Abstract:

RUPPERT, DAVID STRATER. A Study of Osseointegration of Additively Manufactured Implants in Rats through Vibration and Ultrasound. (Under the direction of Paul S. Weinhold, PhD and Ola L.A. Harrysson, PhD)

Amputees frequently develop soft tissue problems on their residual limb due to increased pressure and shear-forces generated at the socket-limb interface. Direct skeletal attachment of prostheses via percutaneous osseointegrated implants provides stable connections while eliminating skin lesions. However, effective osseointegration of implants remains a major clinical challenge. Hastening rehabilitation, as-well-as providing implants for patient specific anatomy, would greatly increase the feasibility of percutaneous osseointegrated prostheses as an alternative to socket prosthetics.

Previous studies indicate that vibration and low-intensity pulsed ultrasound (LIPUS) are beneficial for bone healing. However, the optimal vibration amplitude hasn’t been identified nor has it been shown that LIPUS has a beneficial effect in an intramedullary model to stimulate healing at the bone-implant interface. The primary objective of this work was identifying therapies for accelerating implant osseointegration. Whole body vibration at various amplitudes was investigated to identify the optimal stimuli. Locally applied vibration and LIPUS were studied separately and cumulatively.

Non-patient specific threaded implants are being used in FDA clinical osseointegrated prostheses trials. Additive manufacturing can cost effectively create custom implants to better interface with amputees’ residual bones. A secondary objective of this study was evaluating additive manufacturing as an alternative to commonly used Bränemark threaded implant to produce patient specific implants. This objective was broken into two phases: compare
osseointegration of additive manufactured (AM) implants to threaded implants; compare osseointegration strength of coarse and fine textured AM implants.

The two objectives were carried out through three studies. The first study was split into two cohorts of Sprague-Dawley rats receiving bilateral, titanium implants (AM vs. threaded) in their tibiae. One cohort, comprising five groups vibrated at 45Hz: 0.0 (control), 0.15, 0.3, 0.6 or 1.2g, was followed for 6 weeks. A second cohort, divided into two groups (control and 0.6g), was followed for 24 days. Osseointegration was evaluated through mechanical, µCT and histological evaluations. Bone-volume fraction around the implant increased at 0.6g compared to control. The AM implants exhibited significantly improved mechanical stability relative to their threaded counterparts.

The second study comprised of two cohorts receiving bilateral, titanium implants in their distal femurs and were followed for 4 weeks. The first cohort received coarse AM implants produced by electron-beam melting (EBM) transcortically in one femur and a direct melt laser-sintered (DMLS) fine textured AM implant in the contralateral femur. The second cohort received DMLS implants (either fine textured or coarse textured to mimic EBM) in the intramedullary canal of each femur. Osseointegration was evaluated through mechanical and µCT evaluation. The fixation strength of coarse textured implants provided superior interlocking relative to fine textured implants without affecting bone morphology in both cohorts.

The bilateral femoral intramedullary implant model of the third study looked at effects of local vibration and LIPUS on early osseointegration (4 weeks) and the separate and combined effects of these treatments on midterm osseointegration (8 weeks). Osseointegration was evaluated through mechanical, µCT and histological evaluations. LIPUS produced increased pushout load relative to control at 4 weeks. Both µCT and histology revealed treatment with
either LIPUS or vibration increased bone around the implant relative to controls at 4 weeks. No differences were noted in pushout loads at 8 weeks. The bone gained with LIPUS at 4 weeks was no longer present at 8 weeks. Vibration treatment resulted in greater bone around the implant than all other groups at 8 weeks.

These studies demonstrate the potential benefit of LIPUS as a therapeutic tool for reducing amputees’ rehabilitation period and the use of vibration for preventing bone resorption during the rehabilitation period while the limb isn’t yet loaded. Additive manufacturing was also shown as a viable alternative to current production methods, opening the way to fabrication of patient specific implants.
A Study of Osseointegration of Additively Manufactured Implants in Rats through Vibration and Ultrasound

By
David Strater Ruppert

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Biomedical Engineering

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2017

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Biography:

David Ruppert is a PhD candidate in the NCSU/UNC joint BME program. He has a Masters in mechanical engineering from Virginia Tech where he focused in biomechanics and equine rehabilitation. Ruppert also has over 10 years of experience developing novel technologies for industry. His current research is in improving mechanical stability and accelerating osseointegration of titanium implants.

After earning his Bachelor of Science in Mechanical Engineering from the University of Maine in Orono in 1997 he started his career at Steag Hamatech. As a mechanical engineer he supported designers through analytical calculations, FEA, and experimentation. In 1998 he joined the research and development department where he designed and completed research projects for various processes, designed solutions to improve yields and reliability of machines and managed the summer engineering interns. While at Steag Hamatech he developed a thermal management system to robustly manufacture the emerging DVD technology. He designed a robot handling system to create the most compact CD manufacturing machine on the market. He also worked with a team to produce a repeatable coating process for bonding the DVD substrates.

In 2000 he was invited as a fully funded graduate student to attend Virginia Tech’s Mechanical Engineering Master’s program to focus in biomechanics. He performed equine gait analyses and designed a robotic simulator to test a rehabilitation device he had also conceptualized to earn his degree. While at Virginia Tech, he also conducted undergraduate labs and graded reports for Junior and Senior level students. He was responsible for maintaining the undergraduate laboratory equipment and trained fellow teacher’s assistants.
In 2003 Ruppert joined D2 Inline Solutions, a small startup company, as the senior mechanical engineer providing design-leadership to designers for product development. During his two years there he created revolutionary equipment for the vacuum metallization process. As the senior engineer, he developed the primary intellectual property for the company including a novel valve sealing surface and a helium cooling process for vacuum metallization equipment that went on to be patented and acquired by an international company.

In 2005 he started his own engineering consulting service where he provided to industry: thermal, fluid, and structural analysis, kinematic and dynamic analysis, specification of mechanical components based on load calculations. He recommended initial designs and design modifications to draftspersons in order to document and modify designs. He also checked detail and assembly drawings for correct design intent.

Later in 2005, Ruppert went to Clyde Bergemann Bachmann as their Finite Element Analyst where he provided finite element analysis for all of the engineering teams designing stack, diverter, louvers and dampers for various industries. The analysis included structural analysis per customer specified standards and codes; thermal and fluid flow models; hand calculations as well as checking other engineers’ hand calculations; and providing the knowledge base for technical analysis of structures to engineering teams.

He moved from industry to academia in 2012 to pursue a PhD in Biomedical Engineering through the joint program of the University of North Carolina and the North Carolina State University. He investigated the effects of various amplitudes of whole body vibration, locally applied vibration and low intensity pulsed ultrasound on bone ongrowth in an osseointegration rodent model. His research also spread into the evaluation of various additive manufacturing
processes for improving mechanical stability of osseointegrated implants. His research was funded by the Dean’s Fellowship, a NC Tracs grant, an NSF grant and a teaching assistantship.

He functioned as the lead lecturer for the course Biomedical Engineering Design and Manufacturing II in the spring of 2017. In that role he redesigned the course syllabus from a reverse engineering project to needs-based innovation project. He also organized guest lecturers on various topics from Modern Manufacturing Techniques to Market Analysis and FDA Regulatory Pathways.

Ruppert intends to transition back into industry to develop and design assistive technology for the rehabilitation engineering field.
Acknowledgments:

Sincere gratitude and appreciation goes to Paul Weinhold, Denis Marcellin-Little and Ola Harrysson for their constant support and mentorship.

I’d like to also acknowledge and thank:

Seth Bollenbecker for his countless hours of animal handling in the second cohort of the main study; developing radiographs; collection and preparation of specimens for mechanical, µCT and histologic evaluation; and imaging and evaluation of hisological slides.

Stephen Kallianos for his many hours of animal handling, participation in the operating room and potting of the specimens in the preliminary study as-well-as his many hours of animal handling in the first cohort of the main study.

Jonathan Frank for his guidance and assistance acquiring µCT scans at the Small Animal Imaging Facility at the UNC Biomedical Imaging Research Center.

Melanie Card for her technical assistance with the analysis of µCT DICOM stacks of the first cohort of the pilot study.

Tyler Scheviak for developing Matlab code to calculate cortical thickness at the diaphysis.

Sam Abumoussa for his assistance with the surgeries in the second phase of the pilot study.

Sandra Horton and Nathan Whitehurst for instruction and use of the EXAKT grinding and cutting system at the Histology Lab in the Department of Population Health and Pathobiology, College of Veterinary Medicine, NCSU.

Special thanks goes to Caroline Cross for her emotional support, countless hours editing drafts and putting up with odd working hours.
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List of Abbreviations:

AM: Additive Manufacturing
BIC: Bone Implant Contact
BoTox: Botulinum Toxin
BRIC: Biomedical Research Imaging Center
BV/TV: Bone Volume Fraction
DLAM: Division of Laboratory Animal Medicine
DMLS: Direct Metal Laser Sintering
DSR: Red Fluorescent Protein Filter
EBM: Electron Beam Melting
ELI: Extremely-Low Interstitial Impurity
GFP3: Green Fluorescent Protein Filter
IACUC: Institutional Animal Care and Use Committee
LIPUS: Low-Intensity Pulsed Ultrasound
LMHF: Low-Magnitude High-Frequency
LMMS: Low-Magnitude Mechanical Signals
MTS: Material Testing System
OVX: Ovarectomized
Ra: Surface Roughness
RANKL: Receptor Activator for Nuclear Factor Kappa-B Ligand
Ti6Al4V: Grade 5 Alloyed Titanium
SD: Standard Deviation
WBV: Whole Body Vibration
Introduction:

Research in prostheses design, improvement in traditional socket connections and development of direct transdermal osseointegrated prostheses are frequently investigated due to the prevalence of amputation in the United States alone\textsuperscript{1,2}. Traditional socket-type prostheses are fraught with complications such as dermatitis, infected sores, pain and inefficient connection to the body\textsuperscript{3}. Direct skeletal attachment of limb prostheses through the skin (percutaneous osseointegrated prostheses) overcome shortcomings of socket prostheses through improved control and wider range of motion of the prosthesis, heightened osseoperception (sensory feedback of the environment), increased postural comfort, and reduced soft tissue complications resulting in an overall improvement in quality of life\textsuperscript{4}.

Percutaneous osseointegrated prostheses face several challenges, which prevent their approval by the FDA outside of clinical trials. Some of the major challenges are: osseointegrated implants need to be adapted to patients’ specific anatomy; transcutaneous osseointegrated implants are susceptible to infection; and a measured and gradual rehabilitation period (12 months) is required to avoid fibrous ongrowth onto the implant that can result from excessive loading and micromotion. While osseointegration treatment failures and the infection rate have been reduced\textsuperscript{4}, current research efforts are focused at reducing the rehabilitation time before functional loads can be applied to the prosthesis after surgery\textsuperscript{5}. The field of osseointegrated implants would benefit from enhancing and accelerating the mechanical stability through treatments targeting bone growth as well as implants designed for patients’ specific anatomy.

Low-magnitude high-frequency (LMHF), whole body vibration (WBV) has been shown to improve bone remodeling and enhance callus formation and mineralization through upregulating gene expression related to chondrogenesis and osteogenesis respectively\textsuperscript{6}. Studies
have demonstrated that higher frequencies result in improved bone characteristics\textsuperscript{7,8} while other studies have shown arterial damage with increased frequencies\textsuperscript{9}. 45 Hz has shown positive osteogenic effects while minimize the arterial damage\textsuperscript{8}. Existing osseointegration animal studies have shown early bone ingrowth with LMHF vibration; however, each study only investigated a single vibration amplitude\textsuperscript{10,11}. No dose-response study has determined the optimum amplitude of vibration for stimulating bone ongrowth or whether ongrowth is negatively impacted by higher vibration levels.

Local vibration at site specific locations has been successfully demonstrated to both reduce atrophy of muscles\textsuperscript{12} and increase both bone formation and percent mineralizing surfaces\textsuperscript{13}. However, local application of LMHF vibration has not been studied for stimulation of osseointegration of an implant in an intramedullary implantation model.

Low-Intensity Pulsed Ultrasound (LIPUS) has been shown to be beneficial during the healing of fracture nonunions by upregulating several mRNAs involved in bone healing\textsuperscript{14,15}. LIPUS demonstrates beneficial effects on early stage bone remodeling in both transverse and intramedullary osseointegration animal models\textsuperscript{16,17}. However, it is unclear if the ultrasound will have an effect in an intramedullary model to produce clinically significant osteogenic benefits to improve implant stability of an implant.

The mechanism of vibration’s effects on bone is thought to follow a different pathway than LIPUS. Vibration has been shown to decrease differentiation of stem cells into fat cells while increasing their differentiation into bone\textsuperscript{18} while LIPUS has been shown to upregulate several mRNAs involved in bone healing\textsuperscript{14,15}. By improving bone development along separate conduits, it is logical to expect a cumulative effect of the two treatments. Combining LMHF vibration with LIPUS has not been investigated to determine if there is a cumulative effect.
While the primary goal of the research is addressing the lengthy osseointegration time, a secondary goal is improving bone-implant stability through implant design. Threaded Brânemark implants have been used predominantly in clinical cases of extremity osseointegrated implants. These threaded cylindrical implants are unable to conform to patient specific anatomy. Additive manufacturing offers the means to create custom, organic geometries to match patient-specific anatomy increasing the potential for initial bone-implant contact area.

Many arthroplasty implants are post-processed in order to create a textured surface for improved osseointegration. Rough implants have been shown to improve osseointegration demonstrating higher bone volume fractions (BV/TV) compared to smooth implants due to lower bone resorption in early stage bone remodeling. Few comparative studies have evaluated the mechanical stability of threaded implants to porous surface implants; especially designs developed by new additive manufacturing (AM) technology. AM textured surface designs of implants may better allow bone ongrowth and accelerate the mechanical stability of the implant as compared to standard threaded surface designs through their extensive network of interstices.

Electron beam melting (EBM) and direct metal laser sintering (DMLS) are the two primary methods for producing AM titanium implants for osseointegration. The two methods use different powder sizes and processes for fusing the metal together resulting in different surface roughness in the “as-built” implants. Due to DMLS’s superior spatial resolution an implant could theoretically be manufactured by DMLS having a similar surface roughness as the EBM implants while allowing for a more detailed geometry. Studies have shown that the roughness of implants affects the mechanical stability of the bone-implant interface. There have been few studies comparing DMLS to EBM for osseointegration.
Specific Aims and Associated Hypothesis:

The first aim of this research was to determine the most effective magnitude of LMHF WBV for improving osseointegration of an implant. Several peak acceleration amplitudes (0, 0.15, 0.3, 0.6, & 1.2 g) were investigated to determine the most effective level of LMHF vibration. The hypothesis was that bone ongrowth and torsional stability would be improve through the increasing LMHF vibration, but would eventually be impaired at higher vibration levels (> 1 g) due to excessive micromotion.

The second aim was to determine if the rough texture of an implant produced through additive manufacturing—specifically electron-beam melting—was superior to a conventional threaded design for improving mechanical stability of osseointegrated implants. It was hypothesized that a textured AM surface profile that enables bone to grow within the interstices of its surface would yield greater bone ongrowth and torsional stability compared to a threaded implant. The second aim was explored in parallel with the first aim in a transcortical, tibial, rodent model. A transcortical, tibial model was utilized to allow for the incorporation of a percutaneous component in future work without amputation. Interactions between implant type and vibration amplitude weren’t expected to be significant. However, it was anticipated that the porous surface design would show improved BV/TV, max torque, and % bone-implant contact (BIC) at all vibration levels including the control.

The third aim of the research was to compare the osseointegration strength of two AM methods for producing textured implants: EBM and DMLS. We hypothesized that the coarse surface texture (Ra = 23 µm) inherent of EBM results in superior osseointegration compared to the fine surface texture (Ra = 10 µm) of DMLS. Due to DMLS’s superior spatial resolution an implant can theoretically be manufactured by DMLS having a similar surface roughness as the
EBM implants while allowing for a more detailed geometry. It was believed the fixation strength of EBM and DMLS implants having similar surface roughness would be similar.

The final goal of this study was to compare the relative effectiveness of LMHF vibration and commercial LIPUS alone and combined on regional bone mass and bone ongrowth, through an in-vivo rat study. Clinically, vibration would be introduced by local vibration stimulators secured to the limb or resting the limb on a stimulator. An unloaded intramedullary implant model in the rat with locally applied LMHF vibration was utilized to more accurately simulate the initial clinical ongrowth period with vibration stimulation. A mechanical actuation system was developed and validated for delivery of controlled LMHF vibration with optimized amplitude and frequency to the pelvic limbs of rats. It was hypothesized that LIPUS and local LMHF vibration would both independently improve osseointegration through increased mechanical fixation properties as well as BV/TV and % BIC. LIPUS and LMHF vibration have been shown to stimulate osteogenesis through different pathways. Therefore, it was believed that there would be a cumulative benefit to applying dual LIPUS and LMHF vibration for stimulating osseointegration of an intramedullary implant.

This research focused on shortening the rehabilitation time for transdermal osseointegrated prosthetics while also increasing the bone density of the amputated limb. The results from the proposed research project will greatly improve the post-surgical rehabilitation of a wide variety of traditional osseointegrated implants.
Chapter 1: Background Literature Search

Amputation

Approximately 1 in 190 Americans in 2005 were amputees and that number is expected to double by 2050\(^1\). Of the current amputees, 704 of 1568 thousand (45\%) are due to trauma\(^1\). Based on these statistics, there are currently 755 thousand trauma amputees living in the US\(^{23}\). This number could rise to as many as 1.9 million by 2050 when the population of the US is expected to reach 401 million\(^{24}\).

Socket-type prostheses are the most commonly used amongst amputees. An optimal fit is difficult to achieve with socket-type prostheses often resulting in undesirable stresses on tissues most frequently resulting in painful lesions, bursae, inflammatory edema, soft-tissue calcification and neuromas\(^{3,25}\). Socket prosthetic devices also lack stability due to their inefficient integration with the body. Thus, there is interest in improving methods of attaching prosthetic devices to amputees.

An approach that is gaining popularity is direct transdermal osseointegrated prostheses. Osseointegration allows for a direct structural and functional connection between living bone tissue and the surface of a load carrying implant\(^{26}\). This direct osseointegrated interface in amputees allows for a more stable connection enabling greater control of the prosthesis and heightened osseoperception (sensory feedback of the environment) while eliminating the problems associated with the socket device such as pain and skin irritation, as well as an overall improvement in quality of life\(^4\).

Amputation in patients with diabetes and vascular disease are primarily due to poor metabolic control making them inappropriate candidates for osseointegrated prostheses\(^{27}\).
whereas, younger trauma, cancer or congenital defect patients provide a good patient base for osseointegrated prostheses.

