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PULSED ULTRASOUND FOR BONE REGENERATION – OUTCOMES AND HURDLES IN THE CLINICAL APPLICATION: A SYSTEMATIC REVIEW

R. Puts[1,*], L. Vico[2], N. Beilfuß[1], M. Shaka[1], F. Padilla[3,4] and K. Raum[1]

[1] Charité-Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, BCRT – Berlin Institute of Health Centre for Regenerative Therapies, 13353 Berlin, Germany
[2] INSERM, U1059, University of Lyon, University of Saint-Etienne, F-42270 Saint-Etienne, France
[3] Focused Ultrasound Foundation, Charlottesville, VA 22903, USA
[4] Department of Radiology, University of Virginia School of Medicine, Charlottesville, VA, USA

Abstract

Impaired bone-fracture healing is associated with long-term musculoskeletal disability, pain and psychological distress. Low-intensity pulsed ultrasound (LIPUS) is a non-invasive and side-effect-free treatment option for fresh, delayed- and non-union bone fractures, which has been used in patients since the early 1990s. Several clinical studies, however, have questioned the usefulness of the LIPUS treatment for the regeneration of long bones, including those with a compromised healing. This systematic review addresses the hurdles that the clinical application of LIPUS encounters. Low patient compliance might disguise the effects of the LIPUS therapy, as observed in several studies. Furthermore, large discrepancies in results, showing profound LIPUS effects in regeneration of small-animal bones in comparison to the clinical studies, could be caused by the suboptimal parameters of the clinical set-up. This raises the question of whether the so-called "acoustic dose" requires a thorough characterisation to reveal the mechanisms of the therapy. The adequate definition of the acoustic dose is especially important in the elderly population and patients with underlying medical conditions, where distinct biological signatures lead to a delayed regeneration. Non-industry-funded, randomised, double-blind, placebo-controlled clinical trials of the LIPUS application alone and as an adjuvant treatment for bones with complicated healing, where consistent control of patient compliance is ensured, are required.

Keywords: Low-intensity pulsed ultrasound, bone regeneration, surgery, acoustic dose, non-union, age, osteoporosis, compliance.

***Address for correspondence**: Regina Puts, Berlin Institute of Health Centre for Regenerative Therapies, Charité-Universitätsmedizin, Föhrer Straße 15, 13353 Berlin, Germany.
Telephone number: +49 30450539506 Email: regina.puts@charite.de

List of Abbreviations

BMD	bone mineral density
BMP	bone morphogenetic protein
CT	computed tomography
DC	duty cycle
DKK-1	Dickkopf-1
DXA	dual-energy X-ray absorptiometry
ECM	extracellular matrix
HIF-1α	hypoxia-inducible factor 1α
IM	intramedullary
I_{SATA}	spatial average temporal average acoustic intensity
LIPUS	low-intensity pulsed ultrasound
MMP	matrix metalloproteinase
MSC	mesenchymal stromal cell
NO	nitric oxide
ORIF	open reduction internal fixation
PRF	pulse repetition frequency
PRISMA	preferred reporting items for systematic reviews and meta-analyses
RCT	randomised double-blind clinical trial
Runx-2	Runt-related transcription factor 2
STD	standard deviation

TB	Twin-Block
TMJ	temporomandibular joint
TRUST	trial to re-evaluate low-intensity pulsed UltraSound in treatment of tibial fractures
VEGF-A	vascular endothelial growth factor A

Introduction

According to the USA National Health Interview Survey, more than half of all chronic medical conditions reported in 2012 were associated with musculoskeletal problems (Hauser *et al.*, 2016). The bone is an organ able to regenerate after a fracture to its full functional integrity without scar formation. However, approximately 10 % of all fractures do not heal without complications (Volpin, 2014). These cases, also known as delayed- and non-union bone fractures, are accompanied by the life burdens of limited or no mobility, pain and psychological stress (Lerner *et al.*, 1993; Mitchell *et al.*, 2018). Moreover, the median total costs for treating a non-union in the USA was calculated to be USD 25,556 (Antonova *et al.*, 2013). With progressing age, the odds of a complicated bone healing abruptly increase (Clark *et al.*, 2017). Since the proportion of ageing population continually grows, especially in the developed countries, the advances in novel technologies for efficient fracture regeneration are especially urgent.

In 1983, Duarte showed that stimulation of osteotomised rabbit fibula and femur bones with LIPUS enhanced callus formation (Duarte, 1983). Currently, a device employing LIPUS is manufactured under the brand name of Exogen® (Bioventus LLC, Durham, NC, USA), which emits pulsed sine waves at an ultrasound frequency of 1.5 MHz, a PRF of 1 kHz and a 20 % DC, generating a I_{SATA} of 30 mW/cm^2 (Pounder and Harrison, 2008). Exogen® is used across the globe for the treatment of fresh fractures, delayed- and non-union bones and, so far, no negative side effects have been reported. The device is fully portable and does not require medically qualified staff for its operation. The treatment can be applied by the patient at home and lasts 20 min/d for the prescribed period. However, the question of the efficiency and suitability of the LIPUS technique for fracture healing remains open for debate (Busse *et al.*, 2014; Garner, 2017; Griffin, 2016; Griffin *et al.*, 2014; Poolman *et al.*, 2017; Schandelmaier *et al.*, 2017a; Tarride *et al.*, 2017; TRUST Investigators writing group *et al.*, 2016).

Once a bone fracture occurs, the orthopaedic surgeon has to decide the suitable type of treatment for the patient, with surgery being increasingly the first choice (Courtney *et al.*, 2011; Fernandez, 2005; Schmidt *et al.*, 2003). Should complementary methods, such as LIPUS, be used as an adjuvant to the conservative option with cast or to surgery? Can LIPUS be beneficial for bones with complicated healing? The purpose of the present review is to provide the reader with an impartial opinion on the above questions.

Materials and Methods

Search and retrieval of scientific studies was conducted in accordance with the PRISMA (Moher *et al.*, 2009). Studies published between December 1950 and April 2021 were collected from PubMed and Web of Science databases using as keywords "low-intensity pulsed ultrasound" and "bone fracture". Search duplicates were first identified using EndNote software. Then, these were verified and further removed manually. Articles, that were not peer-reviewed, without a full-text option or written in a language other than English were excluded. Studies describing *in vitro* findings and studies in animal models were not retained for the main data analysis. Additionally, articles irrelevant to ultrasound, using ultrasound for other purposes than LIPUS stimulation or describing LIPUS application in other organs than bone were excluded.

Results

A PRISMA diagram describing the identification of manuscripts for the data analysis is depicted in Fig. 1. The search queries identified 449 and 357 search results using PubMed and Web of Science databases, respectively. 6 publications, meeting all the inclusion criteria, were found in a Google Scholar free search and designated in the PRISMA chart as "other sources". EndNote software identified 134 duplicates and an additional 95 were excluded upon manual verification, resulting in 583 search results. A restriction of the search results based on full-text peer-reviewed articles in English language excluded 43 additional studies. LIPUS application *in vitro*, *in silico* and in animal models accounted for 88, 2 and 139 entries, respectively. These were identified following thorough screening of the full-text articles. Studies, irrelevant to ultrasound techniques (27), irrelevant to bone fracture stimulation (10) or describing other ultrasound methods (111) were screened out manually and excluded from the analysis. Finally, 163 articles met all the set criteria. Out of them, 77 and 24 were review articles and case studies (data not shown), respectively. Finally, 62 articles (Table 1-3) reporting original findings were included in the present review. Most of the clinical studies identified employ Exogen® or Exogen®-like stimulation devices, with the clinical acoustic parameters of 1.5 MHz, 1 kHz PRF, 20 % DC and 30 mW/cm^2 I_{SATA}. These are summarised in Table 1-3. 9 studies use LIPUS parameters that are different from the conventionally used ones or are not clearly specified (Arima *et al.*, 2017; Bawale *et al.*, 2020; Gan *et al.*, 2014; Gopalan *et* *al.*, 2020; Liu *et al.*, 2014; Ozdemir *et al.*, 2008; Patel *et al.*, 2015; Santana-Rodríguez *et al.*, 2019; Warden *et al.*, 2001).

LIPUS and fresh fractures: surgery *vs.* cast

There are several hurdles that the application of LIPUS in a clinical setting encounters. The first is the definition of a fresh fracture, which discriminates cases older than 1 week (Heckman, 2017; Zura *et al.*, 2017). This might prevent some potential candidates from receiving non-invasive treatment strategies such as LIPUS. Furthermore, a large number of studies dedicated to LIPUS stimulation of fresh fractures are either based on case studies (data not shown), retrospective studies (Akiyama *et al.*, 2014; Arima *et al.*, 2017; Kinami *et al.*, 2013; Ota *et al.*, 2018; Ota *et al.*, 2017; Song *et al.*, 2019; Zura *et al.*, 2015b) or prospective trials conducted in an unblinded manner and/or without sham controls (Arimoto *et al.*, 2019; Brand *et al.*, 1999; Dudda *et al.*, 2011; El-Mowafi and Mohsen, 2005; Gan *et al.*, 2014; Gold and Wasserman, 2005; Gopalan *et al.*, 2020; Leung *et al.*, 2004b; Liu *et al.*, 2014; Patel *et al.*, 2015; Salem and Schmelz, 2014; Santana-Rodríguez *et al.*, 2019; Tsumaki *et al.*, 2004; Urita *et al.*, 2013) (Table 1), challenging the credibility of the LIPUS therapy. Additionally, the small size of patient cohorts of several prospective, randomised, double-blind, placebo-controlled trials diminish the importance of their findings (Emami *et al.*, 1999; Handolin *et al.*, 2005a; Handolin *et al.*, 2005b; Raza *et al.*, 2016).

