GENERAL SUMMARY

The bones in our body form a unique organ providing structural support, protection of vital organs, housing of marrow, and participation in mineral homeostasis. Bone is a living tissue and is characterized by the constant adaptation to its mechanical loading environment. Bone cells are sensitive to strains caused by physical loading. Mechanoreceptors convert biophysical stimuli into biochemical responses that alter gene expression and cellular adaptation. Fracture healing is a complex process requiring the recruitment of the appropriate cells and expression of the appropriate genes at the right time in the right place. The majority of clinical fractures heal spontaneously after initial surgical or non-surgical treatment, nevertheless 5-10% of fractures show a delay in healing. Fracture healing is modulated in response to external stimuli, such as growth factors, hormones, and mechanical forces. Insight in the molecular biological mechanisms involved in fracture healing has resulted in the development of new treatment modalities for impaired fracture healing, such as bone morphogenic proteins, extracorporeal shock wave treatment, electro-stimulation, and low-intensity pulsed ultrasound (LIPUS). LIPUS (30 mW/cm^2) is a form of mechanical energy transmitted transcutaneously by high-frequency acoustic pressure waves, which may provide a surrogate for the forces normally applied on bone by physical loading according to Wolff's law. LIPUS has shown to enhances and/or accelerates (impaired) clinical fracture healing, and the positive effect of LIPUS on fracture healing may be caused by a stimulation of the different cellular processes involved in fracture repair and bone formation, such as angiogenesis, chondrogenesis, and intramembranous and endochondral ossification. The exact mechanism by which LIPUS affects clinical bone healing is however still unknown.

This thesis examined the effectiveness of LIPUS-treatment on delayed and/or impaired clinical fracture healing at the tissue level, as well as determined its effectiveness on acceleration and healing of delayed and/or impaired clinical fractures, in order to make suggestions for future treatment considerations and clinical applications. We hypothesized that low-intensity pulsed ultrasound accelerates and/or enhances delayed and/or impaired fracture healing in patients at the bone tissue level by positively affecting different stages of cellular processes involved in fracture repair and bone formation. To test this hypothesis, we addressed the following scientific questions:

1. Is LIPUS effective in the treatment of tibia nonunions in current clinical practice?
2. Is LIPUS as only treatment effective in the acceleration and/or enhancement of fracture union in patients with a delayed union of the non-fixated osteotomized fibula?

3. How does LIPUS affect bone healing at the tissue level in patients with a delayed union of the osteotomized fibula?
4. Is the presence of RUNX2-immunopositive osteogenic cells affected by LIPUS treatment in delayed unions of the osteotomized fibula?
5. Does LIPUS increase vascularity and blood flow in delayed-unions of the osteotomized non-fixated fibula?
6. Does current evidence support the use of LIPUS for the treatment of fracture healing, and if so for which indication is LIPUS most beneficial?

To find answers to these questions we started by investigating the effectiveness of LIPUS treatment on established nonunions of the tibia in current clinical practice, since tibia nonunions have a high occurrence rate and significantly impair daily functioning. All consecutive LIPUS treated established nonunion cases from January 2000 until February 2003 in The Netherlands were evaluated in a self-paired study design. LIPUS was the only new treatment, and strict criteria of enrollment minimized any spontaneous healing chance. The overall healing rate was 52 of 71 cases (73%) as result of LIPUS treatment, showing a significant higher healing rate compared to spontaneous healing ($p<0.0001$). The long-term follow-up showed high compliance rate and no re-fractures. LIPUS is therefore effective in the treatment of established tibia nonunions and can be regarded as a good, safe, and cost-effective alternative to surgery (Chapter 2).

In contrast to fresh fracture healing, a randomized placebo controlled trial on the isolated effect of LIPUS on impaired bone healing may be seen as unethical, because this withholds the sham-treated control patient from any other form of treatment. The osteotomized non-fixated fibula, as part of a high tibial osteotomy, shows a tendency of delayed union and forms a unique clinical model enabling placebo treatment without direct negative consequences and/or conflicting ethical issues. By utilizing the delayed union osteotomized non-fixated fibula as a clinical model, we investigated the isolated effect and outcome of LIPUS treatment on delayed and/or impaired clinical fracture healing in a randomized double blind trial. No difference was seen in number of healed cases between LIPUS-treated (n=7) and sham-treated controls (n=8). Independent radiographical assessment showed that LIPUS decreased healing time by 29% (not significant) at 5 months, and by 57% ($p=0.023$) at 1 year after the start of LIPUS-treatment. LIPUS-treatment is therefore effective in accelerating delayed and/or impaired fracture healing, but as a single treatment option it does not directly contribute to improved fracture union when sufficient stability is absent as in the non-fixated delayed union of the fibula (Chapter 3).