When an osseointegrated implant is surgically placed the pre-existing bone matrix is stimulated and the bone changes following a 3-stage biologically-determined evolution. During the first stage, low-density woven bone tissue is formed and grows rapidly from the residual bone towards the implant. Woven bone formation occurs within the first four to six weeks following surgery. The second stage begins during the second month after surgery. During that stage, woven bone tissue is reinforced and replaced with stronger lamellar bone. The final stage of osseointegration starts around the third month post-surgery. It involves bone remodeling, a lifelong process where new bone tissue is simultaneously formed (regenerated) and removed (resorbed). The physical loads exerted on the bone dictate whether regeneration or resorption dominates. When bones are subject to increased loading, regeneration occurs, and when loading decreases or is absent, resorption occurs. The process by which a bone adapts to the loads placed upon it is known as Wolff’s law. During the period between initial amputation and surgery for placement of an osseointegrated implant, bone remodeling decreases the bone density in the unloaded limb reducing the available bone stock. Also, during rehabilitation it is critical to prevent the loading of the implant from increasing to a level which causes micro motions greater than 20 µm at the bone implant interface. Developing bone exposed to high micro motions (≥ 150 µm) will differentiate into fibrous bone resulting in implant loosening. Therefore a gradual rehabilitation regimen is required to successfully integrate an osseointegrated prosthesis. It is desirable to minimize the amount of bone loss prior to achieving full load bearing on the osseointegrated implant while allowing for the development of non-fibrous bone-implant interface. This extended rehabilitation time (18 months) for bone ongrowth before functional
loads can be applied to the prosthesis is a major disadvantage of osseointegrated prostheses which has resulted in current research efforts to solve this problem\textsuperscript{5}. Transcutaneous osseointegrated implants are susceptible to infection\textsuperscript{31}. An infection at the transdermal interface will lead to an increased level of monocytes as an innate immune response. The low level inflammation of monocytes contributes to bone resorption\textsuperscript{32} further hindering osseointegration.

It is important to develop and validate the most effective methods for accelerating rehabilitation and improving the mechanical stability of the osseointegrated prostheses in order to decrease the recovery period of osseointegration patients and allow them to realize the benefits of osseointegrated prostheses over the socket-type prostheses. Low-magnitude mechanical signals (LMMS) trigger bone regeneration\textsuperscript{33}. Site-specific LMMS could be used to improve the quality of bone stock adjacent to osseointegrated implants and to promote bone ongrowth onto the internal prosthesis. Site-specific LMMS would represent an innovative and cost-effective method used to shorten the rehabilitation period of patients with osseointegrated implants. We intend to evaluate separately and in combination two modalities that will deliver low-magnitude mechanical stimuli: a Low-Magnitude, High Frequency (LMHF) mechanical vibrating system developed in-house and a Low-Intensity Pulsed Ultrasound (LIPUS) device that has proven clinical benefits and is commercially available (Exogen unit, Bioventus, Durham, NC).

**LMHF Vibration**

The LMHF vibration device will deliver localized LMHF vibration to the implant site. LMHF vibration has been shown to improve bone remodeling and enhance callus formation and mineralization through upregulating gene expression related to chondrogenesis and osteogenesis respectively\textsuperscript{6}.
LMHF vibration has also been demonstrated as a successful treatment for fracture healing with several animal studies showing increased callus formations\textsuperscript{34,35} and higher mineral contents\textsuperscript{33,36}. In addition, LMHF vibration has been shown to improve balance, bone mineral density and muscle mass in human subjects\textsuperscript{37-39}.

Researchers have looked at the effects of the frequency of LMHF vibration treatment. Low frequencies ($\leq$10Hz) have failed to show improvement\textsuperscript{40}. However, several studies have demonstrated the ability of LMHF ground-based vibration to increase bone mass or bone volume in normal animals\textsuperscript{7,41-44} and increase bone bending strength and bone formation rates in animal models\textsuperscript{8,45}. Increased bone ingrowth into porous coated titanium alloy implants has been identified in a turkey model\textsuperscript{46}. Studies investigating various frequencies demonstrated that higher frequencies result in improved bone characteristics\textsuperscript{7,8}. However, other studies have shown a worsening of arterial damage with increasing vibration frequencies\textsuperscript{9}. Therefore, our team has chosen 45 Hz, which has been shown to increase periosteal bone formation relative to lower frequencies\textsuperscript{8} yet attempts to minimize the arterial damage.

Vibration therapies have been applied at site specific locations. One study successfully demonstrated the ability to significantly reduce atrophy of the soleus muscle through local vibration of the limb\textsuperscript{12} while another study showed a local 45 Hz stimulation to the tibia is an anabolic stimulus to osteoblasts increasing both bone formation and percent mineralizing surfaces\textsuperscript{13}.

Studies of intact bone suggest that higher vibration amplitudes induce bone formation over a greater extent of bone surfaces\textsuperscript{47,48}. However, too strong a vibration may cause excessive implant micromotion resulting in fibrous encapsulation and impaired implant stability\textsuperscript{49}. Existing osseointegration animal studies have shown bone ingrowth with LMHF vibration at a single
vibration amplitude\textsuperscript{10,11}. Others have confirmed in a non-fracture study that there is a difference in bone remodeling based on vibration amplitude yet they failed to identify an optimum value\textsuperscript{50}. Therefore, we conducted an \textit{in vivo} study in rats aimed at identifying the optimal vibration intensity for osseointegration in a rat model.

Although the mechanism of vibration’s effects on bone is not completely clear, it has been shown that vibration decreases differentiation of stem cell into fat cells while increases the differentiation into bone\textsuperscript{18}. Vibration has been shown to produce fluid shear in bone marrow which may lead to bone anabolism\textsuperscript{51}. Increased arteriole shear stress has been shown to lead directly to increased prostaglandins and subsequent vasodilation\textsuperscript{52}.

**LIPUS**

While LMHF vibration has been shown to improve bone quality; a more widely used and FDA-approved modality for bone regeneration, particularly for healing of fracture nonunions, is LIPUS. LIPUS has been shown to upregulate several mRNAs (alkaline phosphatase, osteocalcin, insulin-like growth factors and bone sialoprotein) involved in bone healing\textsuperscript{14,15}. By causing inflammation, angiogenesis, chondrogenesis, intramembranous ossification, endochondral ossification and bone remodeling, LIPUS demonstrates that it acts on a cellular level at each phase of bone healing\textsuperscript{53}. Combining LMHF vibration with LIPUS has not been investigated to determine if there is an additive effect. It is logical to expect an additive effect with both LMHF and LIPUS treatments since it is believed that vibration increases osteogenic gene expression and acts on mesenchymal stem cells while LIPUS elevates the mRNAs responsible for bone healing.

Patients with potential or actual delay in fracture healing may be treated with LIPUS to boost the bone regeneration process and accelerate fracture healing. Contradictory reviews of several clinical studies\textsuperscript{54,55} have confirmed and refuted that brief exposure to LIPUS has a
significant effect on the healing time of fresh fractures, encourages healing of delayed unions and non-unions and results in stronger, stiffer bones. Furthermore, LIPUS has been shown to lead to an increase in bone density and bone mineral content\textsuperscript{56,57}. The process of osseointegration has been compared to fracture healing\textsuperscript{4,28}. Since LIPUS accelerates fracture healing\textsuperscript{58}, it follows that LIPUS will have a positive impact on bone ongrowth onto osseointegrated implants and will therefore accelerate the rehabilitation process after surgery. LIPUS already demonstrates beneficial effects on early stage bone remodeling in both transverse and intramedullary osseointegration animal models\textsuperscript{16,17}. However, studies have failed to perform mechanical tests and/or demonstrate a mechanical stability benefit from LIPUS treatments\textsuperscript{16,59-61}. Furthermore, it is unclear if the ultrasound will produce beneficial effects in the medullar cavity to produce clinically significant improvements in intramedullary implant stability. The mechanical stability of the implant is critical in the rehabilitation of osseointegration patients and the effects of applying LIPUS prior to and after surgery should be investigated through mechanical testing.

The combined effects of LIPUS and vibration on regional bone mass and bone ongrowth have not been evaluated in an animal model. Rehabilitation time could potentially be reduced for osseointegration amputee patients as well as increasing the bone density of the limb through the combined benefits of LIPUS and vibration. This research will also have a direct impact on improving the post-surgical rehabilitation of a wide variety of traditional osseointegrated implants.

\textbf{Implant Design}

While threaded Brånemark implants have been the dominant choice in clinical cases of extremity osseointegrated implants\textsuperscript{19}, few studies have compared the mechanical stability of these implants
to porous surface implants; especially designs developed by new additive manufacturing technology\textsuperscript{21}. Implants designed using AM have a similar surface texture as porous implants and may accelerate bone ongrowth and mechanical stability compared to standard threaded surface designs\textsuperscript{62}. Furthermore, comparative studies have not been carried out to establish if new additive manufacturing porous surface designs of implants may better allow bone ongrowth and accelerate the mechanical stability of the implant as compared to standard threaded surface designs\textsuperscript{4}.

Alloyed titanium (Ti6Al4V) has been shown to integrate with bone tissue comparably to commercially pure titanium through mechanical testing, histomorphometric and histologic analysis\textsuperscript{63}. This allows for a wider range of fabrication methods including additive manufacturing. Furthermore, studies have demonstrated no difference in cellular response to alloyed titanium implants produced through electron beam melting (EBM) additive manufacturing and extruded titanium\textsuperscript{64}. Implants fabricated using additive manufacturing technology can confidently be compared with threaded implants.

The Bränemark implants are straight and cylindrical by design, limiting their adaptability to the patients’ residual bones. Additive manufacturing methods address the need of the implant design to meet patient specific anatomy and allow for custom surface texture along the implant. The advantages of utilizing additive manufacturing for fabricating osseointegrated implants need to be validated.

Cutting flutes are commonly used in bone screws to aid in advancing the screw through the cortices while maintaining a tight fit. The need to include cutting flutes on the threaded implant was investigated since their addition significantly increases cost and lead-time of the implants. Investigators have looked at optimizing thread design and methods of insertion of bone
screws. Studies show that screws with no cutting flutes tend to strip the near side cortex while attempting to engage the far side cortex and result in lower pullout strength compared to screws with 3 and 4 cutting flutes\textsuperscript{65}. However, pre-tapping the cortices causes the least amount of morphological alteration and lowest insertion torque compared with thread-cutting and thread-forming screws\textsuperscript{66}. Lowering the insertion torque when surgically installing the screw in a bone where the screw to cortex ratio is high would decrease the likelihood of fracturing the bone during insertion. Finally, there is no difference in cortical pullout strength between self-tapping and non-tapping screws when the bone is pre-tapped for the non-tapping screws\textsuperscript{67}. Therefore, pre-tapping the holes for non-tapping threaded implants is the optimal option for minimizing the implant cost, obtaining the best surgical fit and increasing the success rate of the surgery.

Studies show that altering the surface of an implant can improve its mechanical stability, thereby increasing the required torque to remove the implant. Medium-particle (75\,\mu m) blasting results in improved torque resistance compared to fine (25\,\mu m) and coarse (250\,\mu m) particle blasting, indicating that a moderate surface texture results in better mechanical stability\textsuperscript{68,69}. AM manufactured osseointegrated implants inherently have a moderate surface texture with a roughness of 35 ±12\,\mu m\textsuperscript{64} and can be manufactured to match an infinite range of residual bone geometries making them a logical consideration for osseointegrated implants. Further studies have shown that acid etching of implants improves the holding torque and increases bone-implant contact area (BIC)\textsuperscript{70-72}.

When titanium is exposed to air a thin oxide layer is formed. The oxygen is freed from the surface to form water when the oxidized titanium is submerged in sulfuric acid (48\% H\textsubscript{2}SO\textsubscript{4}), a common solution used for acid etching of titanium. Consequently, titanium is also removed in the form of titanium-sulfide leaving behind pits in the surface. Finally, freed hydrogen in the
solution bonds with the titanium surface to form titanium-hydride. The resulting surface of titanium etched in sulfuric acid has both a desirable roughened surface and demonstrated biocompatibility.\textsuperscript{73} The etching time of sulfuric acid and titanium has been investigated by others\textsuperscript{74} and per their result the desired etched surface can be achieved by agitating the implant for 30 minutes in a 48% sulfuric acid (H\textsubscript{2}SO\textsubscript{4}) bath at an elevated temperature of 60°C (140°F).

**Additive Manufacturing**

Traditionally, parts are produced by the removal of material via lathes, mills, grinders, etc. These methods require either the part or tool to be spun about an axis significantly limiting the complexity and geometry of features. Alternatively, casting or forging of parts can be achieved through the use of molds or dies. However, the construction of these molds and dies are again limited by traditional fabrication techniques or requires time consuming and costly methods of building them. Casting and forging is ordinarily reserved for the mass production of parts to achieve a cost benefit.

Additive manufacturing (AM) is the process of building a part through incremental addition of material until the entire three-dimensional part is completed. AM doesn’t require a costly mold as in casting to generate complex parts; thereby making the fabrication of unique and complex parts a reality. Furthermore, the AM process allows for a greater range of features and shapes which are not inhibited by the typical constraints of subtractive manufacturing; opening the door to organic—3 dimensional—features and complex scaffold structures.

Due to manufacturing limitations of traditional fabrication techniques, implants are either built as cylinders or are offered in a few ‘best-fit’ options\textsuperscript{75}. It can be difficult to find an implant to conform to the unique morphology of a patient’s residual bone using such implants resulting
in limited performance. Custom implants designed from medical imaging of a patient’s bone reduce micromotion and dynamic rotation relative to conventional implants increasing initial stability\textsuperscript{76}. It has also been shown that patient specific implants significantly reduce the duration of surgical procedures\textsuperscript{77,78}. AM implants can be designed based on medical images (i.e. CT or MRI) of patient specific anatomy to optimize the initial contact of the implant with the cortical shell through its entire length within the intramedullary canal. Optimizing the implant fit will increase the bone-implant contact providing a more stable integration.

Conventional fabrication techniques also limit the ability of optimizing the implant stiffness relative to the surrounding bone. While attempting to create a ‘best-fit’ implant, the stiffness of the implant can be compromised: stiffer implants result in higher levels of stress shielding while a flexible implant allows for more uniform strain along the length of the bone and consequently increased bone retention, bone-mineral content and bone-mineral density\textsuperscript{79}. Stress shielding of osseointegrated implants has been raised as a point of concern for bone resorption, implant loosening and periprosthetic fractures\textsuperscript{80,81}. Bone resorption has been seen at the distal end of the residual bone due to unloading\textsuperscript{75} and bony fractures are a concern at the terminal end of the implant\textsuperscript{79}. Also, external fixation plates have been shown to incur cortical bone loss under the plate\textsuperscript{82}. Improper fit and large differences in implant and bone stiffness can result in unloading of bone and the natural physiological response of bone resorption through reduced bone strain. An optimal design for a medullary implant might minimize stress shielding throughout the length of the implant while maximizing the physiological load on the residual bone. AM implants can be designed with a scaffold structure and/or varied thicknesses to customize the stiffness of the implant along its length. Optimizing the implant stiffens to the surrounding bone will reduce unloading of the residual bone and minimize stress shielding.
Furthermore, external features can be considered through AM to aid in seating the distal end of the implant to minimize resorptions as seen in another study\textsuperscript{75}.

AM also allows for the design of implants to integrate with the periosteal surface of the bone to aid or replace an intramedullary implant in the event that the length of the canal is insufficient to properly design an intramedullary implant due to encroaching on the proximal epiphyseal line. Patient specific transdermal osseointegrated implants for amputees produced with the available AM technologies should be investigated for osseointegration, stability and efficacy.

Many AM processes have been developed and optimized to produce parts in a wide array of materials ranging from rubber to Cobalt-Chromium. Many of the materials have been developed for medical application. Some metals have already been approved by the FDA for use in medical devices and implants. Arcam AB has received FDA approval for creating implants using their system with extremely-low interstitial impurity (ELI) Ti6Al4V.

Two common methods for additively manufacturing Ti6Al4V parts are electron-beam melting (EBM) and direct metal laser sintering (DMLS). Material properties vary greatly between the two processes from a micro-hardness of 358–387 HV for EBM and 479–613 HV for DMLS to an ultimate strength of 830–1150 MPa for EBM and 1250–1267 MPa for DMLS\textsuperscript{83-88}. The fatigue strength for $10^7$ cycles of Ti6Al4V parts fabricated using the DMLS process is 210 MPa\textsuperscript{89}. However, the EBM process produces parts with a $10^7$ cycle fatigue limit of 600 MPa\textsuperscript{90}. To achieve the EBM fatigues strength the parts must undergo hot isostatic pressing (HIP) at 920°C & 1000 bar for 120 minutes\textsuperscript{91}. EBM parts which underwent hot isostatic pressing have a superior fatigue strength than machined Ti6Al4V (500 MPa at $10^7$ cycles\textsuperscript{92}). However, Ti6Al4V fatigue strength can be increased to 700 MPa through a 900°C to 955°C solution
treatment and then aging at 540°C. It is unclear as to the specific benefits DMLS parts may experience with similar post processing.

The interstices created by the incomplete melting of the powder at the part surface may also decrease the fatigue limit through “notch sensitivity”. As the radius of a notch decreases the stress in the local area rises and increases the probability of crack development and propagation. The radius of the notch on the finished AM part is directly related to the initial powder size. Typical EBM powder ranges from 45μm to 105μm while DMLS ranges from 25μm to 45μm. Therefore, the small notch radius resulting from the smaller powder size may also attribute to DMLS’s inferior fatigue limit. Another possible difference between the processes that may be adding to the drastically low fatigue limit for DMLS parts is that EBM fabricates parts in a vaccum with no oxygen present while DMLS parts are fabricated under inert gas with a low percentage of oxygen. Therefore, it is possible that brittle titanium-oxide is present throughout the part and negatively affecting its fatigue characteristics. Finally, development of the microstructure within the part, which considerably affects material properties, is dependent on cooling rates, which intrinsically vary between the two processes. The higher cooling rates endured in DMLS yield a harder martensitic phase than those resulting from the lower rates of EBM. Further investigation should be conducted to determine the root of the lower fatigue strength. Also, biocompatible alloy powders for AM implant fabrication could be investigated which reduce the modulus of elasticity without compromising the fatigue strength—like Ti-13Nb-13Zr.

Open porous structures have been shown to support bone migration on and through an implant allowing for more complete osseointegration. Both the pore size and the architecture of the pore have been shown to affect cell proliferation and migration. AM can generate these
scaffold structures to both optimize implant stiffness while reducing stress shielding and improve osseointegration through the encouragement of bone ongrowth and ingrowth. A study was designed to compare similar porous structures fabricated through EBM and DMLS\textsuperscript{99}. The comparison of the EBM process to the DMLS process demonstrated similar bone in-growth through histological evaluation and mechanical pushout supporting either method for implant fabrication.

Several in-vitro studies investigating surface morphology of titanium implants have demonstrated that a textured surface promotes bone proliferation and adhesion with increased improvement corresponding to an increased roughness\textsuperscript{100,101}. A defining characteristic of AM parts is the rough surface texture produced through incomplete melting of powder as described earlier. EBM implants with this rough native surface texture have been shown to experience similar levels of bone-implant contact (24–25\%) as the well-established method for encouraging bone-implant contact—titanium plasma sprayed implants\textsuperscript{102}. Furthermore, titanium screws produced by EBM demonstrated superior osseointegration measured by bone volume/total volume, bone surface area/bone volume, and trabecular number and torsional resistance to machined titanium screws in a sheep cervical vertebrae model\textsuperscript{103}. It is not clear whether the mechanism of increased torque resistance is due to the increased osseointegration, increasing the surface area of the screw with the numerous interstices or a combination of both. Further investigation is needed to compare AM implants to traditional smooth threaded implants to discover the source of benefits seen in earlier studies. These finding suggests AM can be used as an alternative method for osseointegration of implants and a better understanding of the mechanism of osseointegration will allow for improved design of AM implants.
Contradictory results have been seen in the research with surface treatment of implants for osteo-conduction. One study demonstrated improved osseointegration through sandblasting and acid etching of an DMLS implant in a rabbit model, citing the reduced surface roughness as means for improved osteo-conduction\textsuperscript{104}. Another study demonstrated only negative proliferation response to surface roughness above 24.9um of human osteoblasts on EBM Ti6Al4V surfaces\textsuperscript{105}. Yet another study resulted in increased osseointegration of an EBM implant compared to a smooth implant in a sheep model\textsuperscript{103}. It is unclear from the literature if a rough surface—and at what magnitude—is beneficial to osseointegration of Ti6Al4V implants. Continued research must be completed to validate the shape and size of scaffold structures and the roughness and chemistry of surfaces. This study will focus on determining optimal surface morphology as a means of accelerating and increasing stability of osseointegration.