The discussion on whether LIPUS should be used as an alternative or an adjuvant therapy to surgical intervention has become more intense recently, especially since the results of the multicentre randomised, blinded, sham-controlled clinical trial TRUST was published in 2016 (Busse *et al.*, 2014; TRUST Investigators writing group *et al.*, 2016). The study enrolled 501 patients with tibial fractures treated surgically and fixed with an IM nail. No effect of LIPUS stimulation on the radiographically indicated healing time and restoration of full bone-functionality was observed. The data were published soon after as a BMJ Rapid Recommendations article (Poolman *et al.*, 2017), advising the removal of LIPUS from clinical practice. A systematic review (Schandelmaier *et al.*, 2017a) further analysed 26 randomised trials on the use of LIPUS therapy in all types of fracture, concluding that only 3 unbiased studies (Busse *et al.*, 2014; Emami *et al.*, 1999; TRUST Investigators writing group *et al.*, 2016) have been published, with two of them being the results of the TRUST study. LIPUS treatment in these studies was not found to accelerate bone healing. The high risks of bias were defined as i) the lack of a blinded expert, ii) non-identically looking sham device, iii) a

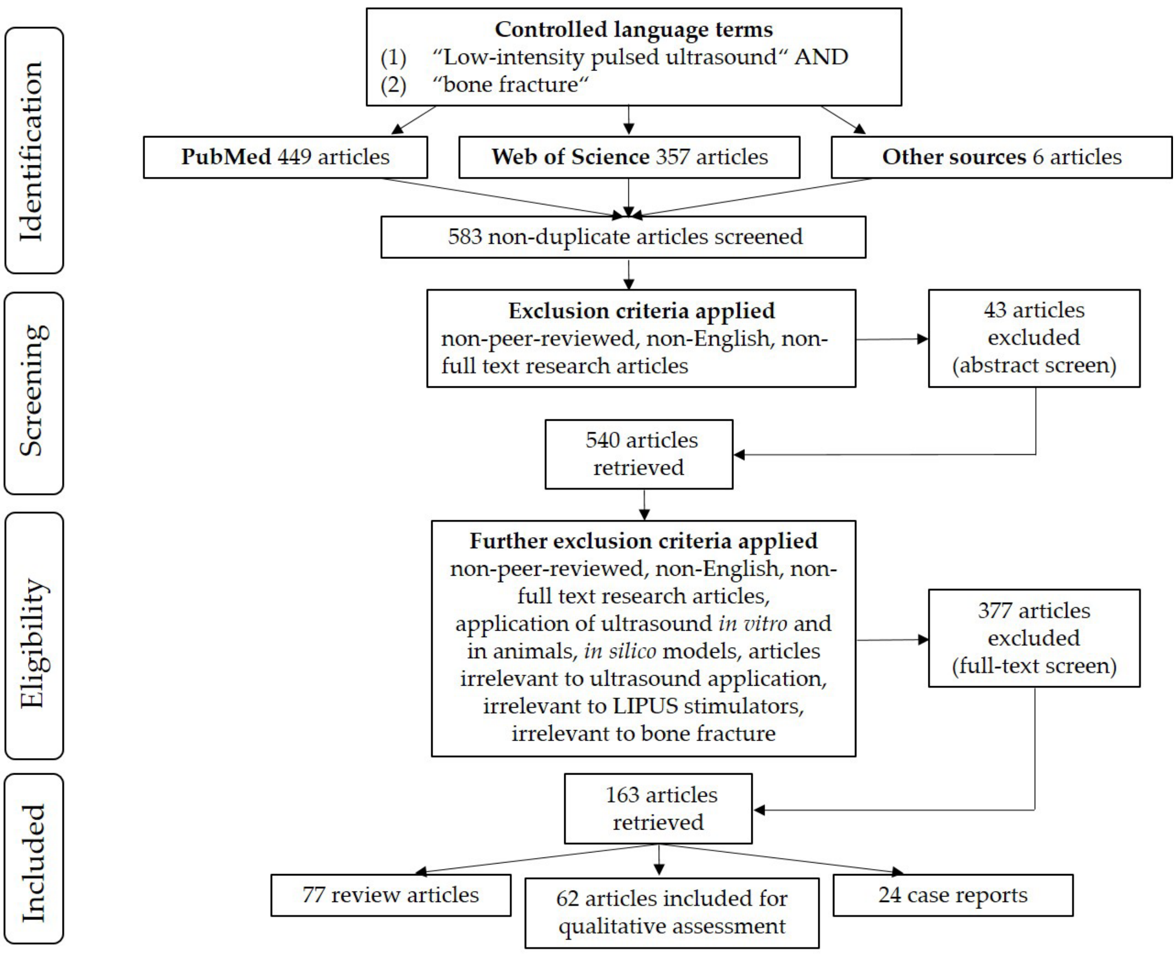

Fig. 1. PRISMA diagram of search inclusion and exclusion criteria. The search yielded 62 scientific studies published between December 1950 and April 2021 that were analysed in the present review.

Table 1. LIPUS for fresh fractures and distraction osteogenesis.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Akiyama *et al.*, 2014	Retrospective, comparative	Femoral reconstruction using a cortical-only strut allograft	35 patients LIPUS 14, mean: 63 years old (23-79 years old) Control 21, mean: 65.8 years old (45-84 years old)	Exogen®	No	Not reported	Early and complete radiographic bridging was 60-65 % faster in LIPUS group	LIPUS, mean 29 months Control, mean 75 months No complications	Retrospective study, without sham control; small patient cohort
Arima *et al.*, 2017	Retrospective, comparative	Paediatric lumbar spondylosis treated conservatively (brace)	13 patients LIPUS 6 (14.7 ± 2.2 years old) Control 7 (14.6 ± 2.9 years old)	1.5 MHz, 200 ms, 1 kHz, I_{SATA} = 60 mW/cm^2	No	Follow up rate 86.7 % LIPUS application performed by medical staff Compliance not specified	66.7 % of defects healed in LIPUS group *vs* 10 % in control group Time to healing was shorter in the active group	CT scans were performed every 1.5 months	Retrospective study, without sham control; small patient cohort
Arimoto *et al.*, 2019	Prospective, randomised patients' distribution and blind assessment of images	Intraoral vertical ramus osteotomy, mandibular	21 patients LIPUS 12 Control 9 16 to 54 years old, not specified between groups	Exogen®	No	Patients were treated for 3 weeks with LIPUS Not assessed beyond 3 weeks	LIPUS improved bone density	At 1 month, 6 months and 1 year postoperatively	Small patient cohort; no sham treatment
Brand *et al.*, 1999	Prospective, observational	Tibial stress fractures	8 patients, high-school or college students	Exogen®	No	Not specified	All but 1 fractures healed	4 weeks	Lack of any controls; small patient cohort
Busse *et al.*, 2014	Prospective, multicentre, double-blind, randomised, placebo controlled	Tibial facture fixed using a reamed IM (pilot study)	51 patients LIPUS 23 (39.0 ± 13.6 years old) Control 28 (39.6 ± 13.6 years old)	Exogen®	Yes	76 % fully compliant and 24 % more than 50 % compliant	No improvement after LIPUS therapy	At 1 year, follow-up rate 84 %	IM provides optimal mechanical conditions
Busse *et al.*, 2016	Prospective, multicentre, double-blind, randomised, placebo controlled	Tibial facture fixed using a reamed intramedullary nail	501 patients LIPUS 250 (37.1 ± 13.2 years old) Control 251 (39.1 ± 14.6 years old)	Exogen®	Yes	73 % administered 50 % of treatments	Addition of LIPUS did not improve healing rate	At 52 weeks	IM provides optimal mechanical conditions; inadequate compliance

Table 1. LIPUS for fresh fractures and distraction osteogenesis.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Coughlin *et al.*, 2008	Prospective, comparative	Hindfoot undergoing subtalar arthrodesis, fixed using a cast	15 patients compared retrospectively to 15 patients without LIPUS No patients' demographics	Exogen®	No	Not specified	Accelerated healing at 9 weeks (measured radiographically)	At 6 and 12 months	Study without sham control; small patient cohort
Dudda *et al.*, 2011	Prospective, randomised, comparative	Distraction osteogenesis of long bones (Ilizarov fixator)	36 patients LIPUS 16 (34.9 ± 14.7 years old) Control 20 (42.2 ± 13.3 years old)	Exogen®	No	Not specified	LIPUS group had shorter healing time, despite bigger distraction gaps	Every 3-4 weeks until healing	No sham control; small patient cohort; unblinded design
El-Mowafi and Mohsen, 2005	Prospective, randomised, comparative	Distraction osteogenesis of tibia (Ilizarov fixator)	20 patients LIPUS 10 Control 10 Mean: 35 years old (18 to 45 years old) Age distribution between groups not specified	Exogen®	No	Not specified	LIPUS shortened time needed for bone consolidation	Every week until healing	No sham control; small patient cohort
Emami *et al.*, 1999	Prospective, randomised, double-blind, placebo controlled	Tibial facture fixed using statically locked or reamed intramedullary nails	32 patients LIPUS 15 (39.9 ± 16.2 years old) Control 17 (34.3 ± 14.1 years old)	Exogen®	Yes	91.4 % compliance recorded by device LIPUS applied only 53 % of the time until healing	No effect of LIPUS on healing time	Every 3 weeks until healing and at weeks 26 and 52	IM provided optimal mechanical conditions; inadequate compliance; small patient cohort
Gan *et al.*, 2014	Prospective, randomised, double-blind, placebo controlled	Lower limb, bone stress injuries	23 patients LIPUS 10 (32.7 ± 10.6 years old) Control 13 (28.6 ± 13.3 years old)	1.5 MHz, 1 kHz PRF, 200 ms pulses, I_{SATA} = 30 mW/cm^2	Yes	Not measured	No effect of LIPUS	At 4, 8, 10 and 12 weeks LIPUS applied for only 4 weeks	Good spontaneous healing rate of bone stress injuries; small patient cohort

