shoulder tendinopathy (Holmgren *et al.*, 2012). Hence, LLLT may have a more pronounced effect on shoulder function if the benefit of pain relief is used specifically to optimize parameters of exercise. It seems reasonable that the effectiveness

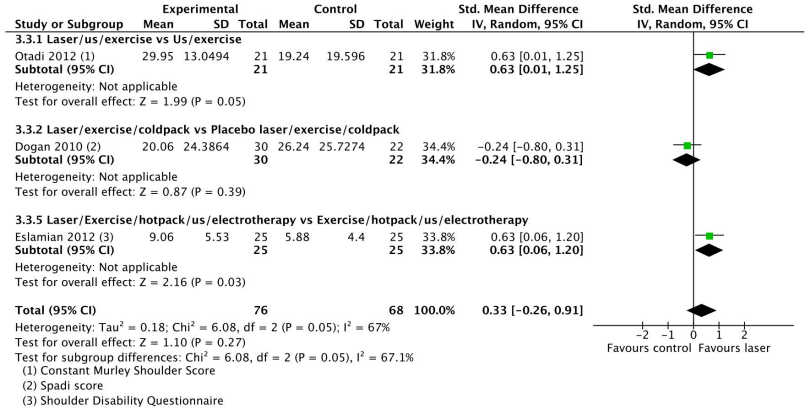
Comparison: Laser alone vs Placebo

Outcome: shoulder function



Comparison: Laser as an adjunctive in a physiotherapy regime

Outcome: shoulder function



Comparison: Trials with inadequate LLLT dosage

Outcome: shoulder function

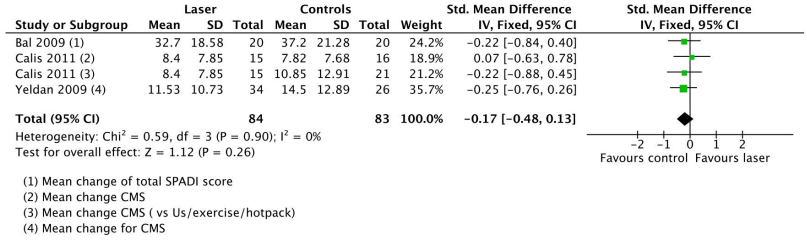


Figure 5. End of treatment results for low-level laser therapy (LLLT) measured as the standardized mean difference for shoulder function. Trials were sub-grouped by control treatment, and the overall effect is shown at the bottom of the table. Plots on the right hand side of the middle line indicate that the effect of LLLT is superior to the control treatment

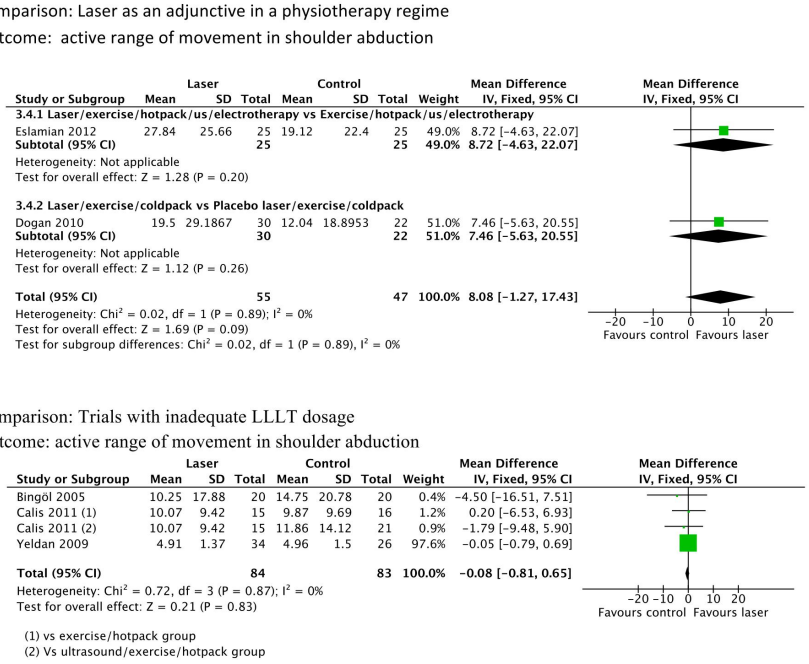


Figure 6. End of treatment results for low-level laser therapy (LLLT) measured as the weighted mean difference for active shoulder abduction. Trials were sub-grouped by control treatment, and the overall effect is shown at the bottom of the Table. Plots on the right hand side of the middle line indicate that the effect of LLLT is superior to the control treatment

of the intervention should be evaluated based on the anticipated impact on the pathophysiology it is targeting (Lopes-Martins *et al.*, 2007), rather than all constructs of a disease. Indeed, pharmaceutical pain treatment methods are usually evaluated by their effects primarily on pain (Van Der Sande *et al.*, 2012).