**Ex vivo \(\mu\)CT**

Osseointegration has been evaluated by medullary bone volume fraction (BV/TV) surrounding the implant and the \% BIC along the entire implant length using \(\mu\)CT in several species including humans\textsuperscript{106,107}, dogs\textsuperscript{108-110} and rats\textsuperscript{111-113}. \(\mu\)CT offers the ability to non-destructively and quantitatively access the trabecular and cortical bone of a specimen allowing for subsequent mechanical testing\textsuperscript{114}. Multiple independent metrics can be acquired through the use of \(\mu\)CT prior to mechanical testing. Studies have shown that the beam artifact is typically concentrated within 45-50 \(\mu\)m of the implant surface requiring an exclusion zone of this region\textsuperscript{110,112}. Previous research has shown a correlation between BIC determined through \(\mu\)CT with an exclusion zone of 5-7 pixels (45-63 \(\mu\)m) and histology\textsuperscript{110}. However, others have shown that there isn’t a correlation between \(\mu\)CT (3 pixel (48 \(\mu\)m) exclusion zone) and histological results\textsuperscript{112}. 
We investigated the possibility of using \( \mu \)CT for determining BIC since there was contradictory results in the literature. All of our specimens were scanned using a \( \mu \)CT (40 model specimen CT, Scanco Medical, Brüttisellen, Switzerland) with a 16mm field of view on medium resolution with a voxel size of 16\( \mu \)m at the UNC Biomedical Research Imaging Center (BRIC). Per BRIC recommendations, the X-ray power setting was 70 kVp, 114 \( \mu \)A, and 8 W; the scans had an integration time of 300 ms and were averaged once; the bone was also kept moist during the scan time through humidifying the X-ray tube with the solution it was soaked in. The resulting \( \mu \)CT scans were analyzed using software developed for processing medical images (Mimics 16.0, Materialise, Plymouth, MI). Threshold values for the scans within our study were held constant. One study found the lower threshold of trabecular bone to be 456 mgHA/cm\(^3\) while the lower bound of cortical bone was 813 mgHA/cm\(^3\)\(^{115}\). Another study determined mineralized bone to be 687-1505 mgHA/cm\(^3\)\(^{116}\). Our threshold values were chosen to optimize the amount of newly mineralized bone. The bone for our study was segmented out using a low and high threshold of 389 and 1615 mgHA/cm\(^3\) respectively. The implants were segmented using a threshold \( \geq 2249 \) mgHA/cm\(^3\) determined through quantitative interpretation of the implant geometry. Based on the prescribed thresholds, the implant was dilated by five pixels (80 \( \mu \)m) to exclude the metal-induced artifact. Our results do not support the use of \( \mu \)CT for determining BIC with implants which produce beam artifact due to the lack of correlation to the well establish histological measurements for BIC.

Vibration has been shown to have an effect on BV/TV and increased levels have been detected at significant levels in a region surrounding the implant out to 500 \( \mu \)m\(^{11}\). The same study also demonstrated that difference in BV/TV could be noted out to 1000 \( \mu \)m. However,
these differences didn’t prove to be significant. Therefore, the region of interest evaluated in similar μCT scans should not exceed 500 μm from the surface of the implant.

**Mechanical Testing**

*Resonance*

Vibration response testing has been utilized in research studies in an attempt determine the axial resonance frequency of structures non-destructively prior to histomorphometric evaluations and other mechanical tests. The resonance frequency is directly proportional to the stiffness of an implant-bone interface\(^ {117}\). However, others have found no statistical difference in resonance frequency while they did find significant differences in other mechanical measures\(^ {63,118}\). There are very few non-destructive methods for evaluating the mechanical stability of an osseointegrated implant. Therefore, it is of great interest to be able to utilize resonance frequency analysis as an additional indication of osseointegration. It is unclear from the literature if resonance frequency analysis is a robust metric for determining mechanical stability of an osseointegrated implant.

**Fluorescence Evaluation**

Fluorochrome labeling is a proven method for investigating bone formation and bone remodeling dynamics. Labeling with calcium binding fluorescent dyes enables one to determine the onset time and location of osteogenesis. Calcein green and alizarin red have both been used successfully to assess osteogenesis. It is recommended to dose rats at a rate of 10mg/kg and 30mg/kg of calcein green and alizarin red, respectively. Calcein dissolves in a 10-20mg/ml solution while alizarin red dissolves in 15-30 mg/ml. They are considered unstable in aqueous solutions; therefore, long-term storage is not appropriate. Using a buffered media with 1.4%
NaHCO\textsubscript{3}, fluorochromes can be dissolved to the correct concentrations. It is important to make sure the final solution has a neutral pH of 7 prior to filter sterilization through a 0.22 µm filter. Administration of fluorochrome labels should either be accomplished by subcutaneous, intraperitoneal or intravenously injection. One study recommends a label interval of 2-3 weeks. However, another study determined that fluorochromes could be discriminated with only a 3 day injection interval\textsuperscript{119}. It is very important not to decalcify the specimens prior to evaluation as this will dissolve the fluorochrome label. The specimens should be formalin-fixed, paraffin-embedded, sectioned and polished to a 75µm thickness for imaging of the fluorochrome labels\textsuperscript{120}. Fluorochrome labels re-emit light when excited at a particular wavelength. Calcein dye excites and emits at 436-495 nm and 517-540 nm respectively and alizarin red dye excites and emits at 530-580 nm and 600-645 nm respectively. Therefore, sectioned specimens can be viewed by fluorescent microscopy to evaluate the portion of labeled mineralizing surface\textsuperscript{121}.

**Histological Staining**

Several researchers have successfully used toluidine blue staining to perform histomorphometric measurements at the bone-implant interface\textsuperscript{62,63,122-126}. These studies, using toluidine blue, investigated the mineralized-bone around the implant to determine the BIC. However, toluidine blue has failed to provide sufficient contrast of mineralized tissue in another study\textsuperscript{127}. Other methods, including methylene blue and basic fuschin\textsuperscript{128}, Stevenel’s blue and Von Gieson’s picrofuchsin red\textsuperscript{11} and toluidine blue and basic fuchsine\textsuperscript{129}, have also been used to differentiate between tissues around the implant. Adding basic fuchsin produces a good contrast between mineralized and non-mineralized tissue. Acid fuchsin has been used in conjunction with methylene blue to produce a vivid contrast between bone and soft tissue\textsuperscript{130}. Finally, a research
group which investigates osseointegration of dental implants regularly uses acid fuchsine in conjunction with basic fuchsine and toluidine blue to develop a vivid contrast tissue\textsuperscript{131}. 
Chapter 2: Preliminary Study—Osseointegration of Additively Manufactured and Threaded Implants in Rats through Whole Body Vibration

Summary:
Transcutaneous osseointegrated prostheses provide stable connections to the skeleton while eliminating skin lesions experienced with socket prosthetics. Additive manufacturing creates custom implants interfacing with amputees’ residual bones. Our objective was to compare osseointegration of textured surface implants made by electron beam melting (EBM), an additive manufacturing process, to machine threaded implants. Whole body vibration was investigated to accelerate osseointegration. Two cohorts of Sprague-Dawley rats received bilateral, titanium implants (EBM vs. threaded) in their tibiae. One cohort comprising five groups vibrated at 45 Hz: 0.0 (control), 0.15, 0.3, 0.6 or 1.2 g was followed for six weeks. Osseointegration was evaluated through torsional testing and bone volume fraction (BV/TV). A second cohort, divided into two groups (control and 0.6 g), was followed for 24 days and evaluated for resonant frequency, bone-implant contact (BIC) and fluorochrome labeling. The EBM implants exhibited significantly improved mechanical stability independent of vibration, highlighting the benefits of using EBM to not only produce custom designs, but also custom surfaces. Bone formation on and around the EBM implants increased compared to machined implants, seen by BIC and fluorescence. No difference in torque, BIC or fluorescence among vibration levels was detected. BV/TV significantly increased at 0.6 g compared to control for both implant types.
Introduction:

In 2005, approximately 1 in 190 Americans were amputees. That ratio is expected to double by 2050\(^1\). Transcutaneous osseointegrated implants hold the potential to overcome several shortcomings of traditional socket prostheses. An optimal fit is difficult to achieve with socket-type prostheses, often resulting in undesirable stresses on tissues most frequently resulting in painful lesions, bursae, inflammatory edema, soft-tissue calcification or neuromas\(^3,25\). Socket prosthetic devices also lack stability due to their inefficient connection to the body.

Osseointegration allows for a direct structural and functional connection between living bone tissue and the surface of a load carrying implant\(^26\). This direct osseointegration interface in amputated limbs allows for a more stable connection enabling greater control of the prosthesis and heightened osseoperception, while eliminating problems associated with socket devices such as pain and skin irritation and improving overall quality of life\(^4\). Transcutaneous osseointegration faces several challenges. Osseointegrated implants need to be adapted to patients’ specific anatomy. The commonly used Brånemark osseointegrated implants, however, are threaded rods that do not always match the anatomy of the femur in which they are inserted. To avoid fibrous ongrowth onto the implant that could result from excessive loading and micromotion, a controlled and gradual rehabilitation period lasting 12 months is required\(^30,132\).

Osseointegration of a metal implant requires some motion at the bone-implant interface for proper healing\(^133\), but excessive micromotion can lead to the development of a fibrous tissue interface rather than a bone interface. Fibrous interfaces are not mechanically stable in the long term. Another challenge facing transdermal osseointegrated implants is that the transcutaneous interface is prone to infection. More than 48% of patients with transcutaneous osseointegrated implants experienced an infection within three years of implantation. Infection at the skin
interface can lead to bone infection. In turn, bone infection may result in implant loosening and removal of the osseointegrated implants\textsuperscript{31,134}. Threaded Brånemark implants are the type of implant that is predominantly used in clinical cases\textsuperscript{19}. These implants are straight and cylindrical by design, limiting their adaptability to the patients’ residual bones. Transdermal implants with a curved and non-cylindrical shape would have a more precise endosteal fit. While conventional manufacturing methods (forging or casting) are used to fabricate curved and non-cylindrical implants for arthroplasty, these methods cannot be realistically used to make the patient-specific transdermal osseointegrated implants. Additive manufacturing (AM) methods can be used to make textured non-cylindrical implants addressing patient specific anatomy and allow for custom surface texture along the implant. Few studies have compared the mechanical stability of threaded implants to textured surface implants; especially implants developed by AM technology\textsuperscript{21}. Implants designed using AM have a similar surface texture as porous implants and may improve mechanical stability compared to standard threaded surface designs\textsuperscript{62}.

Low magnitude high frequency (LMHF) vibration has been shown to enhance bone mass and could prevent bone loss in a residual limb or accelerate bone ingrowth into a prosthesis to shorten the rehabilitation period\textsuperscript{35}. A previous animal study identified improved mechanical stability of osseointegrated implants using LMHF vibration\textsuperscript{122}. The study investigated the effects of LMHF vibration on implant push-out, which is indicative of the ‘property of surrounding bone’\textsuperscript{135}. The study didn’t investigate torsional stability which represents the ‘interface mechanics’, a critical aspect of osseointegrated prostheses stability\textsuperscript{135}. No dose-response study has determined the optimum amplitude of vibration for stimulating bone ongrowth or whether ongrowth is negatively impacted by higher vibration levels.
The goal of this project was to evaluate osseointegration of implants of varying design. It was hypothesized that a textured AM surface profile would yield greater bone ongrowth and torsional stability compared to a threaded implant. A parallel objective in this study was to identify if the mechanical stability of an osseointegrated implant can be improved dependent on the magnitude of LMHF vibration. Our hypothesis was that bone ongrowth and torsional stability would improve through a range of increasing LMHF vibration but would be impaired at higher vibration levels (>1 g).

**Materials and Methods:**

*Phase 1 Animals*

Animal work was approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee and performed in accordance with ARRIVE guidelines. Female retired breeder Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) with a mean age of 24 weeks were used. The rats were housed and cared for by the Division of Laboratory Animal Medicine (DLAM) in individually ventilated cages (Seasafe Plus Rat, Techiplast, Italy). Rats were caged in pairs and given *ad libitum* access to food and water with a 12-hr light/dark cycle (7 a.m. to 7 p.m.) throughout the study.

A power analysis assuming a significance level of 0.05 and power of 0.80 for comparing 5 groups was completed to compute minimum sample sizes. Sample sizes were calculated for each main outcome measure for expected mean differences and standard deviations (SD) between groups relative to the control: 25% improvement in the fraction of medullary bone volume to total volume (BV/TV) (n = 10/group) with SD = 15%\(^{11}\), 40% improvement in max torque (n = 15/group) with SD = 30%\(^{70}\), 50% improvement in % bone-implant-contact (BIC) (n
= 6/group) with SD = 25%\(^\text{11}\). Therefore, 80 rats were used for the five treatment groups (n = 16/group).

**Implant Model**

Implants were made of grade 5 titanium (Ti6Al4V). Titanium rods were threaded to M2-0.4 by machining (Allied Titanium, Inc., Lewes, DE). 2-mm diameter titanium rods were produced using electron beam melting (EBM) (Arcam A2, Arcam, Mölndal, Sweden) using a powder size ranging from 45 to 105 µm. A 1.39 mm flat-to-flat hex was machined on the last 2.86 mm of the 10-mm-long implants for insertion and torque-out testing (Figure 1).

![Image of threaded and EBM osseointegrated implants before surgical implantation.](image)

Implants were ultrasonically cleaned in a 1% Alconox 10 gm/L solution at 65°C for 15 minutes. The implants were rinsed twice with 65°C deionized water for ten minutes under ultrasonic agitation. All implants were textured by acid etching in a 48% sulfuric acid (H\(_2\)SO\(_4\))
bath at 60°C and were agitated with a stir bar for 30 minutes. The implants were rinsed in deionized water, dehydrated in a 70% ethanol solution, and allowed to air dry before packaging for sterilization by autoclave. The surface topography was optically evaluated using a Hirox KH-7700 microscope (Hirox-USA, Inc., River Edge, NJ). Surface roughness (Ra) was obtained through linear regression of the resulting spatial map.

*Treatment*

Seventy-two hours prior to undergoing surgery, the Sprague Dawley rats were given 2.4 mg/ml acetaminophen solution so they become accustomed to its taste. Under isoflurane anesthesia (5% for rapid induction and 2.25% for the duration of surgery), implants were surgically placed bilaterally in the proximal tibiae through a lateral skin incision. The transcortical model was utilized to facilitate comparison to previous studies investigating the effect of vibration on bone ongrowth and to facilitate future studies with transcutaneous implants in the same model. The right limb received the threaded implant while the contralateral limb received the EBM implant. Toe pinch was use to insure proper loss of consciousness prior to initiating the procedure and respiratory rate and quality was monitored during surgery for signs of distress. An electric hot pad insulated by a towel was used to maintain the animals' body temperature while subjected to anesthesia. Aseptic techniques were used to expose the anterior-lateral aspect of the proximal tibia thru a 1.5 ± 0.5 cm skin incision. Blunt dissection was used to separate the soft tissue overlying the implantation site on the tibia. A custom guide was developed to consistently align the implant relative to the anteromedial surface of the tibia and locate the implant relative to the patellar tendon insertion (Figure 2). The guide was designed to accommodate the range of expected tibia sizes. Using the guide, 1.5 mm-diameter pilot holes were drilled under saline
irrigation distal to the insertion of the medial collateral ligament and then either tapped with an M2 tap or drilled to 1.9 mm for the threaded and EBM implants, respectively. Once the implant hole is effectively prepared, a threaded and EBM 2 mm diameter, 10 mm long, titanium, transcortical implant were inserted by manual torque into the drilled hole in the right and left limb respectively. The incisions were closed using wound clips (Autoclips, MikRon Precision, Gardena, CA) and tissue adhesive (TA5, Med Vet International, Mettawa, IL). The clips were removed seven to 14 days after the surgery when radiographs were made. All animals were given a 0.8 mg/kg subcutaneous injection of sustained-release buprenorphine and were given *ad libitum* access to acetaminophen-doped drinking water (2.23 mg/ml) for seven days after surgery for amelioration of postsurgical pain. A 10ml lactated ringer’s subcutaneous injection was also given to each animal following surgery. The animals' body temperature was maintained with a heating pad during the recovery period. The rats were returned to their cages after awakening and monitored closely for signs or symptoms of excess infection or poorly controlled pain. To minimize the risk of infection, 0.15 ml of Ceftriaxone injection was given subcutaneously daily for three days post operatively.
Vibration treatments began 7 days after surgery. The rats were allowed to stand on a four-chamber platform (Figure 3) during their normal light cycle for treatment. The groups subjected to whole body vibration (WBV) were shaken vertically with a 45 Hz sinusoidal acceleration while the control groups were placed in the chamber on an isolated table. The enclosed box was opaque on the top and sides, but the bottom was transparent so the experimenter could observe the status of the animal within. Since the box and the platform are fused, the entire box vibrates with the accelerating platform. The vibrated platform was oscillated with an electromagnetic shaker (Model N-300, Agac-Derritron, Alexandria, VA) driven by a function generator in series with a power amplifier. The static load of the platform was balanced with support springs in each corner to maintain axial displacement of the shaker armature\textsuperscript{136}. The acoustic noise produced at a 2 g acceleration level has been found to be approximately 72 db. Seventy two decibels is only slightly greater than the range of normal human conversation [sixty to seventy decibels].
Although specific data concerning the noise tolerance of adult rats was unavailable, this level of noise is clearly tolerable to humans and there is no evidence that the same noise parameters would be stressful or harmful to the rats. To confirm the rats were being subjected to the desired vibration amplitudes, an accelerometer was secured to the top of the chambers to measure the acceleration as the system was adjusted initially and at the end of the treatment period. Prior to euthanasia, an accelerometer was mounted to both the top of the chambers and to the implant through an incision in order to determine the transmissibility of the vibration.

Figure 3: Vibration table with four enclosed boxes on the accelerating platform.