Table 1. LIPUS for fresh fractures and distraction osteogenesis.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Gold and Wasserman, 2005	Prospective, comparative	Distraction osteogenesis of tibia (large bone defect; Ilizarov fixator)	20 patients LIPUS 8 Control 12 Mean: 34 years old (18-50 years old) Compared retrospectively	Exogen®	No	Not specified	The external fixation index was reduced by 17.2 % (statistically non-significant) as a result of LIPUS therapy	Weekly for 4 weeks, twice a month for 2 months and once a month until healing	Lack of any control; small patient cohort
Gopalan *et al.*, 2020	Prospective, randomised, single-blind, comparative	Mandibular fracture	40 patients LIPUS 20 (28.0 ± 7.3 years old) Control 20 (26.8 ± 8.7 years old)	1.5 MHz, I_{SATA} = 30 mW/cm^2 (rest not specified), on days 4, 8, 14 and 20	No	100 % LIPUS applications performed by medical staff	LIPUS reduced pain and improved fracture healing (measured radiographically)	Pain: on days 5, 9, 15 and 21 Images: at weeks 4, 8 and 12	No sham control
Handolin *et al.*, 2005a	Prospective, randomised, double-blind, placebo controlled	Screw-fixed, lateral malleolar fracture	22 patients LIPUS 11, mean: 37.5 years old (18-5 years old 4) Control 11, mean: 45.5 years old (26-59 years old)	Exogen®	Yes	Not specified	No effect of LIPUS on bone healing (measured radiographically)	At weeks 2, 6, 9 and 12	Small patient cohort; possibility of early weight bearing
Handolin *et al.*, 2005b	Prospective, randomised, double-blind, placebo controlled	Screw-fixed, lateral malleolar fracture	30 patients LIPUS 15, mean: 41.4 years old (19-65 years old) Control 15, mean: 39.4 years old (18-59 years old)	Exogen®	Yes	Not specified	LIPUS did not speed up fracture healing; however, more frequent callus formation was observed in LIPUS group	At weeks 2, 6, 9 and 12	Small patient cohort; possibility of early weight bearing
Heckman *et al.*, 1994	Prospective, multicentre, randomised, double-blind, placebo controlled	Tibial fracture, fixed using a cast	66 patients with 67 fractures LIPUS 33 (36 ± 2.3 years old) Control 34 (31 ± 1.8 years old)	Exogen®	Yes	89.5 % of patients returned to follow-ups Exact device usage is not specified	LIPUS accelerated bone healing, when assessed both clinically and radiographically	At weeks 10, 12, 14, 20, 33 and 52 Final follow-up at 24 months	Compliance was not descriptively specified but seemed rather low

Table 1. LIPUS for fresh fractures and distraction osteogenesis.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Kinami *et al.*, 2013	Multicentre, retrospective, comparative	Femur or tibia, managed surgically	LIPUS 78, mean: 48.7 years old (16-95 years old) Control 63, mean: 46.9 years old (16-94 years old)	Exogen®	No	Not specified	LIPUS accelerated by 30 % healing of stable comminuted fractures, but not of simple and wedge ones	Every month until bone union LIPUS therapy administered at least for 3 months	Retrospective design
Kristiansen *et al.*, 1997	Prospective, multicentre, randomised, double-blind, placebo controlled	Distal radius fracture, fixed using a cast	61 fractures in 60 patients LIPUS 30 (54 ± 3 years old) Control 31 (58 ± 2 years old)	Exogen®	Yes	By device: average for LIPUS 62 d (29-77); average for placebo 65 d (39-76.	LIPUS accelerated healing by 30 %	At weeks 1, 2, 3, 4, 5, 6, 8, 10, 12 and 16	Compliance was not descriptively specified but seemed rather low
Leung *et al.*, 2004b	Prospective, randomised, single-blind, placebo controlled	Complex, open tibial fractures, surgically fixed	28 patients with 30 fractures LIPUS 16 Control 14 Mean: 35.3 years old (22-61 years old)	Exogen®	Yes, differs from active device	Not specified	LIPUS improved fracture healing, as assessed clinically, radiographically, and biochemically.	At weeks 3, 6, 9, 12, 18, 24, 32, 40 and 48.	Unblinded study design; small patient cohort per group
Liu *et al.*, 2014	Prospective, randomised, single-blind, comparative	Distal radius fixed using a cast	81 patients LIPUS 41 (67.9 ± 5.6 years old) Control 40 (65.7 ± 6.1 years old)	Most likely Exogen®, PRF not specified, 15 min/d	No	Not specified	LIPUS accelerated fracture healing	Every week until healing	No sham group; single-blind design
Lubbert *et al.*, 2008	Prospective, multicentre, randomised, double-blind, placebo controlled	Midshaft clavicle fracture treated non-operatively	101 patients LIPUS 52 Control 49 Age distribution between groups not specified	Exogen®	Yes	Not specified.	LIPUS did not accelerate fracture healing when accessed clinically	At weeks 1, 2, 4, 6 and 8.	Good spontaneous healing of clavicle fractures

Table 1. LIPUS for fresh fractures and distraction osteogenesis.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Maurya *et al.*, 2019	Prospective, randomised, double-blind, placebo controlled	TMJ with a fixed functional appliance	40 patients LIPUS 20, mean: 14.1 years old Control 20, mean: 14 years old	Exogen® 10 d in a row and 3 times a week after	Yes	100 % compliance, LIPUS applications performed by medical staff	LIPUS improved TMJ remodelling and condylar head position and joint space, as assessed by CT scans	Not specified; assumed to be on days of LIPUS application	Small patient cohort per each group
Namera *et al.*, 2020	Prospective, randomised, single-blind, placebo controlled	TMJ with a functional TB appliance	45 patients LIPUS 15 (TB) TB 15 Control 15 (untreated) 10.5-14 years old, age distribution between groups not specified	Exogen® 21 d in a row and every 3 weeks after	Yes, medical staff unblind	100 % compliance; LIPUS applications performed by medical staff.	LIPUS reduced functional treatment and stimulated growth during correction	Every 3 weeks	Unblinded study design; small patient cohort per group
Nolte *et al.*, 2016	Retrospective, observational	Metatarsal fractures treated either with cast and LIPUS or surgery	Patients evaluated through propensity matching, using registry of 594 LIPUS-treated fractures	Exogen®	No	Not specified	LIPUS accelerated healing of fractures less than 1 year old; these results were comparable to surgery	Not specified	Retrospective study without sham control
Ota *et al.*, 2017	Retrospective, comparative	Surgically fixed with IM nail; radius or ulna in children	44 patients LIPUS 25 (8.9 ± 3.1 years old) Control 19 (9.7 ± 3.2 years old)	Exogen®	No	No loss to follow-ups; compliance not specified.	LIPUS reduced healing time; all fractures achieved functional recovery	Every week until healing	Retrospective study without sham control
Ota *et al.*, 2018	Retrospective, comparative	Displaced mallet finger fractures either LIPUS-stimulated or pinned	19 patients LIPUS 8, mean: 13 years old (11-15 years old) Control 11 (pinned), mean: 13.5 years old (11-15 years old)	Exogen®	No	Not specified	LIPUS provided excellent functional recovery, although at cost of longer application, when compared to pinning following the Ishiguro's method	Every week until bone union and every 2 weeks until functional recovery	Retrospective study without sham control; small patient cohort

Table 1. LIPUS for fresh fractures and distraction osteogenesis.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Patel *et al.*, 2015	Prospective, comparative	Minimally displaced mandibular fracture through intermaxillary fixation	28 patients LIPUS 14 Control 14 15-35 years old, age distribution between groups not specified	1 MHz, I_{SATA} = 1.5 W/cm^2, PRF not specified	No	Performed by medical staff, compliance is not specified	LIPUS-accelerated healing and improved clinical mobility were observed in the sonicated group	Every week	Study without sham control; small patient cohort in each group
Raza *et al.*, 2016	Prospective, randomised, double-blind, placebo controlled	Torque on tooth root during orthodontic procedure	10 patients LIPUS 10 Control 10 (left or right) 15.5 ± 5.5 years old	Exogen®	Yes	Not specified	LIPUS decreased root damage (lower number of resorption lacunae)	At 4 weeks, evaluated by micro-CT	Very small patient cohort
Salem and Schmelz, 2014	Prospective, randomised, comparative	Distraction osteogenesis of tibia (Ilizarov fixator)	21 patients LIPUS 12, mean: 32 years old Control 9, mean: 29 years old Rest not specified	Exogen®	No	Not specified	LIPUS shortened healing time, as measured both clinically and radiographically	Every 2 weeks clinical follow-ups, and every 4 weeks radiographic evaluation	Unblinded study design; lack of sham control; small patient cohort
Santana-Rodríguez *et al.*, 2019	Prospective, randomised, double-blind, comparative	Rib fracture	47 patients LIPUS 24 (64.0 ± 13.1 years old) Control 23 (58.9 ± 17.3 years old)	1 MHz, 0.5 W/cm^2, DC 10 %, 1 min/d, PRF not specified	No	100 % compliance; LIPUS applications performed by medical staff	LIPUS decreased pain and intake of pain medication Accelerated callus healing and return to life activities	At months 1, 3 and 6	No sham control
Simpson *et al.*, 2017	Prospective, multi-centre, randomised, double-blind, placebo controlled	Distraction osteogenesis of tibia (Ilizarov fixator)	55 patients LIPUS 30 (37.2 ± 12.9 years old) Control 25 (38.4 ± 12.0 years old)	Exogen®	Yes	75 % of patients were 50 %-compliant	LIPUS did not accelerate bone healing	Every 4 weeks until healing, as measured radiographically and by weight-bearing	Inadequate compliance

Table 1. LIPUS for fresh fractures and distraction osteogenesis.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Song *et al.*, 2019	Retrospective, comparative	Bilateral tibial lengthening over nail (also fixed using an Ilizarov fixator)	30 patients LIPUS 15, mean: 22.1 years old (17.5-34.0 years old) Control 15, mean: 20.6 years old (17.9-25.4 years old)	Exogen®	No	Not specified	LIPUS enhanced callus formation and accelerated bone healing, as assessed radiographically	At weeks 1, 2, 3 and 4, and monthly until healing	Retrospective study without sham control
Tsumaki *et al.*, 2004	Prospective, randomised, comparative	Bilateral one stage opening – wedge high tibial osteotomy by hemicallotasis	21 patients Left or right were randomly with/without LIPUS, mean: 68 years old (53 to 78 years old)	Exogen®	No	100 % compliance; LIPUS applications performed by medical staff	LIPUS accelerated callus maturation in elderly patients, as assessed radiographically Earlier removal of pins in active group	Every week	No placebo control and unblinded study design
Urita *et al.*, 2013	Prospective, randomised, single-blind, comparative	Shortening osteotomy of ulnar or radius	27 patients LIPUS 14, mean: 52 years old (34-70 years old) Control 13, mean: 44 years old (20-56 years old)	Exogen®	No	Not specified	LIPUS accelerated bone healing, as assessed radiographically Clinical parameters were not improved	At weeks 2, 4, 6, 8, 12, 16 and 24.	No placebo control and unblinded study design
Zacherl *et al.*, 2009	Prospective, randomised, double-blind, placebo controlled	Chevron osteotomy for *hallux valgus*	52 osteotomies in 44 patients LIPUS 26, mean: 51 years old (20-77 years old) Control 26, mean: 54 years old (28-77 years old)	Exogen®	Yes	Checked weekly Non-contact with device produced sound 92.3 % completed > 78.6 % of treatments	LIPUS had no effect on radiographic and clinical healing Placebo group had more frequent relapse (statistically significant) in distal metatarsal articular angle at 6 weeks	At 6 weeks and 1 year	None
Zura *et al.*, 2015b	Retrospective, observational	Fractures at various locations	4190 patients (43.3 ± 18.2 years old)	Exogen®	No	Only compliant patients were included in the study; details not specified	96 % of fresh fractures healed Shorter time to treatment correlated with positive outcome	Not specified	Retrospective study without any controls

less than 90 % compliance without the appropriate sensitivity analyses. The present review excluded two well-controlled studies, in which fresh tibial fractures (closed or open grade 1) (Heckman *et al.*, 1994) and fractures of the distal radius metaphysis (dorsally angulated, negative volar) (Kristiansen *et al.*, 1997) were immobilised in a cast and treated by LIPUS. Both studies reported that the radiographically assessed healing time was significantly decreased by the LIPUS treatment; however, they were excluded based on a low compliance of 69 % (Heckman *et al.*, 1994) and 72 % (Kristiansen *et al.*, 1997).