The accelerating effect of LIPUS on fracture healing may be caused by a stimulation of the different cellular processes involved in fracture repair and bone formation, such as angiogenesis, chondrogenesis, and intramembranous and endochondral

ossification. To better understand how LIPUS affects delayed and or impaired bone healing, biopsies of delayed unions of the human osteotomized fibula after a high tibial osteotomy were obtained. By using histology and histomorphometric analysis, bone formation and bone resorption parameters were determined at the tissue level. In LIPUS-treated delayed unions, endosteal callus formation by direct bone formation without a cartilage intermediate as well as indirect bone formation was observed, while in untreated controls only indirect bone formation was observed. In the area of new bone formation, LIPUS significantly increased osteoid thickness by 47%, mineral apposition rate by 27%, and bone volume by 33%. No increase in the number of blood vessels was seen in the newly formed bony callus. Our results sugest that LIPUS accelerates clinical fracture healing of delayed unions of the fibula by increasing osteoid thickness, mineral apposition rate, and bone volume, indicating increased osteoblast activity, at the front of new bony callus formation. Improved stability and/or increased blood flow, but probably not increased angiogenesis, might explain the differences in ossification modes between LIPUS-treated delayed unions and untreated controls. (Chapter 4).

Osteogenic cell proliferation and differentiation play an important role in adequate fracture healing, and is target for osteoinductive therapies in delayed fracture healing. Mechanical adaptive modeling can promote bone tissue formation by a proliferative response or by a direct anabolic effect on bone cells. RUNX2 is a transcription factor, and is regarded as the master gene of osteogenic cell differentiation and bone matrix production. Immunolocalization of RUNX2 as an early bone cell marker was performed to determine the effect of LIPUS on the presence of osteogenic cells located within and around the newly formed woven bone at the fracture end (area of new bone formation), and up to 3 mm distance of the fracture end. LIPUS treatment of fibula delayed unions significantly reduced the number of RUNX2 immunopositive cells within the soft connective tissue at the fracture ends, whereas the number of RUNX2 immunopositive cells at the bone surface was not affected. The number of RUNX2 immunopositive cells were similar for the atrophic and hypertrophic delayed unions. Immunolocalization of RUNX2 positive cells in delayed unions of the fibula reveals that delayed clinical fracture healing does not result in impairment of osteogenic cell proliferation and/or differentiation at the tissue level, even if delayed unions are clinically regarded as atrophic. Reduced numbers of osteogenic RUNX2 immunopositive cells within the soft connective tissue, and unchanged numbers of RUNX2 immunopositive cells at the bone surface, implicates that LIPUS does not increase osteogenic cell presence, but likely affects osteogenic cell differentiation. (Chapter 5).

Vascularity is a key factor in fracture healing, and angiogenesis is required for osteogenesis. Re-establishment of the circulation is essential in the early stages of fracture healing. Whether LIPUS stimulates vascularity by angiogenesis and/or blood flow to the fracture site in delayed and/or impaired fracture healing is still largely

unknown. Parameters of blood vessel formation were measured and related to histomorphometric bone characteristics of newly formed bone in histological sections of fibular biopsies. The volume density of blood vessels was increased in LIPUS-treated delayed-unions compared to sham-treated controls. LIPUS did not change blood vessel number, but significantly increased blood vessel size. Healed delayed-unions (LIPUS-treated and sham-treated controls) showed significant correlations between blood vessel size and osteoid volume. Our results therefore suggest that LIPUS enhances vascularity in delayed-unions of the osteotomized fibula by increased blood flow and perfusion through increasing blood vessel size. LIPUS only enhances fracture healing in patients showing a correlation between vascularization and osteoid volume at the tissue level, but not in patients in which this correlation is lacking (Chapter 6).

Evidence-based medicine forms an important basis to evaluate clinical therapies and guide to clinical application. Overall consensus on the effectiveness and indication for usage of LIPUS in bone healing remains absent. To obtain the best current evidence of LIPUS' clinical healing capacity, as well as to specify its clinical role by identification of those fracture cases most beneficial to LIPUS treatment, we carried out a systematic review and meta-analysis of randomized controlled trials. LIPUS is effective in reducing time-to-radiographical fracture union. In 9 trials the time to radiographic fracture union was the primary outcome measure evaluated. In these trials LIPUS treatment resulted in an overall beneficial effect ($p=0.0006$) after pooling the time-to-radiographical healing data in the combined patient population ($n=402$), i.e. LIPUS resulted in a mean reduction in healing time of 42.2 days (95% confidence interval 18.1 to 66.3 days; $I^2=94\%$; heterogeneity $P<0.00001$). Most reduction in time-to-radiological union by LIPUS was seen in fractures with long natural healing tendency, i.e. non-fixated fibula osteotomies[37], and (complex) fractures of the tibia. Only few studies reported on the effect of LIPUS on functional recovery in fracture healing, but could not demonstrate a beneficial effect. Although decreased time-to-radiographical union may allow for earlier functional use, future studies of high quality should focus on reporting functional outcome needed to firmly establish the clinical effectiveness of LIPUS in fracture healing (Chapter 7).

In conclusion, LIPUS treatment provides a valuable contribution to enhancing delayed bone healing and/or fractures with long natural healing tendency by increasing bone formation and blood flow at and to the fracture site, resulting in accelerated bone healing and significant reduction in time-to-healing. Co-factors negatively influencing fracture healing, such as insufficient stability, may diminish the enhancing capacities of LIPUS on fracture repair (Chapter 8).