The animals were divided into a control group (0.0 g) and four groups that underwent vibration amplitudes of 0.15, 0.3, 0.6, and 1.2 g peak acceleration. Mean group weights were matched. Body mass was recorded upon arrival, immediately before surgery, and after euthanasia. The rats were followed for six weeks after surgical placement of implants. Treatment lasted 15 minutes a day, five days a week. Each rat received a unique tail “tattoo” via “Sharpie” markers so that it could be quickly and easily identified from its cage mate and the other rats in the study. The animals were humanely euthanatized 6 weeks after surgery by CO2 overdose for a minimum of eight minutes followed by thoracotomy as prescribed by DLAM. Blood (approximately 3 ml)
was drawn from the heart after and thoracotomy. Blood was used to assay serum prostaglandin E2 levels as one proposed mechanism for vibration's anabolic effects on bone is that fluid-induced shear stresses of vibration cause vascular endothelial cells to increase prostaglandin production. Tibiae were collected, wrapped in saline-soaked gauze and stored at -20°C until testing.

Radiographs

Radiographs were made using a cabinet radiographic unit (HP 43804 X-Ray System Faxitron, Hewlett Packard, Palo Alto, CA) at 7 to 14 days after surgery using dental x-ray film (DX-42, Henry Schein, Melville, NY) at 35 kV with a 12-second aperture exposure. Rats were positioned in the radiograph chamber in a supine position to expose the medial aspect of each limb subsequent to inhalation of isoflurane anesthesia for approximately five minutes. The radiograph film was then developed for 15 seconds in developer, given a five second water rinse, then 45 seconds in fixer, a 15 second water rinse and finally, 30 seconds in free running room temperature water. Radiographs (Figure 4) were used to confirm the implant location and assess the tibia for fractures. The acetaminophen solution was provided for the remainder of the study to animals that incurred a fracture during surgery. A second set of radiographs was made 40 to 44 days after surgery.
Ex vivo µCT

Ten tibia pairs from each group were scanned using a µCT (40 model specimen CT, Scanco Medical, Brüttisellen, Switzerland) with a 16 mm field of view on medium resolution with a voxel size of 16 µm. The X-ray power setting was 70 kVp, 114 µA, and 8 W. The scans had an integration time of 300 ms and were averaged once. Specimens were soaked in a 1:100 dilution of protease inhibitor cocktail (Sigma-Aldrich #P8340, St. Louis, MO) with saline prior to µCT. The distal end of the tibia of each specimen was removed using a fine toothed rotary bone saw to an overall length of 14 mm, which included the condyles and cartilage. Shortening of the specimens was necessary for them to fit in the µCT, 0.13 mm beryllium coated, X-ray tube. The resulting µCT scans (Figure 5) were analyzed using software developed for processing medical images (Mimics 16.0, Materialise, Plymouth, MI). The implant was dilated by five pixels (80 µm) to exclude the metal-induced artifact as determined by a prior study\textsuperscript{112}. The bone was segmented out using a low and high threshold of 389 and 1615 mgHA/cm\textsuperscript{3}, respectively. The implants were segmented using a threshold \( \geq 2249 \) mgHA/cm\textsuperscript{3}. The total area of the implant was obtained and divided by its length. This area per unit length was used later for normalizing the torque results.
Figure 5: Typical ex-vivo µCT scan of specimen with EBM implant.

**BV/TV**

The BV/TV within 500 μm of the implant was calculated. A cylinder was constructed within the cortices (medullary cavity) that had a diameter 1,000 μm larger than the mean diameter of the implant. The volume of bone within this region was divided by the volume of the cylinder minus the dilated implant volume within the cylinder. The resulting percentage represented the medullary bone volume fraction.
BIC area

The BIC was calculated along the length of the implant bound by the cortices as well as within the medullary canal. Using the dilated implant, the region was dilated again one pixel to define a total region of interest. The number of pixels defined as bone within the region of interest was obtained and divided by the total number of pixels within that region to obtain the percent BIC.

Mechanical Testing

Torque

Torsional testing on all specimens was performed to assess osseointegration by evaluating the stiffness and torque of the bone-implant interface. Specimens were potted in a custom tapered mold using a polymer resin (number 265, 3M Bondo Corp., Atlanta, GA) and allowed to cure for 20 minutes while in a fixture to insure proper orientation. The potted specimens were kept hydrated by covering them with saline soaked gauze. Mechanical testing was carried out with a material testing system (MTS) (8500 Plus, Instron Corp., Norwood, MA). The uniaxial servohydraulic motion of the MTS was transferred to a rotary motion through a custom fixture with a chain and sprocket. Angular rotation was measured using a potentiometer (Series P2201, Novotechnik U.S., Southborough, MA) and torque was measured with a 350 N•mm torque cell (Model 2105-50, Honeywell Sensotec, Columbus, OH). The hexagonal head of the implant was secured into the jaws of a Jacob’s chuck attached to the torque cell and then slid along a linear slide so that the potted tibia was captured by a receptacle on the rotary sprocket similar to the tapered mold (Figure 6). Maximum torque and stiffness were shown to be independent of angular velocity in the range of 3–12 °/s angular velocity in prior research. Specimens were preloaded with 3 N•mm and torqued at a constant rate of 6 °/s until failure of the bone-implant
interface was reached or rotation exceeded 25°. Rotations greater than 25° without reaching a peak torque were considered as fibrous encapsulation. Maximum torque and stiffness were determined from the resulting torque and rotational displacement data using a program developed using Labview (Labview 6.0, National Instruments, Austin, TX). Stiffness was determined by the slope of the regression line of the torque–deflection angle curve between 25 and 75% of the maximum torque. The maximum torque was normalized by dividing the resulting value by the product of the implant’s surface area in the tibia and the mean radius of the implant. This normalization was done to calculate the equivalent shear stress at the bone-implant interface.

Figure 6: Torque load cell, implant clamp and receptacle similar to the tapered mold on the rotary sprocket.
Resonance

Vibration response testing was used to determine the axial resonance frequency of all of the implants non-destructively before torsional or histological testing (Figure 7). A broadband random excitation signal (0-8,000 Hz) was generated by an electromagnetic minishaker (Brüel & Kjær, Nærum, Denmark) and charge amplifier through a piezoelectric impedance head transducer. The signal was applied to the head of the implant in the tibia through a suspended balance beam-type reaction mass system. The driving head of the impedance head, which sensed the dynamic transmitted force and acceleration at the contact point, was preloaded with 3.13 N (319 gm) to maintain system stability. The specimens, having been potted for subsequent torsional testing, were clamped into a jig with M2 screws and washers and positioned to receive an axial excitement to the implant. The acceleration and force signals were processed by a dual-channel fast Fourier transform analyzer similar to past work in order to identify the resonant frequency of the system. 139.
Figure 7: Tibia-clamp on base plate with electromagnetic minishaker for non-destructive resonance vibration testing of the second cohort specimens.

Statistical Analysis

A two-way repeated measures analysis of variance with a one-factor repetition (Implant type) was performed using a statistical analysis program (SigmaPlot v11.0, Systat Software, San Jose, CA) with Holm-Sidak posthoc mean comparison testing for all outcome measures.
Phase 2 Animals

A second cohort of animals was followed for 3 weeks using the same protocol as the first cohort to collect early osseointegration information on the vibration group that demonstrated the greatest mean stiffness after preliminary analysis of the data. Based on power analysis, 30 rats (n = 15/group) were used in the second cohort of animals that included a control group (0.0 g) and a vibration group (0.6 g) with matched mean weights. Manufacturing and implant preparation identical to the first cohort were used. Due to a limited supply of threaded implants, half of the vibrated animals in the second cohort received an EBM implant in both limbs. The second cohort also received identical surgeries and treatment. However, only one radiograph was made at the early time point due to the shorter study duration. Specimens were soaked in 70% ethanol in preparation for histology instead of the 1:100 protease inhibitor cocktail prior to µCT. Non-destructive vibration response testing was performed by securing the specimens to a steel plate in a modified spring clamp, which captured the proximal end of the tibia approximately 1mm from the implant. The statistical analyses used to compare the results of the second cohort were identical to the first cohort.

Histology

*In vivo* double fluorochrome labeling was administered to the second cohort of animals. Subcutaneous injections of 10 mg/kg calcein and 30 mg/kg alizarin red (Sigma-Aldrich, St. Louis, MO) were given 14 and 21 days after surgery, respectively. Ten control and seven vibrated pairs of specimens where randomly selected for sectioning. The proximal 14 mm of each tibia was 70% ethanol-fixed, paraffin-embedded, sectioned and polished to a 75 µm thickness for imaging of the fluorochrome labels. The plane of the section was defined by the
axis of the implant and the longitudinal axis of the tibia. The longitudinal sections of the implant-bone interface were initially viewed by fluorescent microscopy to evaluate the portion of labeled surface. Labeled surface was calculated as the percent area fluorescing between the cortical walls and within 250 µm of the implant.

Multiple images of the implant-bone interface were acquired using an upright dissecting microscope (Leica DM RSXA2) with a diffraction-limited objective (0.63X PLAN APO Leica, model 10447051) set to a magnification of 20 with appropriate excitation/emission filters. The resulting pixel size of the images was 5µm per pixel. A green fluorescent protein filter (GFP3) was used to excite the Calcein dye (excites and emits around 495 nm and 515 nm respectively). A red fluorescent protein filter (DSR) was used to excite the Alizarin Red dye (excites and emits around 530/560 nm and 580 nm respectively). The exposure of the GFP3 and DSR filters was set to 0.030098 sec and 0.300098 sec respectively through Simple PCI software both with a gain of 255. Images were spliced to form a composite image. The composite image was separated into red and green images and segmented at a threshold of 30 using ImageJ (1.47v). The percent areas of each fluorescent label of the segmented image were measured in a region defined by the intersection of the medullary canal and a cylinder with a diameter 500 µm larger than the mean implant diameter using modeling software (Mimics v16.0, Materialise, Plymouth, MI).

The longitudinal sections were then polished to a 50 µm-thickness and stained with toluidine blue for 20 minutes followed by basic fuchsin for 4 minutes\textsuperscript{129}. Due to insufficient collagen staining; the sections were additionally stained with acid fuchsin for 4 minutes\textsuperscript{140}. The sections were imaged through a 40x lens on the Leica DMIL with a Q-imaging Micropublisher 3.3RTV digital camera.
Multiple images were collected to capture the entire bone-implant region. The images were merged and analyzed using imaging software (ImageJ, NIH, USA). A “freehand” line was traced for the perimeter of the implant within the cortical walls using a digitizing tablet (CTH680, WACOM, Vancouver, WA). “Freehand” lines were also traced of the implant perimeter where stained bone was in contact. The lengths of the bone-implant interface perimeters were summed and divided by the total perimeter length of the implant to calculate the % bone-implant contact area.

Results:

Mechanical Testing

The EBM implants (331 ±40 N•mm) resisted twice as much torque (200%, P < 0.001, Figure 8) as threaded implants (152 ±42 N•mm) regardless of vibration level. Normalized torque data showed that the EBM design supported twice the equivalent shear stress at the bone-implant interface than the threaded design (2.2 ±0.3 N/mm² and 1.1 ±0.3 N/mm², Table 1). Also, the bone-implant interfaces of EBM implants (33 ±8 N•mm/deg) were 49% stiffer (P < 0.001) than the interfaces of threaded implants (23 ±8 N•mm/deg) for all treatment groups. All of the threaded implants failed at the bone-implant interface while the bone-implant interface of nine of the EBM implants exceeded the strength of the bone. The bone-implant interfaces of EBM implants demonstrated an upward trend in stiffness for increased vibration levels (Figure 9). These effects occur despite the fact that the average surface area of threaded implants was slightly greater than that of EBM implants (8.89 mm²/mm vs. 8.33 mm²/mm, Δ6.7%) based on µCT measurements which included the micro-undulations. The mean torsional stiffness in the vibrated groups was slightly larger (+14%) than the control group, though this was not statistically significant. Maximum torque did not differ statistically among vibration amplitudes.
Figure 8: Box plot comparison of maximum torque between threaded implant (●) and EBM implant (▲) as a function of vibration amplitude. Maximum removal torques of EBM implants were 200% greater than threaded implants (P < 0.001).

Figure 9: Box plot comparison of torsional stiffness between threaded implant (●) and EBM implant (▲) as a function of vibration amplitude. The bone-implant interfaces of EBM implants were 49% stiffer than threaded implants (P < 0.001).
Table 1: Resulting maximum torque to remove implant, normalized torque as equivalent shear, stiffness of bone-implant interface, axial resonant frequency of the implants and the axial resonant frequency of the implants (Data are in mean ± SD; Different lowercase letters within a column indicates significant difference. Different uppercase letters within a row indicates significant difference; ‘Combine groups’ includes the ‘Control’, ‘0.15 g’, ‘0.3 g’, ‘0.6 g’ and ‘1.2 g vibration’ groups.).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.15 g vibration</th>
<th>0.3 g vibration</th>
<th>0.6 g vibration</th>
<th>1.2 g vibration</th>
<th>Combine groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maximum Torque (N*mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Threaded</td>
<td>173.7 ± 38.5a</td>
<td>153.2 ± 37.7a</td>
<td>151.7 ± 60.8a</td>
<td>142.8 ± 35.9a</td>
<td>164.1 ± 36.1a</td>
<td>157.3 ± 42.5a</td>
</tr>
<tr>
<td>EBM</td>
<td>332.2 ± 36.4b</td>
<td>335.9 ± 46.5b</td>
<td>336.8 ± 38.3b</td>
<td>318.8 ± 38.8b</td>
<td>330.7 ± 41.9b</td>
<td>330.5 ± 39.6b</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>253.0 ± 88.8</td>
<td>248.6 ± 102.1</td>
<td>244.2 ± 106.8</td>
<td>230.8 ± 96.8</td>
<td>238.1 ± 92.5</td>
<td>242.6 ± 96.1</td>
</tr>
<tr>
<td><strong>Normalized Torque (N/mm²)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Threaded</td>
<td>1.18 ± 0.21a</td>
<td>1.08 ± 0.28a</td>
<td>1.10 ± 0.45a</td>
<td>0.98 ± 0.24a</td>
<td>1.13 ± 0.30a</td>
<td>1.09 ± 0.30a</td>
</tr>
<tr>
<td>EBM</td>
<td>2.28 ± 0.25b</td>
<td>2.26 ± 0.25b</td>
<td>2.29 ± 0.39b</td>
<td>2.13 ± 0.32b</td>
<td>2.19 ± 0.26b</td>
<td>2.23 ± 0.30b</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>1.73 ± 0.61</td>
<td>1.69 ± 0.66</td>
<td>1.70 ± 0.74</td>
<td>1.56 ± 0.64</td>
<td>1.60 ± 0.60</td>
<td>1.65 ± 0.64</td>
</tr>
<tr>
<td><strong>Stiffness (N*mm/deg)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threaded</td>
<td>22.3 ± 9.5a</td>
<td>24.1 ± 7.7a</td>
<td>23.0 ± 8.6a</td>
<td>23.1 ± 9.9a</td>
<td>24.1 ± 7.6a</td>
<td>23.3 ± 8.5a</td>
</tr>
<tr>
<td>EBM</td>
<td>30.4 ± 8.2b</td>
<td>32.8 ± 9.4b</td>
<td>32.6 ± 9.0b</td>
<td>35.4 ± 7.2b</td>
<td>35.7 ± 7.5b</td>
<td>33.4 ± 8.2b</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>26.3 ± 9.6</td>
<td>28.6 ± 9.5</td>
<td>27.8 ± 9.9</td>
<td>29.3 ± 10.6</td>
<td>29.3 ± 9.5</td>
<td>28.3 ± 9.7</td>
</tr>
<tr>
<td><strong>Resonance frequency squared (KHz²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threaded</td>
<td>2.11 ± 0.41</td>
<td>2.01 ± 0.41</td>
<td>2.09 ± 0.33</td>
<td>2.17 ± 0.51</td>
<td>2.09 ± 0.39</td>
<td>2.09 ± 0.39</td>
</tr>
<tr>
<td>EBM</td>
<td>1.85 ± 0.34</td>
<td>2.17 ± 0.75</td>
<td>1.99 ± 0.58</td>
<td>1.95 ± 0.28</td>
<td>1.93 ± 0.38</td>
<td>1.98 ± 0.49</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>1.98 ± 0.38</td>
<td>2.09 ± 0.59</td>
<td>2.04 ± 0.45</td>
<td>2.06 ± 0.41</td>
<td>2.01 ± 0.38</td>
<td>2.04 ± 0.44</td>
</tr>
</tbody>
</table>
Resonance frequency testing from the second cohort revealed an increased axial resonance frequency of the EBM implants relative to the threaded implants for all cases (0.845 KHz\(^2\) and 0.767 KHz\(^2\) respectively). Resonance frequency was significantly greater (P = 0.016) regardless of vibration amplitude, showing a main effect between implant types (Table 2) not differ statistically among vibration amplitudes. No difference in treatment group or implant type was detected in the resonance frequency testing of the first cohort (Table 1).