It should be further noted that all three unbiased studies (Busse *et al.*, 2014; Emami *et al.*, 1999; TRUST Investigators writing group *et al.*, 2016), as defined by Schandelmaier *et al.* (2017a), investigated the healing of fresh tibial fractures fixed using only a reamed IM nail. Fractures treated this way are known to have a very low complication rate (Coles and Gross, 2000) and the weight bearing with this type of fixation can start relatively early, due to the immediately acquired stability with the preservation of subtle interfragmentary movement within the fracture gap (Perren, 2002; Schmal *et al.*, 2020). Similarly, a lack of beneficial LIPUS effects was observed in screw-fixed lateral malleolar fractures, providing a possibility of early weight bearing (Handolin *et al.*, 2005a; Handolin *et al.*, 2005b). Therefore, one of the reasons for the lack of pro-regenerative effects might be that the LIPUS application cannot override the benefits of the mechanical loading generated by natural skeletal motion (Malizos *et al.*, 2006). This could be also true for defects with high spontaneous healing rates, where addition of the LIPUS therapy becomes redundant (Gan *et al.*, 2014; Lubbert *et al.*, 2008). The fractures immobilised in the cast, on the other hand, might have a suboptimal mechanical environment and more significantly rely on the well-controlled mechanical component of LIPUS and, thus, more profound impacts were observed there (Coughlin *et al.*, 2008; Farkash *et al.*, 2015; Heckman *et al.*, 1994; Kristiansen *et al.*, 1997; Liu *et al.*, 2014; Nolte *et al.*, 2016). These hypotheses should be further tested in preclinical models, using ultrasound set-ups with well-controlled acoustic parameters (see section "Importance of LIPUS acoustic dose based on preclinical studies"), and in future clinical studies.

LIPUS and bones with compromised healing

Fractured bones with impaired healing present several challenging tasks for the orthopaedic surgeon. It starts with the difficulty in defining the onset of a delayed-union or non-union and propagates along the decisions on the selected treatment type and time, which must be compliant with the health status including the physiological, psychological and professional demands of the patient (Stewart, 2019). The non-union bone is defined by the FDA as a fracture with no evidence of progressive healing improvement observed in the last 3 months of a total 9-months post-fracture period (Healy *et al.*, 1990).

Whilst the conduction of a RCT involving alternative treatments such as LIPUS is relatively straightforward for the patients with acute fresh fractures, the same procedure involving a large-patient cohort is more challenging to design for a non-union bone. One of the limiting factors is a lack of global standardised definition of delayed- and non-union fractures, including the absence of a universal agreement on whether radiographic, clinical or both criteria should be used to characterise those bones (Bhandari *et al.*, 2012; Corrales *et al.*, 2008; Özkan *et al.*, 2019). Surgical intervention is a first-line treatment for most bones with impaired healing (Leng *et al.*, 2019; Özkan *et al.*, 2019; Schmal *et al.*, 2020), whereas ultrasound modalities, such as LIPUS, are considered inefficient (Özkan *et al.*, 2019) and even contraindicated by some orthopaedic surgeons (Busse and Bhandari, 2004; Pounder and Harrison, 2008). A prescription of the LIPUS bone-stimulators is usually advised when the surgical intervention carries high risks for the individual (Anderson *et al.*, 2019; Leighton *et al.*, 2017; Zura *et al.*, 2015a). Thus, the to-date evidence for LIPUS effects on delayed- and non-unions (Table 2) mostly relies on either retrospective reports (Adukia *et al.*, 2021; Carlson *et al.*, 2015; Elvey *et al.*, 2020; Farkash *et al.*, 2015; Hemery *et al.*, 2011; Lerner *et al.*, 2004; Mayr *et al.*, 2000; Nolte *et al.*, 2001; Roussignol *et al.*, 2012; Rutten *et al.*, 2007; Teoh *et al.*, 2018; Zura *et al.*, 2015a) or observational studies without placebo controls (Bawale *et al.*, 2020; Biglari *et al.*, 2016; Gebauer and Correll, 2005; Gebauer *et al.*, 2005; Jones *et al.*, 2006; Majeed *et al.*, 2020; Moghaddam *et al.*, 2016).

As far as it can be ascertained, only one multicentre, randomised, placebo-controlled clinical trial evaluating the effects of LIPUS on delayed bone healing (minimal fracture age 4 months) and enrolling a total of 101 subjects with a 91 % final compliance has been performed (Schofer *et al.*, 2010). The study reported an increase in bone-mineral density and a decrease in fracture gap for the LIPUS-active group at the 16-week follow-up, although no statistically significant difference in the number of healed fractures between the groups was found. As it was mentioned by Schandelmaier *et al.* (2017a), this study could have been biased by the age of the fracture at the start of the trial, as the mean age in the LIPUS-treated group was higher. Although the difference in the fracture-age distribution was found to be not statistically significant (Schofer *et al.*, 2010), a similar study with homogenous fracture age groupings for patients with non-union bones will be of great importance.

Two more studies have evaluated biopsies of fibulae with delayed healing within a randomised double-blind, placebo-controlled trial, revealing that LIPUS increased osteoid thickness and bone mineralisation (Rutten *et al.*, 2008), which, most likely, occurred through the locally enhanced osteogenic differentiation of cells (Rutten *et al.*, 2009). However, both studies were based on very small patient cohorts.

Table 2. LIPUS for delayed- and non-union bones.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Adukia *et al.*, 2021	Retrospective, observational	Non-unions at various locations Mostly atrophic	46 patients, 47.0 ± 19.7 years old	Exogen®	No	8 patients were lost during follow-up Not specified how it was measured	Union was achieved in 57.89 % of the cases A small inter-fragmentary gap was a predictor of success	At 6 weeks; 3 and 6 months;1 year	Retrospective study, without sham control
Anderson *et al.*, 2019	Retrospective, observational	Metatarsal fractures with delayed healing (> 14 d)	256 patients, 65.8 ± 11.5 years old	Exogen®	No	Not measured	Delayed healing in young patients with obesity, psychosis, anaemia, chronic lung disease Surgery prescribed to patients who first saw specialist	Not specified	Retrospective study, without sham control If person did not seek treatment after LIPUS, the fracture was assumed to be healed
Bawale *et al.*, 2020	Prospective, observational	Various locations	66 patients, mean 49.2 years old (19-85 years old)	Not specified	No	4 patients excluded due to poor compliance Not specified how it was measured	67 % of compliant patients healed post-ORIF scaphoid fracture and post-ankle joint fusion; non-union did not heal	At 6 months minimum	Study without sham control
Biglari *et al.*, 2016	Prospective, observational	Long bones, non-unions	61 non-unions from 60 patients, 45.0 ± 9.8 years old	Exogen®	No	Not specified	32.4 % healed successfully, the rest had to undergo revision surgery	At 6 and 12 weeks; 4, 5, 6 and 12 months	Study without sham control
Carlson *et al.*, 2015	Retrospective, observational	Scaphoid non-union treated surgically	14 patients, 15.3 ± 1.3 years old	Exogen®	No	Not specified	13 out 14 non-unions healed successfully within a range of 61-217 d	Every 4 to 6 weeks until healing	Without sham control and without non-surgically treated controls; heterogeneous surgical treatments; small patient cohort

Table 2. LIPUS for delayed- and non-union bones.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Elvey *et al.*, 2020	Retrospective, observational	Hand and wrist non-unions	26 patients, 27.7 ± 9.8 years old	Exogen®	No	Not measured	62.5 % of non-unions healed after LIPUS therapy within 12 months	At 12 months	Retrospective study, without any controls
Farkash *et al.*, 2015	Retrospective, observational	Scaphoid delayed union fixed with cast	29 patients; 18-22 years old 1 patient 34 years old	Exogen®	No	Not specified	76 % of delayed-union healed as assessed by X-ray and CT scans LIPUS success was higher in younger fractures	Heterogeneous within cases	Retrospective study, without any controls
Gebauer *et al.*, 2005	Prospective, observational	Various locations	67 non-unions in 66 patients, 46.0 ± 1.9 years old	Exogen®	No	Based on device recording, it was used on average 89 % of the time	85 % of non-unions healed radiographically and clinically	In 1- to 2-month intervals until complete healing	No comparison group; no sham treatment
Gebauer and Correll, 2005	Prospective, observational	Non-unions after long-bones lengthening	17 non-unions in 13 children, 79 ± 22 years old	Exogen®	No	Not specified	All cases healed fully	Every 6 weeks until healing and 4 years later	Small patient cohort; no comparison group; no sham treatment
Hemery *et al.*, 2011	Retrospective, observational	Long bones non-unions	14 patients, 39.1 ± 13.8 years old	Exogen®	No	Not specified	79 % of non-unions healed	Every 3 months	Small patient cohort; no comparison group; no sham treatment
Jones *et al.*, 2006	Prospective, observational (two-centre)	Hindfoot non-unions after revision surgery with internal fixation	13 patients Mean: 51 years old (15-71 years old)	Exogen®	No	Not specified	12 out of 13 cases healed	Radiographs at 3, 6 and 12 weeks; CT scans 3 months after surgery	Small patient cohort; no comparison groups: surgery only, LIPUS only
Lerner *et al.*, 2004	Retrospective, observational	Long-bones high-energy fractures	17 patients with 18 fractures, 32.1 ± 12.2 years old	Exogen®	No	Not specified	16 out of 18 non-unions healed	Not specified	Small patient cohort; lack of any controls