Our assessment of procedural failures related to LLLT treatment resulted in four trials being subjected to a separate analysis. Three of these trials (Bingöl *et al.*, 2005; Bal *et al.* 2009; Yeldan *et al.*, 2009) were performed with the discredited Roland (IR 904) laser device, which displayed reduced power outputs not in accordance to stated specifications (Bjordal, 2010). This manufacturer has since 2011 implemented quality control by testing outputs by an external independent body. Calis *et al.* (2011) used a laser dosage unsupported by the current recommendations for treating tendinopathies with LLLT. No effects of LLLT were seen across these trials. This finding is not surprising, as dosage-dependency of LLLT has been identified in several previous reviews (Bjordal *et al.*, 2008; Tumilty *et al.*, 2010).

For this reason, we were more intrigued to discover a high-quality trial (Dogan *et al.*, 2010) performed with adequate LLLT dosage and application technique, which displayed ineffective results for all outcome measures. Unique for this study was the use of cold therapy as a co-intervention. A possible yet unexplored issue in LLLT research is whether the local slowing of metabolic rate and vasoconstriction induced by cryotherapy (Enwemeka *et al.* 2002) obstruct the delivery or absorption of laser energy. In both cases, a reduced effect of LLLT would be expected. Worth mentioning is also the negative trial result reported by Vecchio *et al.* (1993), where laser dosage was found to be adequate

in the absence of cryotherapy. Our RevMan analysis challenged the conclusions of Vecchio *et al.* (1993), as we found the result to be significantly in favour of LLLT. Similar interpretations have been reported across several other reviews (Van Der Heijden *et al.*, 1997; Bjordal, 2010; Tumilty *et al.,* 2010).

To our knowledge, this is the first systematic review and meta-analysis of LLLT in shoulder tendinopathy. Two previous reviews (Green *et al.,* 2003; Kromer *et al.,* 2009) covered the effect of several physiotherapy interventions and based their conclusions regarding the effectiveness of LLLT for subacromial impinge ment/rotator cuff patients on two (Vecchio *et al.* 1993; Saunders, 1995) and three trials (England *et al,* 1989; Vecchio *et al.,* 1993; Saunders,1995), respectively. Both reviews deemed LLLT ineffective compared with placebo. In the latest review by Tumilty *et al.* (2010), participants suffering from tendinopathy were included, and four trials (England *et al.,* 1989; Vecchio *et al.,* 1993; Saunders, 1995; Saunders, 2003) investigating the effect of laser for shoulder tendinopathy were found. The level of evidence was considered inconclusive in this review, because of what seems to be a negative interpretation of the Vecchio *et al.* (1993) trial.

A further eight trials (Bal *et al.,* 2009; Santamato *et al.,* 2009; Yeldan *et al.,* 2009; Dogan *et al.,* 2010; Abrisham *et al.,* 2011; Calis *et al.,* 2011; Eslamian *et al.,* 2012; Otadi *et al.,* 2012) have been published since the literature search was performed. By not having language restrictions and using a broad range of LLLT related synonyms in our literature search, we found a total of 23 potential RCTs eligible for inclusion in our review. Two trials were excluded for lacking a placebo control group (Montes-Molina *et al.*, 2012b; Montes-Molina *et al.,* 2012a) and another two RCTs for not being full trial reports (Tascioglu *et al.,* 2003; Aydeniz *et al.,* 2011). Other reasons for exclusion were lack of randomization (Bringmann, 1998) and trial duplication (England *et al.,* 1985). These exclusions left 17 eligible studies for our systematic LLLT review, which is substantially more than previously published reviews of LLLT in shoulder tendinopathy.

The mean quality score of the included trials was seven out of 10 on the PEDro scale. This finding is similar to other LLLT reviews on tendinopathy (Bjordal *et al.,* 2008; Tumilty *et al.,* 2010) and shoulder interventions in general (Kromer *et al.,* 2009). Our quality assessment was in line with the PEDro reviews for all but two trials (Taverna *et al.,* 1990; Saunders, 2003). The trial by Saunders (2003) was awarded with two out of 10 points by PEDro reviewers, but our quality score contradicts that of PEDro and is in concordance with other quality assessments (Tumilty *et al.,* 2010). Contrary to PEDro, we awarded points for assessor blinding and baseline comparability, as these aspects were explicitly stated in the paper. Further points were awarded for adequate follow-up and statistical between group comparisons based on reporting of pain measurements and *p*-value analyses for all outcome measures, respectively. Disagreement regarding the Taverna *et al.* (1990) trial was related to patient blinding and intention to treat analysis criteria. We found that all shoulder patients completed the study. Further, using a 904-nm laser device, which emits no visible light in active mode, ensured blinding of subjects. Consequently, we deemed treating controls with the laser switched off adequate for blinding.