Table 2: Axial resonant frequency of the implants (Data are in mean ± SD, ‘**’ indicates a significant increase versus 'Threaded'). ‘Combine groups’ includes both the ‘Control’ and ‘0.6 g vibration’ groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.6 g vibration</th>
<th>Combine groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resonance frequency squared (KHz(^2))</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threaded</td>
<td>0.769 ± 0.098</td>
<td>0.763 ± 0.085</td>
<td>0.767 ± 0.091</td>
</tr>
<tr>
<td>EBM</td>
<td>0.814 ± 0.077</td>
<td>0.866 ± 0.091*</td>
<td>0.845 ± 0.088*</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>0.790 ± 0.090</td>
<td>0.836 ± 0.099</td>
<td>0.813 ± 0.096</td>
</tr>
</tbody>
</table>

*Ex vivo µCT*

BV/TV was increased after 0.6 g vibration relative to controls for both implant types (61.5% and 47.7%, P < 0.001, Figure 10) and after 1.2 g for EBM implants (59.0% and 44.3%, P = 0.006). There was no significant difference between the 0.15 g or 0.3 g treatment and the control. The BV/TV results from the reduced ongrowth period of the second cohort failed to demonstrate a significant difference between either vibration group or implant type (Table 3).
Figure 10: Box plot comparison of BV/TV fraction from *ex vivo* μCT data between threaded implant ( ), EBM implant ( ) and all specimens ( ) as a function of vibration amplitude. ‘*’ indicates a significant difference between groups connected by .
Table 3: Resulting percent area of \( \mu \)CT BV/TV in the medullary canal within 500 \( \mu \)m of the implant and percentage of \( \mu \)CT BIC relative to the available implant length between the cortices (Data are in mean ± SD, ‘*’ indicates a significant increase versus 'Threaded'). ‘Combine groups’ includes both the ‘Control’ and ‘0.6 g vibration’ groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.6 g vibration</th>
<th>Combine groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>( \mu )CT BIC %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Threaded</em></td>
<td>70.8 ± 11.3%</td>
<td>70.1 ± 4.7%</td>
<td>70.5 ± 8.0%</td>
</tr>
<tr>
<td><em>EBM</em></td>
<td>69.5 ± 12.2%</td>
<td>63.5 ± 5.2%</td>
<td>66.2 ± 8.9%</td>
</tr>
<tr>
<td><em>Both (Imp)</em></td>
<td>70.2 ± 10.9%</td>
<td>66.4 ± 5.9%</td>
<td>68.2 ± 8.5%</td>
</tr>
<tr>
<td><strong>( \mu )CT BV/TV %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Threaded</em></td>
<td>44.4 ± 11.5%</td>
<td>44.3 ± 3.4%</td>
<td>44.5 ± 7.9%</td>
</tr>
<tr>
<td><em>EBM</em></td>
<td>47.0 ± 12.0%</td>
<td>41.1 ± 3.9%</td>
<td>43.8 ± 8.5%</td>
</tr>
<tr>
<td><em>Both (Imp)</em></td>
<td>45.7 ± 11.0%</td>
<td>42.5 ± 3.8%</td>
<td>44.0 ± 7.9%</td>
</tr>
</tbody>
</table>

The x-rays from \( \mu \)CT can’t penetrate the titanium implant resulting in a large amount of beam artifact concentrated around the implant. The beam artifact yields higher densities in the voxels near the implant than their true densities. These voxels near the implant surface are unreliable as to their actual density and were excluded from the analysis by dilating the implant. The analysis of the BIC offset 80 \( \mu \)m from the implant surface detected a significant increase for the threaded implants of both the control and combine groups in the first cohort (Table 4). The second cohort demonstrated no differences in \( \mu \)CT BIC between the implant types or the treatment groups (Table 3). Also, there was no correlation between the histology BIC results and the \( \mu \)CT results in the second cohort.
Table 4: Resulting bone volume fraction in the medullary canal within 500 µm of the implant and percentage of BIC relative to the available implant length between the cortices (Data are in mean ± SD; Different lowercase letters within a column indicates significant difference. Different uppercase letters within a row indicates significant difference; ‘Combine groups’ includes the ‘Control’, ‘0.15 g’, ‘0.3 g’, ‘0.6 g’ and ‘1.2 g vibration’ groups.).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.15 g vibration</th>
<th>0.3 g vibration</th>
<th>0.6 g vibration</th>
<th>1.2 g vibration</th>
<th>Combine groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>µCT BIC %</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Threaded</td>
<td>73.6 ± 9.8%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.9 ± 5.6%</td>
<td>74.1 ± 8.2%</td>
<td>75.6 ± 10.5%</td>
<td>73.4 ± 10.0%</td>
<td>74.5 ± 7.6%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EBM</td>
<td>71.2 ± 9.2%&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.4 ± 7.9%</td>
<td>70.7 ± 9.8%</td>
<td>78.8 ± 8.3%</td>
<td>73.8 ± 10.2%</td>
<td>72.2 ± 10.6%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>70.0 ± 9.8%</td>
<td>73.8 ± 8.4%</td>
<td>70.7 ± 7.8%</td>
<td>78.0 ± 7.9%</td>
<td>74.0 ± 10.5%</td>
<td>73.3 ± 9.2%</td>
</tr>
</tbody>
</table>

| **µCT BV/TV %** |         |                   |                 |                 |                 |                |
| Threaded       | 51.1 ± 7.8%<sup>a</sup> | 56.4 ± 11.3%     | 50.9 ± 10.8%    | 60.9 ± 13.2%    | 53.5 ± 12.1%<sup>a</sup> | 54.6 ± 11.4%   |
| EBM            | 44.3 ± 11.6%<sup>b,A</sup> | 55.5 ± 11.9%     | 50.1 ± 10.4%    | 62.0 ± 10.7%<sup>H</sup> | 59.0 ± 13.2%<sup>b,H</sup> | 54.2 ± 12.8%   |
| Both (Imp)     | 47.7 ± 10.2%<sup>A</sup> | 55.9 ± 11.3%     | 50.5 ± 10.3%<sup>A</sup> | 61.5 ± 11.7%<sup>B</sup> | 56.3 ± 12.6% | 54.4 ± 12.1%   |
Histology

BIC, calculated using histomorphometric analysis, was increased in EBM implants relative to threaded implants regardless of the vibration levels (47.6% and 36.8% respectively, \( P = 0.011 \), Table 5). Fluorochrome labeling (Figure 11) also showed more bone remodeling around EBM implants than threaded implants at 14 days for all treatment groups regardless of vibration treatment (33.4% and 24.9% respectively, \( P = 0.029 \), Table 6). At 21 days, EBM implants exhibited more bone formation numerically but not statistically (68.9% and 60.5%, respectively). BIC and fluorescence did not differ statistically among vibration amplitudes. Differences were not detected between implant type nor vibration group in the BV/TV histomorphometric analysis.

Table 5: Resulting percentage of BIC relative to the available implant length between the cortices and BV/TV in the medullary canal within 500 µm of the implant (Data are in mean ± SD, ‘*’ indicates a significant increase versus 'Threaded'). ‘Combine groups’ includes both the ‘Control’ and ‘0.6 g vibration’ groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.6 g vibration</th>
<th>Combine groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histology BV/TV (%)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Threaded</td>
<td>38.2 ± 6.6%</td>
<td>34.8 ± 12.3%</td>
<td>36.7 ± 8.7%</td>
</tr>
<tr>
<td>EBM</td>
<td>42.6 ± 13.0%</td>
<td>31.3 ± 10.0%</td>
<td>37.2 ± 12.7%</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>41.2 ± 11.3%</td>
<td>32.2 ± 10.2%</td>
<td>37.1 ± 11.5%</td>
</tr>
<tr>
<td><strong>Histology BIC %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threaded</td>
<td>36.7 ± 4.7%</td>
<td>37.2 ± 29.4%</td>
<td>36.8 ± 15.2%</td>
</tr>
<tr>
<td>EBM</td>
<td>44.2 ± 11.9%</td>
<td>52.4 ± 6.0%</td>
<td>47.6 ± 10.5%*</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>41.4 ± 10.3%</td>
<td>47.8 ± 16.4%</td>
<td>43.8 ± 13.1%</td>
</tr>
</tbody>
</table>
Figure 11: Typical double fluorochrome labeling of specimen with EBM implant (scale bar = 1mm).

Table 6: Resulting percent area of fluorescence in the medullary canal within 500 µm of the implant (Data are in mean ± SD, ‘*’ indicates a significant increase versus 'Threaded'). ‘Combine groups’ includes both the ‘Control’ and ‘0.6 g vibration’ groups.

<table>
<thead>
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<th>Combine groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcein fluorescence (%)</strong></td>
<td></td>
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</tr>
<tr>
<td>Threaded</td>
<td>24.1 ± 12.2%</td>
<td>26.3 ± 12.9%</td>
<td>24.9 ± 11.9%</td>
</tr>
<tr>
<td>EBM</td>
<td>33.9 ± 7.8%*</td>
<td>32.8 ± 5.8%</td>
<td>33.4 ± 6.8%*</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>29.8 ± 10.8%</td>
<td>30.8 ± 8.6%</td>
<td>30.2 ± 9.8%</td>
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<tr>
<td><strong>Alizarin red fluorescence (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threaded</td>
<td>57.5 ± 13.5%</td>
<td>66.5 ± 11.2%</td>
<td>60.5 ± 13.0%</td>
</tr>
<tr>
<td>EBM</td>
<td>70.3 ± 8.4%</td>
<td>67.2 ± 7.3%</td>
<td>68.9 ± 7.9%</td>
</tr>
<tr>
<td>Both (Imp)</td>
<td>64.9 ± 12.4%</td>
<td>67.0 ± 8.2%</td>
<td>65.8 ± 10.8%</td>
</tr>
</tbody>
</table>
The original staining protocol of toluidine blue and basic fuchsine for the specimens of the second cohort resulted in insufficient staining of the calcified bone (Figure 12). The extended toluidine blue staining saturated the mastocytes in the bone-marrow and resulted in precipitate accumulation near the implant on some specimens. These specimens were excluded from statistical analysis resulting in an N = 9 for the EBM control, N = 8 for the EBM vibrated group, N = 4 for the threaded control and N = 3 for the threaded vibrated group. Re-staining each specimen with the acid fuchsine yielded a vivid contrast between the woven and nonwoven bone allowing for proper analysis (Figure 13).

Figure 12: Typical specimen with EBM implant stained with toluidine blue and basic fuchsine only.
Implants, Animals and Treatments

All of the implants were acid etched to achieve a rough surface texture which has been shown to improve osseointegration\textsuperscript{70,71}. The acid etching process resulted in a loss of 4.4\% of the total mass of the EBM implants and 2.7\% of the mass of the threaded implant. Roughened surfaces were achieved with all implants used in the final surgeries (Figure 14). The threaded implants had a mean Ra value of 62 ±4 µm including global changes due to the thread profile while the EBM design was only 33 ±11 µm. The finished implants, prior to surgical insertion, had mean diameters with a standard deviation of 1.70 ±0.01 mm and 1.86 ±0.01 mm for the threaded and EBM implants respectively. Due to a limited supply of threaded implants, half of the vibrated animals in the second cohort received an EBM implant in both limbs.
Animals in the first cohort had a mean ±SD arrival weight of 313 ±52 gm. The mean group weight was 322 ±8 gm immediately after surgery. The second cohort had an average arrival weight of 362 ±28 gm and mean group weight of 359 ±4 gm post-surgery. There was a mean increase in body weight of 12.8 gm within the first cohort, while the second cohort lost a mean body weight of 10 gm per animal. Vibration had no effect on the change in body weight for either cohort.

Upon initiation of the surgeries it was noticed that a 3% isoflurane ratio resulted in labored breathing of the first rat. The mixture was reduced to 2.25% for the remainder of the surgery and subsequent surgeries. The implants were placed with the custom drill guide distal and cranial to the medial collateral ligament (Figure 15). During the one hour ±20 minute surgery of the first cohort, the tibia was completely fractured on three animals resulting in their immediate euthanasia. Four weeks into the study, an animal developed an infection which resulted in its euthanasia. Eight limbs were also partially fractured on other rats that survived the surgery and didn’t exhibit signs of distress. Data from fractured limbs were excluded from the analysis, reducing the control, 0.15 and 0.3 g groups to N = 13 while leaving the 0.6 and 1.2 g
groups at N = 16. No animal of the second cohort was lost during surgery. Two animals were euthanized during the treatment due to uncontrollable hematomas (N = 1) or inability to bear weight (N = 1). Four limbs sustained partial fractures which resulted in their exclusion from analysis reducing the size of the control and 0.6 g groups to N = 13 and 12, respectively. The rats in their individually ventilated cages (Seasafe Plus Rat, Techiplast, Italy) responded adversely to the original corn-cob bedding secondary to a pica response as result of the sustained-release buprenorphine administered. They were placed on wipes made of wood pulp and binder (White Wypall L40, Kimberly-Clark Corporation, Lexington, NC) for five days post-surgery to prevent impaction of their stomach through ingestion of the corn-cob bedding after which time they were returned to the original bedding.

Figure 15: Typical location of implant in tibia.
Discussion:
The textured surface of the EBM implants led to improved torsional and axial resonant frequency properties relative to threaded implants. The improved torsional properties did not result from an increase in bone contact area, since threaded implants had a slightly higher surface area than EBM implants. Instead, the different properties may be a reflection of how the surface profile interacts with the ongrown bone under torsional loading (Figure 16). Interstices in the EBM design provide more surface area normal to the torsional shear force at the bone-implant interface compared to the threaded design. The larger interspersed area normal to the load likely provides a superior interlocking mechanism between the bone and implant for the EBM design.

Figure 16: Images of specimens post torsional testing showing bone-implant interface. (scale bars = 500 µm). a. threaded implant in tibia, b. enlarged view of boxed section in ‘a’, c. EBM implant in tibia, d. enlarged view of boxed section in ‘c’.
Vibration response testing was used to determine the axial resonance frequency of all implants in the second cohort non-destructively before histology. The resonance frequency is directly proportional to the stiffness of an implant-bone interface. The EBM implants had a stiffer connection to the bone independent of vibration, demonstrating further mechanical advantage compared to the standard threaded implants. This improved mechanical stability highlights the benefits of using EBM not only to produce custom designs, but also custom surfaces for osseointegration. The EBM implants also demonstrated increased early bone formation around the implant. However, it is not clear from this study which mechanism is responsible for the increased bone growth around the textured surface implants. Nevertheless, our findings further support the idea that EBM implants are superior to machined threaded implants for osseointegration. Threaded implants can be fabricated through EBM and have also been shown to osseointegrate more securely than machine threaded implants.

Studies show that altering the surface of an implant can improve its mechanical stability, thereby increasing the required torque to remove the implant. Medium-particle (75 µm) blasting results in improved torque resistance compared to fine (25 µm) and coarse (250 µm) particle blasting, indicating that a moderate surface texture results in better mechanical stability. AM manufactured osseointegrated implants inherently have a moderate surface texture with a roughness of 35 ±12 µm and can be manufactured to match an infinite range of residual bone geometries making them a logical consideration for osseointegrated implants.

This study also revealed that BV/TV around osseointegrated implants is influenced by 45 Hz vibration amplitude and that bone formation was maximal at 0.6 g. Several studies have demonstrated the ability of LMHF ground-based vibration to increase bone mass or volume in normal animals and increase bending strength and bone formation in animal models.
Several studies have investigated various treatment frequencies\textsuperscript{7,8}. However, the optimal vibration amplitude that would accelerate bone ongrowth without inducing fibrous encapsulation (as a result of excessive implant micromotion) has not been previously identified. This study clearly demonstrates that, at 45 Hz, 0.6 g was the most effective amplitude of WBV to improve BV/TV fraction. Contrary to our hypothesis, BV/TV was not impaired at the highest vibration amplitude of 1.2 g. In contrast to the improved BV/TV, no significant differences in torque, resonance frequency, BIC or fluorescence relative to vibration amplitude were present. While past vibration studies have reported increased bone ingrowth into porous coated titanium alloy implants in transcortical turkey and intramedullary rat models\textsuperscript{46,122}, our transcortical rat model did not produce any significant improvements or impairments in bone ongrowth with LMHF vibration at early or late time points.

Previous osseointegration studies investigating LMHF vibration have shown promise in promoting early ingrowth but have failed to determine if this improvement translates to enhanced mechanical stability\textsuperscript{10,11}. This study demonstrates that while BV/TV around an osseointegrated implant may be improved; there is no significant improvement or impairment in mechanical stability of the implant through LMHF vibration.

Previous research has shown a correlation between BIC determine through μCT and histology\textsuperscript{110}. However, others have shown that there isn’t a correlation between μCT and histological results\textsuperscript{112}. The possibility of using μCT for determining BIC was investigated since there was contradictory results in the literature. Our results also do not support the use of μCT for determining BIC with implants which produce beam artifact due to the lack of correlation to well established histological measurements for BIC.
Our study had limitations. While a transverse implantation model was utilized to facilitate comparison to past studies and progression to a transcutaneous osseointegration model, some have questioned its relevance to intramedullary implants because of the potential of a periosteal contribution. However, periosteal responses may be important to the use of collared implant designs intended to maintain distal loading of the residual bone\textsuperscript{141}. Furthermore, the transverse tibial model in some aspects mimics the placement of the proximal part of the tibial component of a total knee replacement as it crosses the cortices and cancellous bone bed. The transverse model displayed initial stability and that stability may have limited the ability to detect the positive effects of vibration on bone ongrowth. Future studies aiming to investigate mechanical properties of osseointegration could evaluate implants that penetrate a single cortex or that are placed in the medullary canal. Reducing the region of intracortical contact around the implant may enhance the ability to detect improvements resulting from vibration.

Another limitation of this study is that a larger variation than anticipated in our stiffness evaluation may have prevented us from detecting a trend for an effect of vibration.

**Conclusion:**

The osteological aspects of the physiology of rats are similar enough to those of humans that interventions found to be effective in rat osseointegration are often shown to be effective in humans. This study has demonstrated that “as built” textured surfaces produced by EBM can osseointegrate to provide enhanced mechanical stability compared to machine threaded implants making it a valid option for the fabrication of customized osseointegrated implants in humans and companion animals. Also, LMHF WBV increases BV/TV around osseointegrated implants more effectively at a specific amplitude of (0.6 g). While vibration increases bone density, it
does not improve bone-implant integration in this transverse implantation model. The increase in BV/TV around the implant as a result of an optimized LMHF vibration amplitude may aid in preventing bone resorption during the rehabilitation period while the limb is not yet loaded.

**Acknowledgements:**
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Summary:
Osseointegrated implants transfer loads from native bone to a synthetic joint and can also function transdermally to provide a stable connection between the skeleton and the prostheses eliminating many problems associated with socket prostheses. Additive manufacturing provides a cost effective means to create patient specific implants and allows for customized textures for integration with bone and other tissues. Our objective was to compare the osseointegration strength of two primary additive manufacturing methods of producing textured implants: electron beam melting (EBM) (mean Ra = 23 µm) and direct metal laser sintering (DMLS) (mean Ra = 10 µm). Due to spatial resolution, DMLS can produce surfaces with a roughness comparable to EBM. Two cohorts of Sprague-Dawley rats received bilateral, titanium implants in their distal femurs and were followed for four weeks. The first cohort animals received EBM implants transcortically in one femur and a DMLS implant in the contralateral femur. The second cohort received DMLS implants (either fine textured or coarse textured to mimic EBM) in the intramedullary canal of each femur. Osseointegration was evaluated through mechanical testing and µCT (bone volume fraction [BV/TV] and bone-implant contact [BIC]). The fixation strength of coarse textured implants provided superior interlocking relative to fine textured implants without affecting BV/TV or BIC in both cohorts. Coarse EBM implants in a transcortical model demonstrated an 85% increase in removal torque relative to the fine DMLS textured implants. The thrust load in the intramedullary model saw a 35% increase from fine to coarse DMLS implants.
**Introduction:**

By 2050, the number of amputees in America is expected to double from approximately 1 in 190 in 2005\(^1\). Direct transcutaneous osseointegrated prostheses are an emerging alternative to traditional socket prostheses that offers a stable connection and the elimination of dermal lesions caused by the socket-skin interface. Osseointegrated implants also transfer loads from the residual native bone to a synthetic joint and back to the opposing bone in total joint replacements. In 2010, an estimated 4.7 million individuals living in the U.S. had a total knee implant and 2.5 million had a total hip implant\(^{142}\). The rate of new total knee and total hip arthroplasties in the U.S. are expected to increase to approximately 3.48 million and 572,000 per year respectively by 2030\(^{143}\). Additively manufactured (AM) implants provide a cost effective means to customize the shape of the implant to interface with a patient’s unique bone morphology and allow for the customization of the surface texture, which integrates directly with the bone and other tissues.

Electron beam melting (EBM) and direct metal laser sintering (DMLS) are the two primary methods for producing AM titanium implants for osseointegration. There have been several studies verifying the biocompatibility of titanium fabricated by these two methods\(^{144-146}\). However, there have been few studies comparing DMLS to EBM for osseointegration\(^99\). The objective of this study is to compare the osseointegration strength of two AM methods of producing textured implants: “as-built” coarse textured (mean ±SD: Ra = 23 ±2.9 µm) implants made by EBM and “as-built” fine textured (Ra = 10 ±0.3 µm) and coarse textured (Ra = 23.1 ±5.0 µm) implants made by DMLS.
Materials and Methods:

Two in vivo studies were conducted with separate cohorts of animals. The first phase was developed to compare implants fabricated from two different additive manufacturing methods (EBM and DMLS) in a bilateral transcortical femoral metaphysis implant model. The transcortical model—similar to a dental implant—was used in the first phase to facilitate a second objective of developing and validating equipment for subsequent study. The second phase compared the fine surface texture of the DMLS implants to DMLS implants fabricated with a surface texture similar to that of an EBM implant. An intramedullary femur implantation model was utilized to better represent percutaneous osseointegrated implant and arthroplasty implant models.