Table 2. LIPUS for delayed- and non-union bones.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Majeed *et al.*, 2020	Prospective, observational	Foot and ankle post-trauma and post-surgery non-unions	47 patients Mean: 56.6 years old (23-76 years old)	Exogen®	No	No losses to follow-ups, all patients completed the treatment	37 out 47 non-inions healed, assessed clinically 26 of healed cases were atrophic	Not specified	Lack of any controls
Mayr *et al.*, 2000	Retrospective, observational	Delayed unions and non-unions at various locations	1317 patients, 20-70 years old	Exogen®	No	Not specified	91 % of delayed-unions and 87 % of non-unions healed	Not specified	Retrospective study without any controls
Moghaddam *et al.*, 2016	Prospective, observational	Long bones non-unions	23 patients, 43.0 ± 13.5 years old Before and after LIPUS therapy	Exogen®	No	Not specified	Healed and failed cases, no differences in cytokine concentrations in blood Decrease in TGF-β1 was observed in healed group at week 1	At 1 and 2 weeks; at 1, 2 and 3 months	Lack of any controls
Nolte *et al.*, 2001	Retrospective, observational	Non-unions at various locations	28 patients (47.0 ± 18.2 years old) with 29 non-unions	Exogen®	No	72 % of cases used device for more than 75 % (recorded by device)	86 % of non-unions healed as assessed clinically and radiographically	Every 6 to 8 weeks until healing	Retrospective study without any controls
Roussignol *et al.*, 2012	Retrospective, observational	Long bones non-unions	59 patients Mean: 43 years old (17-85 years old)	Exogen®	No	Checked at each follow-up Compliance measured: > 95 %	88 % of non-unions healed	Up to 6 weeks, and at 3 and 6 months	Retrospective study without any controls
Rutten *et al.*, 2007	Retrospective, observational	Tibia non-unions	71 patients Mean: 40 years old (17-89 years old)	Exogen®	No	Not specified	73 % of non-unions healed as assessed by radiographic and clinical assessment	Average long-term follow-up 27 years	Retrospective study without any controls
Rutten *et al.*, 2008	Prospective, randomised, double-blind, placebo controlled	Delayed union of osteotomised fibula	13 patients LIPUS 7 (52.3 ± 9.0 years old) Control 6 (52.8 ± 6.1 years old)	Exogen®	Yes	Not specified	LIPUS increased osteoid thickness, mineral apposition and bone volume, as established by histology	Biopsies taken 2 to 4 months after start of therapy	Very small patient cohort

Table 2. LIPUS for delayed- and non-union bones.

Source	Type of clinical study	Fracture details	Patients Mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Rutten *et al.*, 2009	Prospective, randomised, double-blind, placebo controlled	Delayed union of osteotomised fibula	7 patients LIPUS 3 (54.3 ± 10.3 years old) Control 4 (50.8 ± 5.9 years old)	Exogen®	Yes	Not specified	LIPUS reduced number of Runx2-positive cells in soft tissue established by histology	Biopsies taken 2 to 4 months after start of therapy	Very small patient cohort
Schofer *et al.*, 2010	Prospective, multi-centre, randomised, double-blind, placebo controlled	Delayed union of tibia	101 patients LIPUS 51 (42.6 ± 14.6 years old) Control 50 (45.1 ± 11.9 years old)	Exogen®	Yes	91 % compliance if evaluate only 'completers'	LIPUS accelerated healing: improved BMD and reduced gap, as observed by CT No clinical effect at 16 weeks	At 1, 2, 3 and 4 months	Larger (but non-significantly) number of older fractures in LIPUS group
Teoh *et al.*, 2018	Retrospective, observational	Delayed union of fifth metatarsal	30 patients Mean: 39.3 years old (14-76 years old)	Exogen®	No	Not specified	90 % of delayed unions healed after LIPUS therapy assessed both clinically and radiographically	Every 4 weeks	Retrospective study without any controls
Zura *et al.*, 2015a	Retrospective, observational	Chronic non-unions (> 1 year) at various locations	764 patients, 45.8 ± 16.5 years old	Exogen®	No	Not specified	86.2 % of cases healed after LIPUS Patient age: a negative factor for healing Failed mostly in non-compliant patients	Not specified	Retrospective study without any controls

Table 3. LIPUS and osteoporosis.

Source	Type of clinical study	Location of application	Patients mean age ± STD or range	LIPUS parameters	Sham device	Compliance	Outcome	Follow-ups	Limitations
Leung *et al.*, 2004a	Prospective, randomised, comparative	Postmenopausal osteoporosis LIPUS applied at distal radius	20 females, 69.1 ± 7.6 years old Control: contralateral part	Exogen®, 5 times a week for 3 months	No	Not specified LIPUS applied by medical staff	LIPUS had no effect on trabecular and integral BMD assessed by peripheral quantitative CT	At 3 and 6 months	Small patient cohort, short follow-up period
Ozdemir *et al.*, 2008	Retrospective, comparative	Postmenopausal osteoporosis Ultrasound applied at neck and dorsal, shoulders and knees	74 females LIPUS 36 (59.6 ± 5.0 years old) Control 38 (56.9 ± 6.8 years old)	Not specified	No	Not specified	Ultrasound had no effect on BMD assessed by DXA	Not specified	Heterogeneous locations application (within USA): limited number of patients per group
Warden *et al.*, 2001	Prospective, randomised, double-blind, placebo controlled	Osteoporosis following spinal cord injury LIPUS applied at calcaneus	15 males, 23.9 ± 7.3 years old Control: contralateral part	1 MHz 3.3 kHz PRF 3.3 % DC I_{SATA} = 30 mW/cm^2 5 times a week for 2 months	Yes	LIPUS applied by medical staff	LIPUS had no effect on BMD, as assessed by DXA and quantitative ultrasound	At 6 weeks	Small patient cohort; not clear whether staff was blinded towards treatment; short follow-up period

The lack of positive evidence for the LIPUS treatment in fixed fresh fractures, based on the three unbiased studies highlighted above (Schandelmaier *et al.*, 2017a), also advised against the ultrasound technique for patients with non-unions (Poolman *et al.*, 2017; Schandelmaier *et al.*, 2017b). Although one can find this conclusion logical, the biological signatures in acute fractures and chronically impaired non-unions are not alike. These are summarised in the next section.

Biological pathogenesis of non-union bone. Can LIPUS help?

The local biology at the fracture site, systemic conditions of the host and mechanical stability are the key factors defining the outcome of the fractured bone (Harwood, 2010). When the bone fracture is fixed and interfragmentary movement within the gap is sustained in the proper range, a process of endochondral ossification is usually observed. Through interlinked phases of inflammation, callus formation and remodelling, the fractured bone is reconstituted *ad integrum* (Loi *et al.*, 2016; Marsell and Einhorn, 2011). If one or more phases of this well-orchestrated process are compromised, a non-union occurs. Based on radiographic and histological assessments, these non-unions can be further categorised into hypertrophic and atrophic types. For the former, biological aspects are in place, but no adequate stability of the fractured bone exists, resulting in callus formation but hindering callus union, maturation and remodelling. For the latter, the biological components are compromised and, at times, combined with mechanical instability (Volpin, 2014). The hypertrophic non-unions can usually be managed by additional stabilisation of the fractured bone (Nauth *et al.*, 2018), whereas atrophic non-unions are more challenging to treat and complex approaches are often required.

The initial acute inflammation in the bone regeneration process is critical for the resultant organ functionality, as shown in animal studies (Grundnes and Reikeras, 1993a; Grundnes and Reikeras, 1993b; Park *et al.*, 2002). It is usually the strongest within several days to a week and declines with time in a normal healing scenario (Loi *et al.*, 2016). The persistence of an immune reaction can result in chronic inflammation, impaired healing and bone non-union (Bastian *et al.*, 2011; Claes *et al.*, 2012; Hardy and Cooper, 2009; Zura *et al.*, 2016). It has been shown that dendritic cells isolated from bone marrow and stimulated with LIPUS secrete exosomes with enhanced anti-inflammatory potential, which alleviates TNF-α-induced inflammation of endothelial cells (Li *et al.*, 2019). The LIPUS treatment also supports the transition of inflammatory to resident macrophages, enhances gene expression of anti-inflammatory factors and improves spinal fusion in a rat animal model (Zhang *et al.*, 2019). The anti-inflammatory potential of ultrasound stimulation has been as well described in several other studies (da Silva Junior *et al.*, 2017; Li *et al.*, 2003; Nakao *et al.*, 2014; Yang *et al.*, 2017).

When MSCs are isolated from hypertrophic non-union fractures, they show strong differentiation potential into all three lineages *in vitro*, *i.e.* chondrogenic, adipogenic and osteogenic (Iwakura *et al.*, 2009). The same cell type isolated from atrophic non-unions not only undergo senescence and growth arrest but also have a significantly lower osteogenic differentiation potential (Bajada *et al.*, 2009). The co-stimulation of mesenchymal cells isolated from patients with different non-union types with BMP-7 and LIPUS significantly enhances the osteogenic potential of these cells (Koga *et al.*, 2013). Unfortunately, the effect of LIPUS alone is not described. The expression and activation of BMPs and their antagonists are out of balance in both hypertrophic and atrophic non-union human fractures (Fajardo *et al.*, 2009; Kloen *et al.*, 2002; Kwong *et al.*, 2009a; Kwong *et al.*, 2009b). The application of LIPUS enhances expression of BMP-2, BMP-4 and BMP-7 and their receptors in osteoblasts-like cells (Gleizal *et al.*, 2006; Suzuki *et al.*, 2009a; Suzuki *et al.*, 2009b), which might help to compensate for this imbalance.