Our findings demonstrate that LLLT is a safe and effective pain treatment option in shoulder tendinopathy patients. Interesting perspectives regarding conservative pain management arises in this context. The benefit of treating shoulder tendinopathy patients with CSI seems small and short-lived compared with placebo and not superior to NSAIDs (Buchbinder *et al.,* 2003; Koester *et al.,* 2007; Van Der Sande *et al.,* 2012). But even harmful long-term effects of CSI have been reported for other tendinopathy, such as inferior reductions in pain (Coombes *et al.,* 2010). Future research on LLLT should elaborate on this question, by designing high-quality trials where the effect of laser is directly compared with pharmaceuticals. As for now, it seems reasonable that LLLT is offered, before proceeding to potentially harmful drugs.

Limitations of this review

Even though the overall effect for LLLT on pain and global improvement is encouraging in this review, the result should be interpreted with caution. The included trials were subjected to analysis in one out of four different comparison groups, depending on control group measures. Hence, the result for each comparison and outcome arises from a handful of studies. It should also be noted that our outcome measures are based solely on end of treatment data (2–12 weeks) and display the potential short-term effects of LLLT for shoulder tendinopathy. Only three trials (Al-Shenqiti and Oldham, 2003; Bal *et al.,* 2009; Otadi *et al.,* 2012)

provided post-treatment follow-up data, and no robust conclusions can be drawn regarding the long-term effects.

Synthesizing evidence was challenging for numerous reasons. In LLLT research, the validity of a study is based on both methodological quality and the validity of the intervention procedure. Clinical application procedures and laser parameters were poorly or inaccurately described in some studies (England *et al.,* 1989; Logdberg-Andersson *et al.,* 1997; Santamato *et al.*, 2009; Abrisham *et al.,* 2011). In addition, there were large variations in laser wavelength (nm), number of points treated, composition of co-interventions and exercise design across the included studies. Lack of therapist blinding and intention to treat analysis were the two most frequent methodological shortcomings, consequently increasing the potential bias of this review. Although we thoroughly searched databases for available literature, hand-searches of the grey literature were not performed. As negative publication bias has been reported for the LLLT literature (Bjordal *et al.,* 2008), we cannot exclude the possibility that published trials may have been overlooked.

Conclusion and implications

This systematic review and meta-analysis suggests that LLLT is a safe and effective treatment alternative for painful shoulder tendons. The clinical benefit does not seem to be limited to LLLT when used as monotherapy. On the contrary, LLLT seem to induce additive effects in terms of reduced pain and a more rapid improvement, even when used as an adjunct to the gold standard of exercise or physiotherapy treatment regimens. Our results support the growing body of evidence demonstrating that LLLT acts in a dose-dependent manner. The use of cold therapy may negatively influence the effect of LLLT and should be investigated in future laboratory and clinical trials. There is also need for future research comparing LLLT to the most widely used pharmaceutical agents.

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APPENDIX

Assessment of Saunders 2003 trial

Assessed by	1	2	3	4	5	6	7	8	9	10	11	Score
PEDro	–	+	+	–	–	–	–	–	–	–	–	2
review authors	+	+	+	+	–	–	+	+	–	+	–	6

Comments regarding disagreement in Saunders 2003 method score (PEDro scale)

Item	Criteria	Comment
1	Eligibility	List of criteria used to determine who was eligible to participate in the study listed on page 366 under subjects, for example men and women with supraspinatus tendinoses diagnosed on clinical examination (empty can test, palpation tenderness and full passive range of movement). Symptoms of supraspinatus tendinosis for longer than 4 weeks.
4	Baseline comparability	Stated in summary page 369 that the three groups were similar at baseline regarding muscle weakness, functional disability and tendon tenderness. Mean (SD)/median age and symptom duration of study subjects support this conclusion (page 367, Tables 1 and 2).
7	Blind assessors	'The assessor was blind to the treatments received by the patients', stated in the article on page 368 under outcome measures (tenderness).
8	Adequate follow-up	Criteria satisfied if the report states number of subjects initially allocated to group and the number of subjects from whom key outcome measures were obtained. Subjects and outcome for changes in pain accounted for in Figure 2, page 369.
10	Between group statistical comparisons	*P*-values for between group comparison stated for each outcome measure, page 369–370 under results.

PAPER II

PAPER III

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