Phase 1 Animals

Animal work was approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Female retired breeder Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) with a mean age of 24 weeks were used. The animals were housed and cared for by the Division of Laboratory Animal Medicine (DLAM) in individually ventilated cages (Seasafe Plus Rat, Techiplast, Italy). Rats were caged in pairs and given ad libitum access to food and water with a 12-hr light/dark cycle (7 a.m. – 7 p.m.) throughout the study. The rats respond adversely to the original corn-cob bedding in their individually ventilated cages secondary to a pica response as result of the sustained-release buprenorphine administered in prior studies. Therefore, all animals are placed on digestible paper bedding (7070C Diamond Dry Bedding, Diamond Star Products, Poynette, WI) for 5 days post-operatively to reduce the
chance of gastric obstruction through ingestion of the corn-cob bedding after which time they were returned to the original bedding.

Based on previous intramedullary implantation studies done with rats in the literature\textsuperscript{122} and our own experience with a transverse implantation model in the rat tibia a standard deviation of 20 percent of the mean torque-out strength for the electron beam melted implants was estimate. In order to detect a 35 percent difference in strength between the two manufacturing methods with a power of 0.80 and an alpha of 0.05 a power analysis calculated an N of 6 rats was required.

\textit{Phase 1 Implants}

All implants were fabricated using grade 5 Titanium (Ti-6Al-4V). The EBM implants were recovered from the prior study (2mm diameter with a machined 1.39 mm flat-to-flat hex on the last 2.86 mm produced on an Arcam A2 using a powder diameter ranging from 45 to 105 µm), reduced in total length to 7 mm and the soft tissue and bone removed (Figure 17 and Figure 18). The residual bone on the implants was decalcified with a 5% acetic acid solution (white distilled vinegar, Harris Teeter, Matthews, NC) on a stir plate for 36 hours. The implants were ultrasonically cleaned and scrubbed with a nylon brush upon removal from the acetic acid solution. The implants were rinsed with deionized water and then dried with a 70% ethanol solution. The implants were subsequently soaked in a neutralizing disinfectant (HDQ Neutral, Spartan Chemical Company, Maumee, OH) for three hours followed by soaking in an enzymatic cleaner (Endozime Bio-Clean, Ruhof Corporation, Mineola, NY) for one hour. The implants were then scrubbed with an acrylic brush and ultrasonically agitated in the enzymatic cleaner for a 10-minute cycle. The complete cleaning process was performed twice to fully
remove all bone and soft tissue from the implants. Additional titanium rods were produced at lengths of 7 mm and diameters of 2 mm with a 1.39 mm flat-to-flat hex on the last 2.86 mm (Figure 19) through DMLS on an EOS M290 (EOS GmbH, Krailling, Germany) using a powder size of 25 to 45 µm.

Figure 17: Recovered EBM implants prior to cleaning.
Figure 18: Recovered EBM implants post cleaning

Figure 19: Implant model for phase 1.
The DMLS implants were textured by acid etched in a 48% sulfuric acid (H$_2$SO$_4$) bath at 60°C (140°F) and were agitated with a stir bar for 30 minutes. All implants were rinsed in deionized water, dehydrated in a 70% ethanol solution, and allowed to air dry before packaging for sterilization by autoclave. The surface topography of both implant types was optically evaluated using a Hirox KH-7700 microscope (Hirox-USA, Inc., River Edge, NJ) and the surface roughness (Ra) obtained through a linear regression of the resulting spatial map.

**Phase 1 Surgical Model**

Seventy-two hours prior to undergoing surgery, the Sprague Dawley rats were given 2.4 mg/ml acetaminophen solution so they become accustomed to its taste. On the day of surgery each rat was anesthetized by isoflurane inhalation anesthesia by vaporizer (5% for rapid induction and 2.5% for the duration of surgery) for one hour ±20 minutes in the Division of Laboratory Animal Medicine (DLAM) procedure room and weighed. Toe pinch was use to insure proper loss of consciousness prior to initiating the procedure and respiratory rate and quality was monitored during surgery for signs of distress. An electric hot pad insulated by a towel was used to maintain the animals' body temperature while subjected to anesthesia. Aseptic techniques were used to expose the cranial-lateral aspect of the distal femur thru a 1.5 ±0.5 cm skin incision. Blunt dissection was used to separate the soft tissue overlying the implantation site on the femur. An 18-gauge needle (1.16-mm-diameter) was used to start a hole through the medial cortex of the intersection of the distal metaphysis and epiphysis of the femur. The hole was then drilled to 1.9 mm under saline irrigation to a depth of 8mm. Drilling progressively larger holes under saline irrigation is a technique similar to that used by prosthodontists in dental implant procedures. Once the implant hole was effectively prepared, an EBM and DMLS 2 mm diameter, 7 mm long,
titanium, transcortical implants were inserted by manual thrust and torque into the drilled hole in
the right and left limb as randomly determined. The incisions were closed using wound clips
(Autoclips, MikRon Precision Inc, Gardena, CA) and tissue adhesive (TA5, Med Vet
International, Mettawa, IL). The clips were removed 12 days after the surgery at the time of the
initial radiographing. Each rat was given a subcutaneous injection of Buprenorphine SR at 0.8
mg/kg post operatively for amelioration of postsurgical pain. A 10ml lactated ringer’s
subcutaneous injection was also given to each animal following surgery. The animals' body
temperature was maintained with a heating pad during the recovery period. The rats were
returned to their cages after awakening and monitored closely. All rats receive adequate
analgesia during the postoperative period and were monitored closely for signs or symptoms of
excess infection or poorly controlled pain. If rats exhibit such behaviors they would be removed
from the study immediately. The acetaminophen solution was also provided ad libitum for seven
days following surgery. However, the acetaminophen solution would be provided for the
remainder of the study to animals that incurred fractures during surgery. To minimize the risk of
infection, 0.15 ml of Ceftriaxone injection was given subcutaneously daily for three days post
operatively.

The rats’ masses were recorded upon arrival, immediately before surgery, periodically
during recovery and after euthanasia. Each rat received a unique tail “tattoo” via “Sharpie”
markers so that it could be quickly and easily identified from its cage mate and the other rats in
the study. The animals were humanely euthanatized four weeks after surgery by C02 overdose
for a minimum of eight minutes followed by thoracotomy as prescribed by DLAM. Femurs were
collected, wrapped in saline soaked gauze and stored at -20°C until testing.
Phase 1 Radiographs

Lateral radiographs were made of the femurs at 12 days after surgery while rats were positioned in the radiograph chamber in the supine position exposing the medial aspect of each thigh under inhalation isoflurane anesthesia for approximately five minutes. A cabinet radiographic unit (HP 43804 X-Ray System Faxitron, Hewlett Packard, Palo Alto, CA) was used with dental x-ray film (DX-42, Henry Schein, Melville, NY) at 35 kV with a 12-second aperture exposure. The radiograph film was then developed as described in the prior study. Radiographs were used to confirm the implant location and confirm the absence of femoral fractures. An additional radiograph was taken at four weeks post-surgery after euthanasia.

Phase 1 Ex-Vivo µCT

All femur pairs were scanned using a µCT (40 model specimen CT, Scanco Medical, Brüttisellen, Switzerland) with a 16 mm field of view on medium resolution with a voxel size of 16 µm. The x-ray power setting was 70 kVp, 114 µA, and 8 W. The scans had an integration time of 300 ms and were averaged once. Specimens were soaked in a 1:100 dilution of protease inhibitor cocktail (Sigma-Aldrich #P8340, St. Louis, MO) with saline prior to µCT. The proximal end of the femur of each specimen was removed using a fine toothed rotary bone saw to an overall length of 14mm which included the condyles and cartilage. Shortening of the specimens was necessary for them to fit in the µCT, 0.13 mm beryllium coated, X-ray tube as well as the potting jig for mechanical testing.

The resulting µCT scans were analyzed using software developed for processing medical images (Mimics 16.0, Materialise, Plymouth, MI). The implant was dilated by five pixels (80 µm) to exclude the metal-induced artifact as determined by a prior study\textsuperscript{112}. The bone was
segmented out using a low and high threshold of 529 and 1615 mgHA/cm$^3$ respectively. The implants were segmented using a threshold $\geq 2249$ mgHA/cm$^3$. The total area of the implant was obtained and divided by its length. This area per unit length was used later for normalizing the torque results.

The BV/TV within 500 µm of the implant was calculated$^{11}$. A cylinder was constructed in the femur that had a diameter 1000 µm larger than the mean diameter of the implant. The volume of bone within this region was divided by the volume of the cylinder minus the dilated implant volume within the cylinder. The resulting percentage represented the medullary bone volume fraction.

The BIC was calculated along the length of the implant embedded in the femur. The percent bone-implant contact was determined as the ratio of voxel threshold as bone to the total number of voxel adjacent to the dilated implant.

**Phase 1 Mechanical Testing**

Following the µCT evaluation, the mechanical stability of osseointegration of the implants was evaluated through torsional removal. A polymer resin (number 265, 3M Bondo, Atlanta, GA) in conjunction with a tapered mold (20 minute cure time ) was used to pot the specimens in the proper orientation to interface with a material testing system (MTS) (8500 Plus, Instron, Norwood, MA) as in the prior study. The potted specimens were kept hydrated by covering them with saline soaked gauze during curing. A chain and sprocket on a custom fixture were used to transfer the rotary motion of implant removal to the uniaxial servohydraulic motion of the MTS. The angular rotation and torque were measured using a potentiometer (Series P2201, Novotechnik US, Southborough, MA) and a 350 N•mm torque cell (Model 2105-50, Honeywell
Sensotec, Columbus, OH), respectively. A Jacob’s chuck which attached to the torque cell and then slid along a linear slide was utilized to transmit torque to the hexagonal head of the implant while minimizing translational loads to the femur in the tapered mold (Figure 20). A prior study has shown the maximum torque and stiffness to be independent of angular velocity in the range of 3–12 °/s angular velocity$^{137}$. Thus, specimens were preloaded with 3 N\(\text{m}\) and torqued at a constant rate of 6 °/s until failure of the bone-implant interface was reached. Maximum torque and stiffness were determined from the resulting torque and rotational displacement data using a program developed in Labview (Labview 6.0, National Instruments, Austin, TX). Stiffness was determined by the slope of the regression line of the torque–deflection angle curve between limits of 25 and 75% of the maximum torque$^{138}$. The maximum torque was normalized to calculate the equivalent shear stress at the bone-implant interface by dividing the resulting value by the product of the implant’s surface area in the femur and the mean radius of the implant both obtained from the µCT analysis.
Figure 20: Torque load cell, implant clamp and receptacle similar to the tapered mold on the rotary sprocket.

**Phase 1 Statistical Analysis**

A one way repeated measures analysis of variance with one factor repetition (Implant type) with Holm-Sidak posthoc mean comparison testing for all outcome measures was performed on the results using a statistical analysis program (SigmaPlot v11.0, Systat Software Inc, San Jose, CA).

**Phase 2 Animals**

The animal model for the second phase was identical to the first phase. However, rats were caged in groups of three, based on allowable housing density for rat weight. Based on a power analysis, 10 rats were used in the second cohort of animals. This pilot study was performed to also facilitate the development and practice of the intramedullary surgery needed in the following
studies. Therefore, the surgical model was changed from the trans-femoral model of the first phase to an intramedullary model to support the subsequent investigation of LIPUS and local vibration.

*Phase 2 Implants*

Nine femurs of female Sprague-Dawley rats representing similar size of expected animals were radiographed. The radiograph was digitally scanned and scaled using physiological markers. An implant was designed from the digital scan to fit the intramedullary canal (Figure 21).

![Figure 21: Digital scan with implant overlaid in intramedullary canal.](image-url)
To design a roughness to mimic that of the EBM implants several implants were built with various size interstices using DMLS. An average Ra of the various surface designs was calculated using the method described in the first phase of this study. The size of the interstices was extrapolated from the resulting Ra values of the different surface designs.

Implants were fabricated using Grade 5 Titanium (Ti-6Al-4V). The titanium rods were produced at lengths of 20 mm and diameters of 1.5 mm with a 1.6 mm boss on the last 1.5 mm and a dimple in each end to facilitate surgical implantation and mechanical pushout. The implants were built using DMLS on an EOS M280 15° from vertical with beam offset of 0.09 mm using a powder size ranging from 25 to 45 µm. Two different groups of implants were built: one with the fine native surface texture and one with a surface texture designed to simulate the Ra value of the coarse native EBM surface texture (Figure 22). The surface topographies of the DMLS implants were optically evaluated and surface roughness (Ra) was calculated as in the first phase. The implants were cleaned, etched and sterilized as in the first phase.

Figure 22: A. "as-built" surface texture, B. "rough" surface texture.
Phase 2 Surgical Model

The same pre and post-surgical methods as before where followed with the exception of a reduction of the acetaminophen solution to 1.6 mg/ml and Buprenorphine SR dose to 0.5 mg/kg to minimize pica as approved by the IACUC governing board. Implants were surgically placed bilaterally in the intramedullary canal of the distal and epiphysis femur through a cranial-lateral skin incision. The right limb randomly received either a fine or coarse textured DMLS implant while the contralateral limb received the alternative implant texture. After subluxation of the patella, an 18 gauge needle (1.16 mm-diameter) was used to start a hole in the intercondylar notch while the final hole was reamed and extended to a 21 mm depth manually with a 1.5 mm twist bit without irrigation. The DMLS implant was inserted by manual thrust and torque into the drilled hole. The implant was pressed until it was flush with the articular surface. The patella was returned to its original position and the knee joint closed with absorbable suture. The incisions were closed using the same techniques as in the first study. The animals were followed for 4 weeks.

Animal care, radiographs, euthanasia and specimen collection and storage was performed as in the first phase. The statistical analyses used to compare the results of the second phase were identical to the first phase.

Phase 2 Ex-Vivo\(\mu\)CT

Osseointegration was evaluated by BV/TV and BIC along a 2.5 mm length, 2 mm from the proximal end and along a 6 mm length 1.5 mm from the distal end of the implant using \(\mu\)CT (Figure 23) to assess a predominately cortical region versus a trabecular region respectively. All of the femur pairs of the second phase were processed using the same method as the first
phase. However, a 10 mm field of view on medium resolution with a voxel size of 12 µm was utilized due to the implant running along the longitudinal axis of the femur. Therefore, the proximal ends of the femurs were removed using a fine toothed rotary bone saw to facilitate fitting in the 0.07 mm beryllium coated, X-ray tube and allow subsequent mechanical pushout of the implants. The dilation of the implant to five pixels results in a distance of 60 µm due to the reduction in voxel size. This decreased region still agrees with the prior study\textsuperscript{112}. The region of BV/TV surrounding the implant was reduced to 250 µm to decrease deviation induced by the implants random proximity to the cortical shell. Due to a distinct cortical shell seen in the proximal scans, the cortical shell was excluded from both the BV/TV and BIC calculations. However, the cortical region near the epiphyseal line was less distinct and remained in the distal scan analyses. The BV/TV and BIC calculations only included the region proximal to the epiphyseal line due to the change from bone to cartilage at that delineation\textsuperscript{129}.

Figure 23: Distal femur with intramedullary implant.
Phase 2 Mechanical Testing

Following the µCT evaluation, push-out testing on all specimens was performed to assess osseointegration by evaluating the stiffness and the allowable shear of the bone-implant interface. Prior to mechanical testing, the proximal ends of the femurs were removed using a fine toothed rotary bone saw to allow pushout of the implants. Specimens were potted in a custom tapered mold using a self-curing acrylic resin (Ortho Jet BCA, Lang Dental, Wheeling, IL) and allowed to cure for 30 minutes. The axis of the implant was aligned in the direction of push-out with two opposing tapered pins, which rested in the dimple at each end of the implant (Figure 24) while the resin set. A 3 mm diameter, 2 mm thick silicone disk (Figure 25) was used during potting to insure consistent support of the femoral condyles during mechanical testing while allowing an opening for the implant to be pressed through. The specimens were kept hydrated by submerging the potting fixture into a beaker with 250 ml of 27°C saline while the resin cured for 20 minutes followed by wrapping the specimen in saline-soaked gauze for the remaining 10 minutes.
Figure 24: A. opposing pins for alignment of specimens, B. specimen prepared for potting.

Figure 25: Spacer for producing consistent support of condyles.
Mechanical testing was carried out with a material testing system (MTS) (8500 Plus, Instron Corp., Norwood, MA). The uniaxial servohydraulic motion of the MTS was transferred to the implant through a tapered stainless steel pin secured in a Jacob’s chuck (Figure 26). Linear load was measured with a 500 N load cell. The potted specimen was allowed to sit squarely on a platform with a hole in the center for implant pushout. Specimens were preloaded with 5 N and pushed out at a constant rate of 2 mm/min until failure of the bone-implant interface was reached. Maximum load and stiffness were determined from the resulting data using the system software. The maximum load was normalized by dividing the maximum axial load by the implant’s surface area obtained from the µCT analysis. This normalization was done to calculate the equivalent shear stress at the bone-implant interface.

Figure 26: Mechanical push-out fixture with potted specimen.
Phase 2 Statistical Analysis

A one way repeated measures analysis of variance with one factor repetition (Implant type) with Holm-Sidak posthoc mean comparison testing for all outcome measures were performed on the results using a statistical analysis program (SigmaPlot v11.0, Systat Software Inc, San Jose, CA).

**Results:**

*Animal Model*

Animals in the first cohort had a mean ±SD weight at surgery of 342 ±25 g. The mean weight loss between surgery and euthanasia was 12 g. The second cohort had a mean group weight of 341 ±25 g at surgery. Rats lost a mean body weight of 3 g per animal between surgery and euthanasia.

*Implant Model*

The finished implants for the first phase had mean ±SD major diameters of 1.86 ±0.00 mm and 1.91 ±0.03 mm for the EBM and DMLS implants, respectively. The area per length determined through µCT was 9.23 ±0.25 mm²/mm for EBM implants and 8.13 ±0.12 mm²/mm for DMLS implants. The intramedullary implants for the second phase all had the same diameter of 1.50 ±0.00 mm and had an average area per length of 5.57 ±0.09 mm²/mm and 5.75 ±0.04 mm²/mm for fine and coarse implants, respectively. The surface topography analysis revealed that EBM produced implants had a mean ±SD “as-built” surface roughness of Ra = 23 ±2.9 µm (Figure 27). Conversely, DMLS generated implants had a mean “as-built” surface roughness of Ra = 10 ±0.3 µm. The DMLS process for the second phase of the study produced surface roughness of Ra = 7.7 ±1.8 µm and Ra = 23.1 ±5.0 µm for fine and coarse implants, respectively (Figure 28).
Figure 27: EBM (A) versus DMLS (B) implant.

Figure 28: A. surface topography of “as-built” DMLS implants, B. surface topography of “textured” DMLS implant.
**Implant Surgery**

During surgery of the first phase, one limb on one animal had to be re-drilled which resulted in the implant’s failure to osseointegrate. An implant was placed anterior to the desired location for another rat, which resulted in its exclusion from statistical analysis. Finally, an implant was dislodged on the day of euthanasia.

The design of the surgery for second phase (Figure 29 through Figure 33) went without any complications. It was, however, noted that a new 18-gauge needle was necessary for starting each hole in the intercondylar notch.