Mechanical loading in the properly stabilised fracture induces NO production, which in turn modulates bone adaptation to the applied stimulus (Klein-Nulend *et al.*, 2014). NO signalling is especially deregulated in patients with atrophic non-unions (Wijnands *et al.*, 2012). LIPUS stimulation of osteoblasts augments NO release *via* nuclear factor-κB signalling pathway (Hou *et al.*, 2009). NO signalling induces expression of VEGF-A and HIF-1α in LIPUS-treated osteoblasts (Wang *et al.*, 2004). This promotes tube formation by endothelial cells, which is crucial for angiogenesis and is often debilitated in pathological fractures. NO release also activates other pathways, such as canonical Wnt/β-catenin signalling in osteoblasts and osteocytes, which is known to influence bone mass (Krishnan *et al.*, 2006). The secretion of DKK-1, antagonising Wnt-signalling (Pinzone *et al.*, 2009), is enhanced in the culture medium of MSCs isolated from patients with atrophic non-unions (Bajada *et al.*, 2009). LIPUS may be able to counteract this effect, since Wnt-signalling is enhanced in stimulated osteoblasts and osteoprogenitors (Olkku *et al.*, 2010).

The expression of MMPs, regulating cell attachment, migration, release of biologically active molecules and invasion of newly formed blood vessels into the callus is also alleviated in non-union fractures (Ortega *et al.*, 2003). The decrease in expression of MMP-2, -9 and -13 in non-union fractures results in impaired bone remodelling (Ding *et al.*, 2018). LIPUS mechanical stimulus enhances MMP-13 expression in long-term cultured osteoblasts (Unsworth *et al.*, 2007), which could potentially improve ECM turnover, critical for successful tissue regeneration.

The key biological signatures of a non-union fracture and the hypothetical LIPUS effects influencing them are summarised in Fig. 2. Despite the positive evidence of LIPUS stimulation, most of the studies described in this section revolve around cell-lines or cells isolated from bones with uncomplicated healing scenario. Whether LIPUS can have similar effects on cells from atrophic and hypertrophic non-unions is a question worth further investigation that needs to be addressed *in vitro* and in appropriate preclinical models. To the authors' knowledge, only two preclinical *in vivo* studies, investigating the effects of LIPUS on a hypertrophic non-union, have been published so far, demonstrating contradictory findings (Takikawa *et al.*, 2001; Volpon *et al.*, 2010).

LIPUS for aged and osteoporotic patients

With progressing age, the human skeleton undergoes cortical-bone thinning, increased trabecular spacing and expansion of the medullary cavity (Javaheri and Pitsillides, 2019). These morphological changes and overall bone homeostasis are results of systemic changes to biochemical signalling pathways of the human body, eventually leading to impaired mechanoadaptation and compromised fracture regeneration (Haffner-Luntzer *et al.*, 2016). Aged individuals experience a reduction in osteoprogenitor cells (Kasper *et al.*, 2009), with a reduced osteogenic potential (D'Ippolito *et al.*, 1999; Ross *et al.*, 2000) and an altered response to mechanical stimulation (Kasper *et al.*, 2009). Additionally, changes in shape of osteocytes and the number of canaliculi per lacuna are found in aged organisms, which dampens their mechanosensitivity and could result in an inefficient interaction between osteoblasts and osteoclasts (Hemmatian *et al.*, 2017). The mechanical stimulation of chronic non-unions with LIPUS in aged patients has shown certain promise, although the fracture-healing rate declines moderately with increasing age (Zura *et al.*, 2015a). MSCs isolated from aged rats experience enhanced expression of osteogenic markers, *i.e.* Runx-2 transcription factor and osteocalcin, when stimulated with high intensity LIPUS, in comparison to cells isolated from young rats (Puts *et al.*, 2016a). This might imply that due to changes in mechano-responsiveness of the osteoprogenitors with increasing age, an adjustment of the LIPUS-stimulation protocol is required. The accelerated fracture healing following LIPUS exposure was also confirmed in *in vivo* studies performed with aged rodents (Aonuma *et al.*, 2014; Katano *et al.*, 2011); however, the relevance of these results for the clinical setting remains questionable due to the animal size in relation to the area of the transducer (see section "Importance of LIPUS acoustic dose based on preclinical studies").

Osteoporosis is a chronic metabolic bone disorder that more commonly affects postmenopausal women and, given the increasing life expectancy, is becoming a global health challenge (Cauley, 2017). Medication-free therapies for the management of this disease represent a very appealing research topic (Kasturi and Adler, 2011b; Yadollahpour and Rashidi, 2017). Application of LIPUS as a treatment option for postmenopausal bone-loss has been investigated previously and no positive effects on the BMD were observed (Leung *et al.*, 2004a; Ozdemir *et al.*, 2008) (Table 3). Another study in young male patients with spinal cord injury, experiencing up to 70 % bone loss, comparable to 5 years of bone depletion due to osteoporosis, found that LIPUS stimulation of the calcaneus bone did not influence its bone mineral content (Warden *et al.*, 2001). In this study, shorter pulses of ultrasound stimulation were used and the frequency of the sine wave was 1 MHz in comparison to the 1.5 MHz conventional stimulation frequency (Table 3). In contrast, several *in vivo* studies using an ovariectomised rat osteoporosis model have shown the beneficial effects of LIPUS exposure on improvement of the disease markers (Carvalho and Cliquet Junior, 2004; Ferreri *et al.*, 2011; Wu *et al.*, 2009). Given the size of the LIPUS-probe, the anabolic

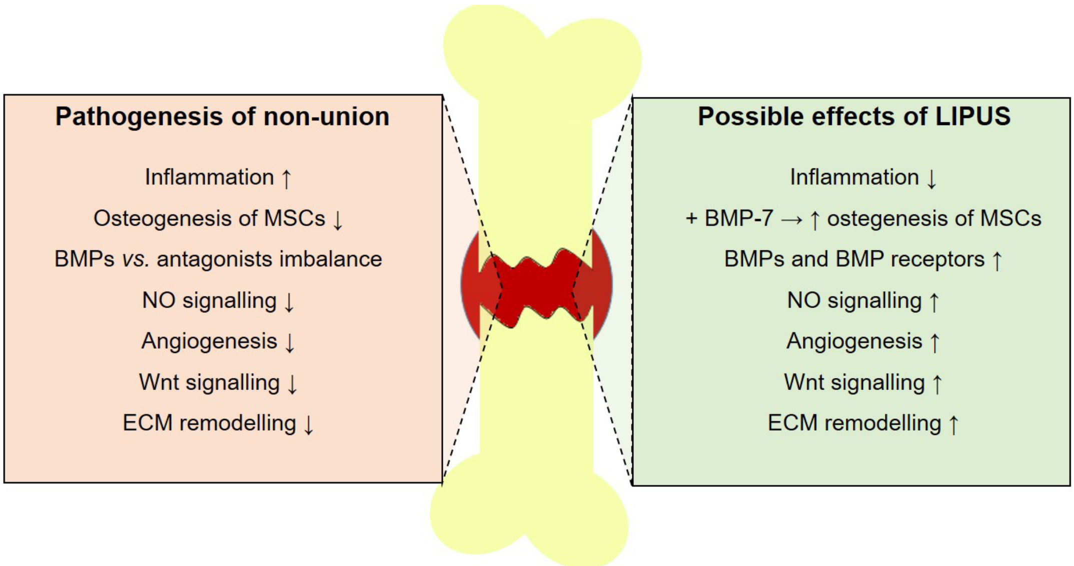

Fig. 2. Can LIPUS help regenerate a non-union? Biological signatures of non-union bone (left) and hypothetical effects of LIPUS-stimulation on non-union regeneration (right).

effects of ultrasound in rodents might partially mimic a low-magnitude high-frequency whole-body vibration therapy, which shows promising results in improving BMD in postmenopausal women (Kasturi and Adler, 2011a; Lai *et al.*, 2013; Rubin *et al.*, 2004; Verschueren *et al.*, 2004).

Although stimulation with LIPUS represents an appealing medication-free treatment for osteoporosis, this chronic metabolic disorder has a systemic nature and will not likely succumb to local stimulation with ultrasound. As discussed by Warden *et al.* (2001), the losses associated with the ultrasound propagation constrain the acoustic stimulation to a very restricted volume. Although the current clinical LIPUS set-up and protocol most likely has limited potential for the treatment of osteoporosis, the investigation of the LIPUS application for regeneration of fractures in aged, osteoporotic patients and patients with other co-morbidities is of great interest.

LIPUS and patient compliance

Patient compliance with the treatment regimen can profoundly affect the outcome of a clinical trial. As was demonstrated by Czobor and Skolnick (2011), non-compliant patients can disguise the efficacy of a tested therapy. In this study, the compliant patients were screened out based on the detection of the drug metabolite in their blood over the course of treatment. A comparison of the compliant patients, which comprised 70 % of the patients, to the placebo group confirmed the drug's efficacy, whereas the non-compliant group did not differ from the control. Moreover, the same compliance assessed by counting consumed pills was more than 92 %. Adherence to the study protocol carries even a bigger challenge for treatments outside the medical facility, resulting in a biased data interpretation (Pounder *et al.*, 2016; Pullar *et al.*, 1989). LIPUS application is usually prescribed to the patients as a long-term treatment and requires a 20 min time window every day. Therefore, motivation and dedication of the patients plays an indispensable role in the study outcome. Certain factors, such as age and fracture site, could significantly affect the adherence to the prescribed LIPUS protocol (Matsubara *et al.*, 2015). The detailed description of patient compliance in the reviewed studies is summarised in Table 1-3.

There is a considerable variability in documentation regarding patients' compliance in LIPUS clinical trials. Some studies reported the number of patients available at the end of the treatment out of the whole sample, whereas others additionally supplied the number of days and min/d of LIPUS application accomplished by the patients. It is not always clear, though, whether the active minutes were counted only when the device was in direct skin contact, as it was described in some studies (Emami *et al.*, 1999; Zacherl *et al.*, 2009). Overall, there is a trend towards positive regenerative outcomes of the LIPUS application in clinical trials with increasing patient device-application compliance (Gopalan *et al.*, 2020; Maurya *et al.*, 2019; Namera *et al.*, 2020; Nolte *et al.*, 2001; Roussignol *et al.*, 2012; Santana-Rodríguez *et al.*, 2019; Schofer *et al.*, 2010; Tsumaki *et al.*, 2004). Studies, where around 30 % of the patients performed less than 50 % of LIPUS applications found LIPUS ineffective (Emami *et al.*, 1999; TRUST Investigators writing group *et al.*, 2016; Simpson *et al.*, 2017). As an example, exclusion of non-compliant patients (as reported by the recordings on the device) in a study of LIPUS-treated non-unions revealed pro-healing effects of sonication comparable to surgical intervention (Bawale *et al.*, 2020). Studies, where the compliance is not descriptively documented are ambiguous regarding the efficacy of LIPUS therapy (Table 1-3).