Figure 29: Petella subluxated.
Figure 30: manual reaming with 1.5mm bit and insertion of intramedullary implant.
Figure 31: Implant flush with articular surface.

Figure 32: Closure of knee joint with absorbable suture.
Figure 33: Closure of incision with wound clips.

*Radiographs*

Implant location and bone response for both phases of the study were assessed using radiographs (Figure 34 and Figure 35). Femoral fractures were not seen in either phase of the study. Therefore, all limb pairs of the first phase and six matched pairs from randomly selected animals of the second phase underwent μCT. Mechanical stability analysis was also conducted on all limb pairs for both phases.
Ex vivo µCT

µCT scans where successfully generated for all specimens (Figure 36 - Figure 38) and subsequently analyzed as described earlier. Two specimens for whom difficulties were encountered with surgical placement and another specimen that was overloaded at dissection displayed poor osseointegration and were excluded from implant comparisons of the first phase.
specimens. Both studies failed to demonstrate significant differences in either BV/TV (Figure 39) or BIC between implant types (Table 7).

Figure 36: Typical µCT scan of phase one specimens viewed in Mimics.
Figure 37: Typical 2.5mm proximal scan of phase 2.

Figure 38: Typical 6mm distal scan of phase 2.
Figure 39: Typical bone volume surrounding transcortical implant.

Table 7: Mean ±SD BV/TV (measured within 500 µm of implants for phase 1 and 250 µm excluding cortical regions of implants for phase 2 scans) and BIC relative to available area of embedded implant. Groups did not differ statistically.

<table>
<thead>
<tr>
<th>Implant Type</th>
<th>BV/TV</th>
<th>BIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>70.3±3.8%</td>
<td>73.5±9.0%</td>
</tr>
<tr>
<td>Coarse</td>
<td>74.9±2.7%</td>
<td>80.4±7.0%</td>
</tr>
<tr>
<td>Phase 2 – Distal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>28.4±7.4%</td>
<td>36.5±5.2%</td>
</tr>
<tr>
<td>Coarse</td>
<td>30.6±7.2%</td>
<td>37.6±4.7%</td>
</tr>
<tr>
<td>Phase 2 – Proximal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>5.6±3.5%</td>
<td>14.4±7.9%</td>
</tr>
<tr>
<td>Coarse</td>
<td>6.1±5.4%</td>
<td>15.9±8.3%</td>
</tr>
</tbody>
</table>

*Mechanical Testing*

In phase 1, maximum removal torque (+85%, P = 0.003) and energy to failure (+109%, P = 0.019) were higher in EBM than fine textured DMLS implants (Figure 40). In phase 2 the
maximum thrust load (+35%, P = 0.007) and energy to failure +41% (P = 0.019) was larger for coarse textured DMLS implants than fine textured DMLS implants (Figure 41). Bone-implant stiffness did not differ for various implant types for both phases of the study. Phase 2 results developed a statistically significant correlation between the max load and stiffness (r = 0.7228, P < 0.001). However, the results of phase 1 did not establish a statistically significant correlation (r = -0.1083, P = 0.78).

Figure 40: Mechanical torque-out results of phase 1 specimens.
The removal loads of both the first and second phase were normalized as equivalent shear-forces to better compare the results between the torsional testing of the transcortical implants and the push-out of the intramedullary implants. In phase 1, the equivalent shear-force was larger (+59%, P = 0.003) for EBM implants than fine textured DMLS implants. The mean equivalent shear-force of the coarse textured DMLS implants of phase 2 showed a +27% increase relative to the fine textured DMLS implants but did not differ statistically (Figure 42).
Figure 42: Maximum equivalent shear-force of implants as a function of implant area.

In the first phase, difficulties were encountered with surgical placement for two specimens and another specimen was overloaded at dissection. Due to the complications these three specimens were excluded from implant comparisons.

Discussion:
The coarse textured surface of the EBM design yielded higher torsional properties compared to the fine textured DMLS design, highlighting the increased interlocking area between the bone and implant for a given implant diameter. Due to the DMLS’s superior spatial resolution it is possible to manufacture an implant by DMLS with a similar surface roughness as the EBM.
implants while allowing for a more detailed geometry. Phase 2 of this study examined the osseointegration of such coarse DMLS implants. The fixation strength of coarse DMLS implants was also proven to provide superior interlocking relative to the fine textured DMLS implants. Such a statistically significant difference in torsional strength between coarse textured implants versus fine textured implants is supported by similar work by Wennerberg et al., where a similar phenomenon was seen when comparing various sand-blasted titanium screw implants\textsuperscript{69}.

Interestingly, implant roughness in both phases of this study didn’t affect the BV/TV or the BIC. This finding supports previous observations that bone-implant contact is unrelated to surface roughness\textsuperscript{68}. In these two studies, BV/TV and BIC did not influence fixation strength. These results are supported by another study demonstrating a significant increase in max push-out force with no histomorphologic differences in BV/TV or BIC\textsuperscript{99}.

It was demonstrated in this study that maximum pushout load correlated to the bone-implant stiffness. However, fixation strength in phase 1 exhibited low variation within the implant types and thus failed to draw a correlation between removal torque and implant stiffness converse to the results of phase 2.

Placing the implants in the intramedullary canal resulted in more variability in cortical contact proximally and subsequently larger variations in equivalent shear-force. It was also noted that the equivalent shear-force for phase 2 was considerably lower than phase 1. This is most likely due to decreased implant contact with the cortical shell for the available surface area. Cortical bone may provide superior mechanical stability in osseointegrated implants compared to trabecular bone. Matching the implant to the residual bone would greatly increase the contact with the cortical shell and therefore should also enhance mechanical stability of osseointegration highlighting the need for patient specific implants.
This study was not without limitations. Histological analysis results in a planar image of the BIC interface, however, excluding information around the remainder of the implant perimeter. µCT evaluation allows for a non-destructive analysis of the BIC interface around the entire implant perimeter. This study’s µCT evaluations were performed with an offset implant surface to exclude the metal-induced artifact determined in a prior study\textsuperscript{112}. The exclusion of information immediately adjacent to the implant may reduce the sensitivity to detect meaningful differences in BIC.

**Conclusion:**

As the trend in amputee prosthetic devices moves toward transcutaneous osseointegrated implants instead of socket-cup fitting prosthetic devices, this study is important in showing that additive manufacturing can provide a means of producing well-fitted osseointegrated implants that can be easily customized. AM implants provide a means to produce customized geometries to match patient specific anatomy as well as customized surface textures for optimizing implant stability. This research indicates that coarse textured surfaces can provide higher interface strength for titanium alloy implants than fine textured surfaces. Future studies should be conducted to determine the optimal roughness for implant fixation. Another question left unanswered is if there is an optimum surface roughness-interaction between cortical bone and an implant compared to trabecular bone and an implant.

**Acknowledgements:**

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Additional Contributions: We thank Melanie Card for her technical assistance with the analysis of µCT DICOM stacks and Sam Abumoussa for his assistance with the surgeries of the second phase. Gratitude also goes to EOS North America for fabricating the DMLS implants on short notice for the first phase of the study.
Chapter 4: NSF Pilot Study—Development of a rat restraint System for the application of local vibration and/or LIPUS to the distal femur.

Summary:
Many therapeutic treatments are being clinically studied and optimized in in-vitro or human studies due to the limitation in rodent restraints for testing locally administered therapies. This study investigated the possibility of a custom restraint system to facilitate the application of local therapeutic stimuli in a rodent model.

Female retired breeder Sprague-Dawley rats were used for tailoring the custom restraint to a live animal. The rats used for fitting had a mean group weight of 341 ±25 g.

A system was developed to adequately restrain the rats for prolonged treatments. The custom restraint was constructed from an adhesive and latex free flexible composite of open-cell foam and soft fabric in conjunction with hook-n-loop fasteners. The restraint system secures the rat with its hind limbs flexed and externally rotated. A hood—equipped with an aperture for breathing—was secured over the rat’s head to minimize struggling and better secure the limb restraint to the body.

The custom, reusable and washable, restraint system allows for the application of treatments to the hind limbs while the animal is conscious. With minimal resistance to being contained, the animal is immobilized enough that treatments can be applied in a continuous and repeatable manner to a desired limb location. The system can also be scaled to accommodate larger or smaller rodents and utilized to explore local therapeutic treatments in a variety of rodent models for a multiplicity of diseases.
Introduction:
Treatments are being developed for a variety of diseases, which include the local application of therapeutic stimulation. These therapies aim to treat diseases including the prevention of non-union in fracture healing\textsuperscript{55}, pain relief of arthrofibrosis\textsuperscript{150} and relief from rheumatoid arthritis\textsuperscript{151} just to mention a few. Limited in-vivo animal studies are performed without anesthetized subjects due to the limitation in rodent restraints\textsuperscript{57,151}. Anesthesia with isoflurane, the preferred method for rodents, requires the use of vaporizers, delivery systems, scavenger or room ventilation systems, isoflurane, oxygen tanks, etc. Therapies are often intended to be applied for 10 to 20 minutes per day.\textsuperscript{152} Also, the animal must be immobilized enough that treatments can be applied in a continuous and repeatable manner to the desired limb location. Therefore, there is a need to facilitate the application of locally applied stimulus to conscious rodents where many subjects will be treated each session.

This study aimed to create a system to explore the effects of sustained locally administered treatments without continuous inhalation anesthesia. A custom restrain system was developed to allow for the application of treatments to the hind limbs. Specifically, we were interested in a system designed to target the distal femurs for accelerating implant osseointegration.

Materials and Methods:
To efficiently administer locally applied experimental treatments to rodents the use of anesthesia must be minimized due to equipment limitations. A rat restraint was developed for locally applied treatments such as LIPUS and local vibration to be administered for prolonged periods without sustained inhalation anesthesia.
A preliminary restraint was commissioned (Phoenix Design Solutions, Ashburn, VA) around an additively manufactured replica of a Sprague-Dawley rat (Figure 43). The prototype was constructed from an adhesive and latex free flexible composite of open-cell foam and soft fabric (Breath-o-prene, TANKindustries, Buffalo, NY) in conjunction with hook-n-loop fasteners (Velcro, Manchester, NH) as shown in Figure 44.

Figure 43: Additively manufactured replica of a rat.
Figure 44: Rat restraint prototype fitted to subject.
Female retired breeder Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) with a mean age of 24 weeks were used for tailoring the restraint to a live animal. These rats were part of an intramedullary implant osseointegration study and the work was approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. The animals were housed and cared for by the Division of Laboratory Animal Medicine (DLAM) in individually ventilated cages (Seasafe Plus Rat, Techiplast, Italy). Rats were caged in pairs and given ad libitum access to food and water with a 12-hr light/dark cycle (7 a.m. – 7 p.m.) throughout the study.

**Results:**

The rats used for tailoring the restraint had a mean group weight of 341 ±25 g. After several iterations of restraint designs, a system was developed to adequately restrain the rats for prolonged treatments. The restraint was constructed from Breath-o-prene and Velcro fasteners (Figure 45). The restraint system secures the rat with its hind limbs flexed and externally rotated. A hood—equipped with an aperture for breathing—was secured over the rat’s head to minimize struggling and better secure the limb restraint to the body (Figure 46).
Figure 45: Rat restraint system for application of local treatments to the hind limb.
Discussion:

A custom restraint system was successfully developed to allow for the application of treatments to the hind limbs. The system designed securely holds the rat on a platform with both of its pelvic limbs immobilized and located relative to the restrain for repeatable application of
treatment. The restrain is capable of securing a LIPUS transducer, allowing a local vibration stimulator access to the knee or simultaneous treatments. The animals exhibited minimal resistance to being contained in the restraint with the hood blacking out their vision of the surrounding laboratory. The restraints are reusable and washable. The hook and loop fasteners securely contained the rats for over three hundred 20-minute-long applications per restraint. However, the restraint systems were clearly tired at the end of the study with over 100 hours of uses per restraint and should be repaired or replaced at that point.

The restraint system developed in this study can be easily modified to facilitate the application of various treatments to different aspects of the pelvic limb of a rat. The system can also be scaled to accommodate larger or smaller rodent. This custom restraint system can be utilized to explore local therapeutic treatments in rodent models for a variety of diseases such as no-union in fracture healing$^{55}$, pain relief of arthofibrosis$^{150}$ and relief from rheumatoid arthritis$^{151}$. 
Chapter 5: NSF Study—Evaluation of the osteogenic benefits of low-intensity pulsed ultrasound and local vibration in an in vivo rodent osseointegration model.

Summary:
Amputees frequently develop skin and soft tissue problems on the residual limb due to increased pressure and shear-forces generated at the socket-limb interface. Direct skeletal attachment of prostheses via percutaneous osseointegrated implants is a feasible alternative with numerous advantages over conventional techniques. However, effective osseointegration of implants remains a major clinical challenge.

Previous studies have indicated that vibration and low-intensity pulsed ultrasound (LIPUS) can be beneficial to bone healing. A 4-week rodent study was conducted in a bilateral femoral intramedullary implant model (control, vibration and LIPUS) to determine the effects of local vibration and LIPUS to stimulate healing at the intramedullary implant interface. A subsequent 8-week study was performed to determine midterm osseointegration and the cumulative effect of combined LIPUS and vibration treatments. Bone-implant integration was evaluated quantitatively through mechanical, μCT and histological evaluations.

Maximum pushout load increased significantly with LIPUS relative to the control group at 4 weeks. Both μCT and histology stained results revealed treatment with either LIPUS or vibration increased bone around the implant in the metaphyseal region relative to the control. No significant differences were noted for maximum pushout load at 8 weeks. The stimulatory effect on bone peri-implant bone mass with LIPUS at 4 weeks was no longer present at 8 weeks. Vibration treatment resulted in greater bone around the implant than all other groups at 8 weeks. The measured stiffness from the combined treatment of LIPUS and local vibration was
significantly lower than LIPUS at 8 weeks. No differences were noted for bone morphology around the implant at the diaphysis or bone remodeling in either study.

Although vibration demonstrated increased bone around implants in the metaphyseal region at 8 weeks; this study establishes LIPUS as superior for accelerating early osseointegration through increased axial load to failure at the bone-implant interface. This demonstrates the potential benefit of LIPUS as a therapeutic tool for reducing amputees’ rehabilitation period.

**Introduction:**

Despite recent advances in prosthetic limb engineering and design, amputees frequently develop skin and soft tissue problems on the residual limb due to increased pressure and shear-forces generated at the socket-residuum interface. Direct skeletal attachment of limb prostheses via skin-penetrating osseointegrated implants is a feasible alternative with numerous advantages over conventional techniques, especially for patients with a very short residuum and high soft-tissue volume. It is believed that skeletal attachment of prostheses provides the amputee a wider range of motion, increased postural comfort, enhanced sensory feedback through osseoperception, improved limb control, and reduced soft tissue complications. However, effective osseointegration of implanted devices remain a major clinical challenge. Recent research has focused on device coatings and metallic porosity to reduce infection and to promote osseointegration.

Previous studies investigating fracture healing have indicated that low-magnitude, high frequency (LMHF) whole body vibration (WBV) and low-intensity pulsed ultrasound (LIPUS) can be beneficial to bone healing through mechanisms not clearly defined. In a previous
study, an optimal amplitude of WBV for stimulating osseointegration was investigated in a rat proximal tibial metaphysis model where the implant was inserted transversely in the bone. Transverse implantation would allow for future investigations of the skin implant interface without amputation of the animal's limb. It was found that with this transverse implantation model that the implant became quickly stabilized in the bone and it appeared that ongrowth in the intracortical region was largely responsible for this stabilization. The effects of WBV on osseointegration were marginal in this model as previous work has suggested vibration primarily affects cancellous bone and endosteal bone surfaces rather than the intracortical region. In this study, a more clinically relevant intramedullary implantation model was used to investigate if local application of vibration can accelerate osseointegration. The most effective vibration amplitude identified in the earlier WBV study was used as a basis for the amplitude of locally applied vibration to the rat's knee.

LIPUS was also investigated as a stimulus for bone in-growth in an intramedullary implantation model. While past studies have shown LIPUS has the ability to accelerate osseointegration in transverse implantation models, it is unclear if sufficient levels of the stimulus can reach the endosteal surfaces to stimulate bone healing. Therefore, an intramedullary implantation model was critical for evaluating the LIPUS treatment for accelerating osseointegration along the intramedullary canal. LIPUS in conjunction with local vibration treatments was also investigated for stimulating osseointegration to determine if the effects were cumulative and could provide further acceleration of osseointegration of intramedullary implants. It was believed the beneficial effects of these individual treatments to osseointegration would be cumulative such that when combined an even greater acceleration of osseointegration would be observed. Our objective was to quantitatively evaluate
osseointegration throughout the study via histological, µCT and biomechanical testing. We hypothesize an increased osseointegration of the bone-implant interface due to the stimulatory effects of locally applied LMHFV and LIPUS would be shown.

**Materials and Methods:**
An initial 4-week-long *in vivo* study (study A) was performed to determine differences in early osseointegration resulting from a control, LIPUS and local vibration in a bilateral intramedullary femoral implant model. Bone ongrowth was evaluated using mechanical pushout, µCT and histomorphometry. A subsequent 8-week-long *in vivo* study (study B) was conducted to determine midterm osseointegration and the combined effects of the treatments. Study B evaluated the bone ongrowth as in study A.

Animal work was approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Female retired breeder Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) with a mean age of 24 weeks were used. The animals were housed and cared for by the Division of Laboratory Animal Medicine (DLAM) in individually ventilated cages (Seasafe Plus Rat, Techiplast, Italy). Rats were caged in groups of three based on allowable housing density for rat weight and given *ad libitum* access to food and water with a 12-hr light/dark cycle (7 a.m. – 7 p.m.) throughout the study. All animals are placed on digestible paper bedding (7070C Diamond Dry Bedding, Diamond Star Products, Poynette, WI) for 5 days post-operatively to reduce the chance of gastric obstruction through ingestion of the corn-cob bedding after which time they were returned to the original bedding.

Based on previous intramedullary implantation studies done in rats in the literature and our experience with a transverse implantation model in the rat tibia, a standard deviation of 25
percent of the mean pushout strength for the control implants was estimated. To detect a 40 percent difference in strength between the 3 treatment groups of study A with a power of 0.80 and an alpha of 0.05, a power analysis calculated an N of 9 rats. To compensate for animals lost due to surgical difficulties, an N of 10 rats per group was used for the study. In order to detect a 40 percent difference in strength between the four treatment groups with a power of 0.80 and an alpha of 0.05 a power analysis calculated an N of 10 rats would be required for study B. Due to the confidence achieved from study A in surgical outcomes, an N of 10 rats per group was utilized.

*Implant Model*

Implants were fabricated using Grade 5 Titanium (Ti-6Al-4V). The titanium rods were produced at lengths of 20 mm and diameters of 1.5 mm with a 1.6 mm boss on the last 1.5 mm and a dimple in each end to facilitate surgical implantation and mechanical pushout. The implants were built using DMLS on an EOS M280 15° from vertical with beam offset of 0.09 mm using a powder size ranging from 25 to 45 µm. The implants were textured by acid etching in a 48% sulfuric acid (H₂SO₄) bath at 60°C (140°F) and were agitated with a stir bar for 30 minutes. All implants were rinsed in deionized water, cleaned in deionized water for ten minutes under ultrasonic agitation, dehydrated in a 70% ethanol solution, and allowed to air dry before packaging for sterilization by autoclave. The surface topographies of the DMLS implants were optically evaluated using a Hirox KH-7700 microscope (Hirox-USA, Inc., River Edge, NJ) and surface roughness (Ra) obtained through a linear regression of the resulting spatial map.
Surgical Model

The bilateral implantation design is commonly used in osseointegration animal studies\textsuperscript{62,122} and allows separate evaluations of push-out strength and undecalcified histology in each animal to minimize overall animal numbers. The primary outcome measure will be push-out strength of the implant-bone interface.