A stringent weekly control of adherence to the prescribed protocol, requiring a minimum 15 min-long skin contact with the device through a coupling gel, resulted in an excellent compliance in 44 patients after chevron osteotomy for *hallux valgus* (Zacherl *et al.*, 2009). A profound impact on bone formation was observed in the LIPUS-active group, whereas a relapse in a first distal metatarsal articular angle 6 weeks after treatment was reported in the placebo group. The active support of patients and communication with the medical personnel seem to improve the compliance significantly, favouring LIPUS therapy (Arimoto *et al.*, 2019; Gopalan *et al.*, 2020; Maurya *et al.*, 2019; Namera *et al.*, 2020; Patel *et al.*, 2015; Santana-Rodríguez *et al.*, 2019; Tsumaki *et al.*, 2004; Zacherl *et al.*, 2009). This should be considered when planning a clinical trial. New generation Exogen® devices might also help raising patients' awareness on the treatment progress and support their motivation through direct feedback of an integrated calendar (Pounder *et al.*, 2016). In summary, an inclusion in the scientific studies of the detailed information on the number of completed days and minutes of LIPUS treatment, along with a population size that was intended to be treated and actually adhered to the protocol, can aid an adequate judgment of LIPUS therapy.

Importance of LIPUS acoustic dose based on preclinical studies

The clinically most used LIPUS parameters [1.5 MHz frequency, 1 kHz PRF, 20 % DC and 30 mW/cm² I_{SATA} (Exogen®)] originate from a preclinical rabbit model (Duarte, 1983). Since then, little effort has been made to optimise this acoustic dose. With the exception of 9 studies (see Materials and Methods, and Table 1 and 3), the rest of the studies applied Exogen®-like parameters.

The current evidence for LIPUS-induced pro-regenerative potential in bone shows pronounced positive effects in cell culture (Padilla *et al.*, 2016; Pounder and Harrison, 2008) and in animal studies (Azuma *et al.*, 2001; Shakouri *et al.*, 2010; Wang *et al.*, 1994). However, it seems that these studies hyperbolise the degree of the LIPUS pro-regenerative potential, which does not coincide with the clinical findings (Emami *et al.*, 1999; Poolman *et al.*, 2017; Schandelmaier *et al.*, 2017a).

The two most described *in vitro* LIPUS set-ups, transmitting ultrasound through gel from the bottom of the tissue culture plate or through the medium from the top of the cells, exposes them to the near field of the transducer, which is prone to large spatial and temporal intensity variations (described in detail by Padilla *et al.*, 2014). Although Harrison *et al.* (2016) argued that the near-field ultrasound-stimulation represents the closest configuration to the clinical setting, the cells and transducer, in those *in vitro* experiments, are usually separated by several mm. This exposes the cells to the most heterogeneous proximal near-field of the transducer (Padilla *et al.*, 2014), whereas the clinical device stimulates the fracture site in the mid or far near-field of the transducer (Harrison *et al.*, 2016), where the amplitude differences are dampened. The *in vitro* configurations with focused transducers or far-field stimulation (Horne *et al.*, 2020; Puts *et al.*, 2016b; Subramanian *et al.*, 2013) can help to account for these variables. Additionally, the most described *in vitro* set-ups (Padilla *et al.*, 2014) can subject the cells to physical artefacts, such as multiple reflections and standing waves (Hensel *et al.*, 2011; Mortazavi *et al.*, 2016), and, especially for the gel-coupled configurations, to temperature elevation (Leskinen and Hynynen, 2012). These are, most likely, hardly present in the clinical configurations and should be further evaluated starting with *in silico* analyses.

The Exogen® LIPUS-probe, widely used in preclinical studies, has a diameter of 22 mm, which exposes the stimulated site to an effective area of 3.88 cm^2. If the probe is applied to the femur of a laboratory Wistar rat for example, whose average femur length is 39 mm (Prodinger *et al.*, 2018), more than 50 % of the bone is coupled with the transducer. In contrast, a human femur is on average 440 mm long (Polguj *et al.*, 2013), which results in a 5 % overlap between the bone and the LIPUS-probe. The femur length of a white New Zealand rabbit, another animal often used in *in vivo* studies showing positive influence of LIPUS (Pilla *et al.*, 1990; Shakouri *et al.*, 2010), is around 94 mm (Polguj *et al.*, 2013) and more than 20 % of the bone overlaps with the gel-coupled stimulating probe. These *in vivo* studies apply LIPUS in a manner exactly opposite to the proportional adjustment of the mechanical dose. Subsequently, the smaller the bone treated with LIPUS is, the larger and more diverse resident cell populations embraced by the mechanical stimulation are – including the ones in the bone epiphyses where a large cancellous bone area, rich in stem cells and vasculature, is observed (Gurevitch *et al.*, 2007). This, in turn, can intensively promote migration of the osteoprogenitors to the fracture site, attract immune cells and induce angiogenesis, promoting osteogenesis (Filipowska *et al.*, 2017; Lancerotto and Orgill, 2014). Additionally, the thin soft-tissue layers and small bone-circumferences of a rat result in a stimulation of the fracture in the most heterogeneous near-field of the transducer. Fig. 3**a**, depicting the numerical simulation of the ultrasound field generated by the Exogen® probe, shows how large the stimulation area of a fractured rat femur with LIPUS is and how high are the intensity fluctuations in the near field of the transducer. When the same femur was positioned in the simulated field of a focused transducer (Fig. 3**b**), the geometrically confined and acoustic dose-controlled exposure of the bone gap region was achieved. The geometry of the simulated field in Fig. 3**b** is similar to the one created by a custom-made scanning acoustic microscope (SAM200 Ex, Q-Bam, Halle, Germany) (Rohrbach *et al.*, 2013).

In contrast to the unproportional scaling down of

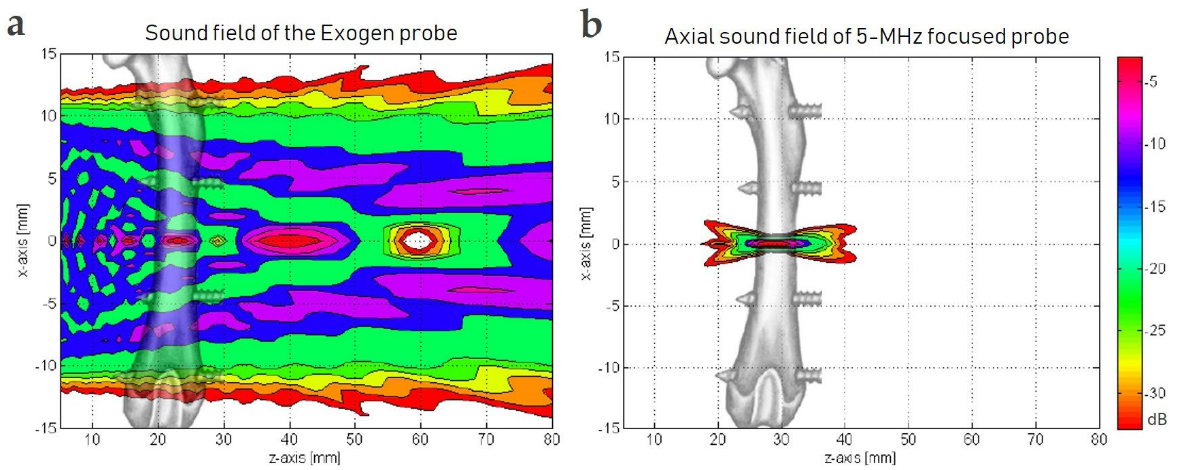

Fig. 3. Schematic drawing of a fractured rat-femur positioned in a simulated sound field produced by (a) a clinically used Exogen® probe and (b) a 5 MHz focused probe producing a − 6 dB spot of 7.4 × 0.6 mm. (a) The fracture or osteotomy gap region was exposed to a highly inhomogeneous near field of the transducer and almost the entire femur received the acoustic stimulation. (**b**) The acoustic energy was deposited in the gap region only. The simulations were performed using Field II program and showed transmit temporal peak intensity. The pin locations of a typically used external fixation device (Rohrbach *et al.*, 2013) are also shown.

the acoustic dose from the clinical setting to *in vivo* and *in vitro*, is the application of BMP-2, a potent growth factor for regeneration of complex bone-injuries and non-unions (Schlundt *et al.*, 2018). The induction of bone healing by BMP-2 in the clinic is performed at a concentration of either 1 mg/mL or 1.5 mg/mL (Carter *et al.*, 2008; Govender *et al.*, 2002; Hwang *et al.*, 2016), whereas the same growth factor is used *in vivo* in rats and rabbits at concentrations ranging from 200 ng/mL to 37.5 μg/mL (Chen *et al.*, 2018; Hyun *et al.*, 2005; Koolen *et al.*, 2019; Seong *et al.*, 2020; Zara *et al.*, 2011; Zhao *et al.*, 2016). *In vitro*, cells are usually stimulated using 50-5,000 ng/mL of BMP-2 (Chen *et al.*, 2018; Chen *et al.*, 2019; Kim *et al.*, 2013; Ning *et al.*, 2019). Although supraphysiological doses of the growth factor are used in clinics, the studies elucidating the mechanisms attempt to adjust the concentration of BMP-2 to the size of the stimulated biological system. Exactly the opposite is done with the LIPUS stimulation experiments. This might explain the significant difference in results obtained from small-animal long bones fixed with an IM nail and stimulated with ultrasound, where pronounced bone-healing effects were observed (Azuma *et al.*, 2001; Wang *et al.*, 1994), and the unsuccessful clinical cases (Busse *et al.*, 2014; Emami *et al.*, 1999; TRUST Investigators writing group *et al.*, 2016). To compare adequately the influence of LIPUS on *in vivo* bone regeneration in small animals and translate these findings to the clinical setting, set-ups with well-controlled physical effects need to be applied (Horne *et al.*, 2020; Puts *et al.*, 2016b; Subramanian *et al.*, 2013). Then, further optimisation of the reproducible clinical acoustic dose might be required (Warden, 2003; Warden *et al.*, 2000). Until it is possible to decipher the essential mechanisms of bone regeneration by the defined acoustic stimulation, using the spatially adjusted set-ups translated from human to preclinical models, *in vitro* and back, the potential benefits of LIPUS will remain underestimated in the clinic.

Discussion

Upon the onset of a long-bone fracture, the orthopaedic surgeon has to make rapid and efficient decisions as to what are the best treatment options for the patient. The new generation of surgeons more frequently refer to invasive treatments with fixation even for uncomplicated fractures (Courtney *et al.*, 2011; Fernandez, 2005; Schmidt *et al.*, 2003). This, on one hand, provides the desired mechanical stability and ensures adequate conditions for bone regeneration. On the other hand, surgical interventions are prone to infections, which ultimately impair bone healing and result in bone non-unions (Coles and Gross, 2000). Not only are these economically burdensome (Hak *et al.*, 2014; Heckman and Sarasohn-Kahn, 1997; Majeed *et al.*, 2020; Teoh *et al.*, 2018) but also the established non-union bone is often hard to diagnose because the blood inflammatory markers remain within the reference levels in up to 20 % of those cases (Bishop *et al.*, 2012; Nauth *et al.*, 2018). Given these and other risks that the surgical procedures have, they cannot be used as a universal treatment solution: elderly individuals with chronic metabolic disorders and other underlying health conditions as well as people with certain lifestyles where the long recovery time is not desired, are the candidates for alternative methods (Anderson *et al.*, 2019; Bawale *et al.*, 2020; Berber *et al.*, 2020; Cook *et al.*, 1997; Leighton *et al.*, 2017; Nolte *et al.*, 2001; Zura *et al.*, 2015a).

Within the process of bone healing, a miscommunication between the components of the "diamond concept" (Fig. 4), essential for successful bone regeneration, could result in a complicated healing scenario (Andrzejowski and Giannoudis, 2019; Giannoudis *et al.*, 2007). When all 4 facets of the concept, *i.e.* cells, matrix, growth factors and mechanical stability, are in balance (Busse *et al.*, 2014; Emami *et al.*, 1999; TRUST Investigators writing group *et al.*, 2016), the LIPUS stimulation will, most likely, not have an additional effect. Furthermore, if an atrophic non-union is established and substantial biological inertness in bone is observed, the fracture deterioration might not be efficiently compensated for by mechanical stimulation with LIPUS (Malizos *et al.*, 2006; Moghaddam *et al.*, 2016; Watanabe *et al.*, 2010). The exposure to micromotion generated by LIPUS (Greenleaf, 2003) might, however, be beneficial for fractures healing with a delay, where biological phenomena are still in place and LIPUS can help supporting the biomechanical environment (Leighton *et al.*, 2017; Majeed *et al.*, 2020; Watanabe *et al.*, 2013). However, these hypotheses require further evaluation in valid *in vitro* and preclinical models, followed by clinical research.

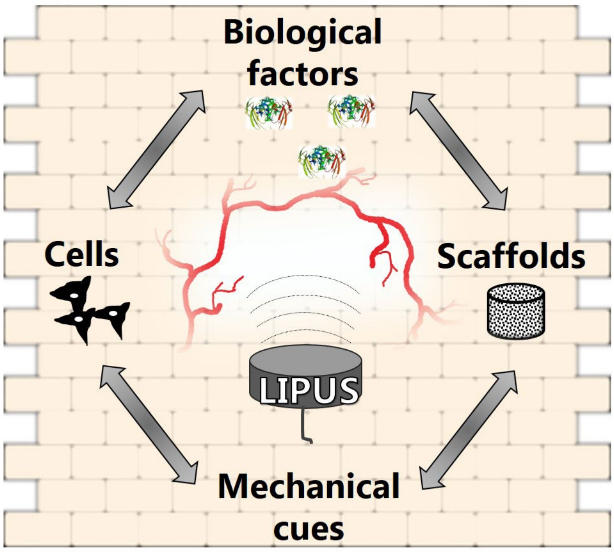

Fig. 4. Role of LIPUS with respect to the "diamond concept" of bone regeneration. Given the fracture stability, LIPUS stimulation might mimic the mechanical cues induced by interfragmentary motion, crucial for successful healing.

Conclusions

The present review attempted to emphasise the limited knowledge on the principal mechanisms of the LIPUS technique and on the lack of adequate clinical evaluation. Research is needed to better understand the *in vitro* and *in vivo* biological and physical mechanisms involved, using set-ups ensuring an adequate translation of the optimal acoustic dose to the clinical setting. Conducting double-blind, randomised, placebo-controlled clinical trials is required for various bone fracture types (fresh, delayed- and non-union), in cast and fixed with implants, for large patient cohorts. Moreover, these studies should ideally be non-industry funded so as to eliminate potential bias. Clinical trials need to be supplied with regular follow-up appointments and easy access to communication with the medical personnel. Detailed documentation of patient compliance is needed, including the population that was intended to be treated originally, the individuals that followed the protocol properly, the number of days LIPUS was applied and the duration of treatment. It should also be specified whether the active minutes recorded by the LIPUS device were counted only when the probe was in direct skin contact. Additionally, investigation and optimisation of LIPUS-treatment protocols for fractures in aged individuals and patients with chronic metabolic disorders, where complementary methods could be used, is worth considering.

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Discussion with Reviewers

Reviewer 1: Do you think an advanced design of an *in vitro* set-up might improve the comparability of the LIPUS stimulation? Would a "tissue-mimicking" *in vitro* approach be an option?

Authors: Due to the complexity of physical phenomena induced by LIPUS, which are highly dependent on the structural and material properties of the interrogated material, the physical sub-mechanisms differ *in vivo vs. in vitro*. A better understanding of which sub-mechanisms are encountered in the clinical setting and their proper translation into advanced *in vitro* and *in vivo* set-ups, supported by *in silico* studies can indeed help to decipher the resulting biological phenomena. Most of the existing *in vitro* set-ups do not allow for controlled transfer of the acoustic dose and, furthermore, introduce physical artefacts, as discussed by Padilla *et al.* (2014). This produces misleading results that most likely do not reflect the clinical reality. Creating tissue constructs, mimicking as closely as possible the material properties of bone and other surrounding tissues could be an excellent way to study the physico-biological mechanisms of ultrasound stimulation and could be object of future research. This research could yield a re-optimisation of the LIPUS acoustic parameters originated from a rabbit animal model (Duarte, 1983). Such studies should be performed using advanced *in vitro* and *in vivo* set-ups.

Reviewer 1: Based on all the information given in the present review, LIPUS might be effective but a good clinical trial is still missing. What could be the main reasons why a well-designed trial was not conducted even through LIPUS has been used since the early 1990s?
Authors: We do not have a clear explanation on why a well-designed trial has not yet been conducted. We can only speculate that clinical trials with non-unions, for example, are unlikely to include LIPUS as a first-line treatment. Patients might be referred to it only after the failure of other type of treatments (*e.g.* surgery). However, for more "simple" fracture types, we believe that the hurdles in conducting such trails might be more related to the difficulty of finding a funding source, especially if companies are not willing to sponsor them. We can only speculate that LIPUS-device manufacturers, principally Bioventus, who sells the Exogen® system, do not see the need of sponsoring further a long and costly clinical trial to improve acceptance and/or rentability of their operations. The device is already approved by several regulatory agencies worldwide and it seems to be commercially successful. Additionally, provider-sponsored trials raise questions of bias, diminishing the concluded findings. We purposely decided not to contact manufacturers on this issue to remain neutral and propose an objective review of published data.

Reviewer 2: What is/are the main future research direction(s) of LIPUS on bone regeneration?
Authors: A thorough characterisation of acoustic dose in preclinical models, followed by its translation to human is an important first step towards the reproducibility and acceptance of the LIPUS therapy. This dose should be further optimised for "special conditions", such as bones with impaired healing, elderly individuals and patients with underlying health conditions. The defined parameters should be tested in preclinical models and verified in well-controlled clinical studies.

LIPUS has been also shown to have synergistic effects *in vitro* and *in vivo*, when used together with other therapies, *e.g.* growth factors such as BMP-2 (Angle *et al.*, 2014, additional reference) and BMP-7 (Koga *et al.*, 2013; Lee *et al.*, 2013, additional reference) and mesenchymal stromal cells (Carina *et al.*, 2017; Chen *et al.*, 2019; Polo-Corrales *et al.*, 2018, additional references), enhancing effects of those treatments. This could be another direction towards exploration of the LIPUS capabilities for tissue regeneration.

Reviewer 2: Is LIPUS scientifically sound for clinical application for bone regeneration?
Authors: There is no doubt that stimulation with LIPUS induces pro-regenerative processes in biological tissues, such as bone, and that this therapy has potential to be used for clinical treatment of bone fractures. However, at this point, randomised double-blind clinical trials with defined and characterised acoustic doses, enrolling large patient cohorts and ensuring patients compliance following support of the medical personnel, are necessary to draw definitive conclusions.

Melanie Haffner-Luntzer: What lessons can we learn from animal models regarding LIPUS application during fracture healing and what might be the limitations?
Authors: Preclinical models are crucial for evaluation of a therapy's efficacy, determination of the underlying mechanisms and optimisation of conditions for its improvement. The use of LIPUS in small animal models, such as rats and rabbits, has shown profound pro-regenerative effects in bone fractures at various locations. However, translation of those findings to the clinical setting, unfortunately, has not always been found successful. One of the biggest limitations to translate preclinical results to the clinical setting could be the fact that the same probes and stimulation parameters were used in most of the preclinical and in the clinical studies, although animal and human proportions, including the soft tissue amount or the bone defect size, differ greatly. This brings us to the question of whether the LIPUS acoustic parameters are directly translatable from preclinical models to patients, or if there is a so-called "acoustic dose" that is suitable for a small animal and which should be then appropriately scaled for a human. Depending on type of fracture, fracture location, patients' characteristics and their medical history, this acoustic dose needs to be standardised and further tested in preclinical models and clinical studies.

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Editor's note: The Guest Editor responsible for this paper was Anita Ignatius.