Seventy-two hours prior to undergoing surgery, the Sprague Dawley rats were given 1.6 mg/ml acetaminophen solution so they become accustomed to its taste. On the day of surgery each rat was anesthetized by isoflurane inhalation anesthesia by vaporizer (5% for rapid induction and 2.5% for the duration of surgery) for 50 ±10 min in the Division of Laboratory Animal Medicine (DLAM) procedure room and weighed. Toe pinch was use to insure proper loss of consciousness prior to initiating the procedure and respiratory rate and quality was monitored during surgery for signs of distress. An electric hot pad insulated by a towel was used to maintain the animals' body temperature while subjected to anesthesia. Each rat was given a 0.5 mg/kg subcutaneous injection of Buprenorphine SR preoperatively for amelioration of postsurgical pain. To minimize risk of infection, 0.15 ml of Ceftriaxone injection was given subcutaneously preoperatively as prescribed by the IACUC governing board. Aseptic techniques were used to surgically placed implants bilaterally in the intramedullary canal of the distal and epiphysis femur through a 2 ±0.5 cm cranial-lateral skin incision. After subluxation of the patella, an 18-gauge needle (1.16 mm-diameter) was used to start a hole in the intercondylar notch while the final hole was reamed and extended to a 21 mm depth manually with a 1.5 mm twist bit without irragation\textsuperscript{147,148}. The DMLS implant was inserted by manual thrust and torque into the drilled hole. The implant was pressed until it was flush with the articular surface. The patella was returned to its original position and the knee joint closed with absorbable suture\textsuperscript{149}.
The incisions were closed using wound clips (Autoclips, MikRon Precision Inc, Gardena, CA) and tissue adhesive (TA5, Med Vet International, Mettawa, IL). A 10ml lactated ringer’s subcutaneous injection was also given to each animal following surgery. The animals’ body temperature was maintained with a heating pad during the recovery period. The rats were returned to their cages after awakening and monitored closely. All rats receive adequate analgesia during the postoperative period and were monitored closely for signs or symptoms of excess infection or poorly controlled pain. The acetaminophen solution was also provided ad libitum for seven days following surgery. The wound clips were removed 12 days after the surgery at the time of the initial radiographing. The animals of study A were followed for 4 weeks and those of study B followed for 8 weeks.

The rats’ masses were recorded upon arrival, immediately before surgery, periodically during recovery and after euthanasia. Each rat received a unique tail “tattoo” via “Sharpie” markers so that it could be quickly and easily identified from its cage mate and the other rats in the study. The animals were humanely euthanatized at the end of their rehabilitation healing period by CO2 overdose for a minimum of eight minutes followed by thoracotomy as prescribed by DLAM. The right femurs were collected, wrapped in saline soaked gauze and stored at -20°C until mechanical testing. The contralateral limb, utilized for µCT and histology, was fixed in 70% ethanol at time of dissection.

*Treatments*

A dual limb local vibration stimulator was constructed from a pair of electromagnetic minishakers (Brüel & Kjær, Nærum, Denmark), signal generator and amplifier. An Endevco-Isotron PE Accelerometer was mounted to the femur of a cadaver rat colinear to the axis of stimulation from the local vibration system. The rat was secured in our custom rat restraint
system to position the knees in a flexed and abducted position. The output amplitude of the local vibration system was then chosen so the magnitude of vibration at the femur matched the magnitude measured at the implant for the optimal *in vivo* WBV amplitude of the “preliminary study” (Figure 47).

Figure 47: Local vibration system.
Study A is designed to compare the efficacy of locally applied LIPUS and locally applied LMHF vibration to accelerate early osseointegration. After the implantation surgeries of the first cohort were performed, animals were randomly divided into 3 groups: control, LIPUS, and LMHF vibration based on maintaining a uniform mean body weight between groups. Treatments were started 7 days post-surgery and the rats received treatment for 20 minutes per day for 5 days per weeks. The animals were secured into the custom restraint system while under isoflurane inhalation induced anesthesia as in the original surgery. Rats were monitored closely to ensure that the hoods wouldn’t restrict breathing or the restraints constrict respiration. Once conscious, the designated 20 minute treatment was initiated (control, local vibration or LIPUS). The 45 Hz vibration stimulus was locally applied to the knee joint using the local stimulator developed in the first pilot study. The local vibration amplitude had been tuned to match the vibration magnitudes measured at the tibia during the most effective WBV of the prior study. The LIPUS applicator was centered over the approximated mid-point of the implant on each limb. The control was secured with a Delrin disc the same size as the LIPUS applicator. Rats quickly became accustomed to the restraint and only struggled minimally (less than 2 minutes) during the initial days of the treatment. At the completion of each treatment, animals were removed from restraints, returned to their cage and provided with three Froot Loops (Kellogg’s, Battle Creek, Michigan, US) per rat. Froot Loops were provided to reduce stress of the rats post restraint and prevent stress associated weight loss.  

The animals in the study B were divided into four groups after surgical implantation: control, LIPUS, vibration, and combination of LIPUS and vibration. This study compares the ability of locally applied LIPUS, locally applied LMHF vibration (as described in the prior
phases of the study) and the combination of these treatments to accelerate midterm osseointegration.

**Mechanical Testing**

Mechanical push-out testing was performed to assess osseointegration by evaluating the stiffness, maximum load to failure and energy to failure. The proximal femors were removed to allow pushout of the implants and the residual specimen potted using a self-curing acrylic resin (Ortho Jet BCA, Lang Dental, Wheeling, IL). During curing of the acrylic resin the implant was aligned in the direction of push-out on a 3 mm silicone disk to insure consistent support of the femoral condyles during mechanical testing.

Mechanical testing was carried out with a material testing system (MTS) (8500 Plus, Instron Corp., Norwood, MA). The uniaxial servohydraulic motion of the MTS was transferred to the implant through a tapered stainless steel pin secured in a Jacob’s chuck. Linear load was measured with a 1kN load cell. Specimens were preloaded with 5 N and pushed out at a constant rate of 2 mm/min until failure of the bone-implant interface was reached. Maximum load and stiffness were determined from the resulting data using the system software.

**Ex-Vivo µCT**

Osseointegration was also evaluated by BV/TV and BIC along a 2.5 mm length, 2 mm from the proximal end and along a 6 mm length 1.5 mm from the distal end of the implant using µCT to assess a predominately cortical region versus a trabecular region respectively. All contralateral femurs were scanned using a µCT (40 model specimen CT, Scanco Medical, Brüttisellen, Switzerland) with a 10 mm field of view on medium resolution with a voxel size of 12 µm. The
x-ray power setting was 70 kVp, 114 µA, and 8 W. The scans had an integration time of 300 ms and were averaged once. The proximal end of the femur of each specimen was removed using a fine toothed rotary bone saw 1 mm proximal to the implant to facilitate placement in the µCT, 0.07 mm beryllium coated, X-ray tube as well as subsequent infiltration of a methacrylate resin.

The resulting µCT scans were analyzed using software developed for processing medical images (Mimics 16.0, Materialise, Plymouth, MI). The implant was dilated by five pixels (60 µm) to exclude the metal-induced artifact as determined by a prior study\textsuperscript{112}. The bone was segmented out using a low and high threshold of 529 and 1615 mgHA/cm\textsuperscript{3} respectively. The implants were segmented using a threshold ≥ 2249 mgHA/cm\textsuperscript{3}.

The BV/TV within 250 µm of the implant was calculated\textsuperscript{11}. Due to a distinct cortical shell seen in the proximal scans, the cortical shell was excluded from both the BV/TV and BIC calculations. However, the cortical region near the epiphyseal line was less distinct and remained in the distal scan analyses. The BV/TV and BIC calculations only included the region proximal to the epiphyseal line due to the change from bone to cartilage at that delineation\textsuperscript{129}. The cortical wall thickness was also evaluated at the mid-shaft (proximal scan) for the µCT scan (Appendix A) to evaluate the effects of the treatments.

\textit{Fluorescence Evaluation}

\textit{In vivo} double fluorochrome labeling was administered to the all animals. Subcutaneous injections of 10 mg/kg calcein green and 30 mg/kg alizarin red complexone (Sigma-Aldrich, St. Louis, MO) were given at 14 and 21 days post-surgery respectively during study A. The subcutaneous injections of 10 mg/kg calcein green followed by 30 mg/kg alizarin red complexone were given at 35 and 42 days respectively post-surgery for study B. All surviving
specimens from each treatment group were sectioned for histology. Subsequent to µCT scanning, the ethanol-fixed femur was plastic-embedded, sectioned and polished to a 75 µm thickness for imaging of the fluorochrome labels\textsuperscript{120}. The plane of the section was defined by the axis of the implant and the femoral notch. The longitudinal sections of the implant-bone interface were initially viewed by fluorescent microscopy to evaluate the portion of labeled surface. Labeled surface was calculated as the percent area fluorescing within 250 µm of the implant.

The specimens were dehydrated with ethanol, stripped of lipids with acetone and infiltrated with a photo-curing methacrylate resin (Technovit 7200 VLC, EXAKT, Oklahoma City, OK, US) under constant agitation via a stir bar in a vacuum desiccator (Table 8). Following the infiltration the specimens were secured in molds at each end of the implant with a custom fixture (Figure 48) to maintain a cranial-caudal orientation along the axis of the implant at the midline of the mold cavity and fully embedded in the methacrylate resin. The top surface of each embedded specimen was ground parallel to the bottom surface with 320 grit sandpaper on a polisher/grinder (EcoMet II, Buehler, Lake Bluff, IL, US) and mounted to a slide with an epoxy (Technovit 4000, EXAKT, Oklahoma City, OK, US) using an EXAKT 401 Vacuum Adhesive Press (Figure 49). The resulting construct was then ground down to the implant mid plane with progressively finer grit paper on a precision grinding system (400 CS, EXAKT, Oklahoma City, OK, US) with a final grit of 2000 (Figure 50). Subsequently, a thin slide was fixed to the specimen block with a photo-curing adhesive (Technovit 7210 VLC, EXAKT, Oklahoma City, OK, US) on an EXAKT 402 Precision Adhesive Press (Figure 51). The sandwiched specimen block was then cut approximately 500 µm from the ground surface of the implant with precision 0.2 mm diamond cutting band saw (310 CP, EXAKT, Oklahoma City, OK, US) and micro-bubbles filled with the photo-curing adhesive. The specimen was next ground to a 75 µm
thickness with progressively finer grit paper on the precision grinding system with a final grit of 2000 (Figure 52).

Table 8: Dehydration & infiltration schedule of tissue sample containing lipids.

<table>
<thead>
<tr>
<th>Step</th>
<th>Treatment</th>
<th>Duration</th>
<th>Step</th>
<th>Treatment</th>
<th>Duration</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>80% Alcohol</td>
<td>3 days</td>
<td>7</td>
<td>100% Alcohol</td>
<td>1 day</td>
</tr>
<tr>
<td>2</td>
<td>95% Alcohol</td>
<td>3 days</td>
<td>8</td>
<td>30% Technovit / 70% Alcohol</td>
<td>3 days</td>
</tr>
<tr>
<td>3</td>
<td>100% Alcohol</td>
<td>3 days</td>
<td>9</td>
<td>50% Technovit / 50% Alcohol</td>
<td>3 days</td>
</tr>
<tr>
<td>4</td>
<td>100% Alcohol</td>
<td>3 days</td>
<td>10</td>
<td>70% Technovit / 30% Alcohol</td>
<td>3 days</td>
</tr>
<tr>
<td>5</td>
<td>100% Acetone</td>
<td>2 days</td>
<td>11</td>
<td>100% Technovit 7200 VLC</td>
<td>2 days</td>
</tr>
<tr>
<td>6</td>
<td>100% Alcohol</td>
<td>1 day</td>
<td>12</td>
<td>100% Technovit 7200 VLC</td>
<td>6+ days</td>
</tr>
</tbody>
</table>

Figure 48: Custom fixture for positioning specimen in mold for embedding in the methacrylate resin (detail of feature for locating implant).
Figure 49: Specimen block mounted to slide with epoxy.

Figure 50: Specimen block ground to mid-plane of implant with 2000 grit.
Figure 51: Sandwich with specimen mounted to final slide.

Figure 52: Specimen ground to required thickness for imaging.
Composite images of the entire implant and surrounding bone were acquired using an upright confocal microscope with a Fluar 5x/0.25 objective on an Axio Imager Z2 (LSM 800, Zeiss, San Jose, CA, US). Appropriate laser lines (488 nm & 561 nm) were used to excite the Calcein dye (excites and emits around 495 nm and 515 nm respectively) and Alizarin Red dye (excites and emits around 530/560 nm and 580 nm respectively). The resulting images were evaluated for percent areas of each fluorescent label within 250 µm of the implant of the composite image (Appendix B).

*Histological Staining*

The longitudinal sections from the fluorescence evaluation were then ground and polished to a 25-µm-thickness and stained with acid fuchsin for 3 minutes\(^{140}\). The sections were imaged through a 10x bright field lens with an exposure of two seconds on the Olympus BX51 with a DP72 color camera. Multiple images were collected on an automated stage and stitched to capture the entire bone-implant region. The resulting images were evaluated for percent BV/TV within 250 µm of the implant and BIC (Appendix C).

*Statistical Analysis*

A one way analysis of variance with Holm-Sidak posthoc mean comparison testing for all outcome measures were performed on the results to assess treatment effects (P < 0.05) using a statistical analysis program (SigmaPlot v11.0, Systat Software Inc, San Jose, CA).
Results:
The intramedullary implants all had the same diameter of 1.50 ±0.00 mm and had an average area per length of 5.57 ±0.09 mm²/mm. The surface topography analysis revealed that DMLS generated implants had a mean “as-built” surface roughness of Ra = 7.7 ±1.8 µm.

The rats were anesthetized for the minimal amount of time (< 30 seconds) to secure them in the restraint system and then allowed to wake up. Each rat was monitored closely for respiration until it was responsive to ensure that breathing was not occluded and subsequently underwent the 20 minute treatment of a Delrin puck (control), LIPUS, local vibration or a combination of LIPUS and local vibration. Once properly secured in the restraint, the rats were unable to escape treatment and only struggled noticeably the first few times being restrained. The vibration axis of the dual limb local vibration stimulators were adjusted to acute angles to the treated femurs—as close to collinear as possible. The rats were positioned to rest on the applicators with their ‘knees’ under their own body weight. Treatments were tolerated well by the rats with minimal resistance.

Animals in study A had a mean ±SD weight at surgery of 317 ±20 g. The mean weight loss between surgery and euthanasia was 18 g. The study B cohort had a mean group weight of 317 ±19 g at surgery. Rats lost a mean body weight of 22 g per animal between surgery and euthanasia.

Only three complications were noted during the surgeries of both phases of this study. Difficulties were experienced in preparing the pilot hole for implant insertion in two limbs of the four-week study, which were excluded from further evaluation. No fractures were seen in the radiographs. However, the radiographs revealed that an implant exited the caudal side of a femur
in one animal resulting in continued supply of acetaminophen elixir and it’s exclusion from evaluation.

*Ex vivo μCT*

In study A, μCT revealed that the LIPUS treatment group had significantly higher BV/TV within 250 μm of the implant in the metaphyseal region than the control group (9.1%, P = 0.003, Figure 53) without resulting in differences in BIC or thickness of the cortical wall in the diaphysis (Table 9). After eight weeks in study B, the BV/TV gain of the LIPUS group seen in study A was no longer present and vibration treatment resulted in greater BV/TV in the metaphyseal region than both the control and LIPUS groups (31.2% average, P = 0.006, Figure 54). Vibration also increased BIC in the metaphyseal region relative to both the control and combined groups [34.8% (P = 0.002) and 32.3% (P = 0.004) respectively, Figure 55]. Interestingly, the LIPUS group experienced a slight decrease in cortical thickness. No differences were seen in BV/TV or BIC in the diaphysis of either study A or B (Table 9 & Table 10).
Figure 53: BV/TV in the metaphyseal region of study A (4 week) as evaluated by µCT.
Table 9: μCT results of study A (4 week). Proximal scans exclude the cortical shell for both BV/TV & BIC. BV/TV evaluated within 250 μm of the implant. *=significantly different than control (P = 0.003).

<table>
<thead>
<tr>
<th></th>
<th>Proximal</th>
<th></th>
<th>Distal</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>BV/TV</td>
<td>BIC</td>
<td>Cortical Thk</td>
<td>BV/TV</td>
</tr>
<tr>
<td>Control</td>
<td>7.7±1.9%</td>
<td>25.7±4.3%</td>
<td>0.629±0.027</td>
<td>29.6±5.9%</td>
</tr>
<tr>
<td>Vibration</td>
<td>6.6±2.3%</td>
<td>19.4±0.6%</td>
<td>0.660±0.045</td>
<td>34.7±5.2%</td>
</tr>
<tr>
<td>LIPUS</td>
<td>6.4±1.7%</td>
<td>24.4±6.0%</td>
<td>0.660±0.060</td>
<td>38.7±1.9% *</td>
</tr>
</tbody>
</table>

Figure 54: BV/TV in the metaphyseal region of study B (8 week) as evaluated by μCT.
**Figure 55:** BIC in the metaphyseal region of study B (8 week) as evaluated by µCT.

**Table 10:** µCT results of study B (8 week). Proximal scans exclude the cortical shell for both BV/TV & BIC. BV/TV evaluated within 250 μm of the implant. †=significantly different than LIPUS (P ≤ 0.007), ‡=significantly different than vibration (P ≤ 0.006).

<table>
<thead>
<tr>
<th></th>
<th>Proximal</th>
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<th>Distal</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BV/TV</td>
<td>BIC</td>
<td>Cortical Thk</td>
<td>BV/TV</td>
</tr>
<tr>
<td>Control</td>
<td>6.6±5.2%</td>
<td>14.3±8.7%</td>
<td>0.693±0.062 †</td>
<td>27.8±8.4% ‡</td>
</tr>
<tr>
<td>Vibration</td>
<td>5.6±3.5%</td>
<td>18.8±14.2%</td>
<td>0.682±0.035</td>
<td>36.0±6.6%</td>
</tr>
<tr>
<td>LIPUS</td>
<td>4.9±4.3%</td>
<td>14.5±14.7%</td>
<td>0.624±0.050</td>
<td>27.2±3.6% ‡</td>
</tr>
<tr>
<td>Combined</td>
<td>3.8±3.5%</td>
<td>4.6±4.6%</td>
<td>0.698±0.046 †</td>
<td>30.2±4.9%</td>
</tr>
</tbody>
</table>
**Mechanical Testing**

Maximum pushout load increased for both LIPUS and vibration groups relative to the control group in study ‘A’ [37.7% (P = 0.002) and 20.2% respectively, Table 11] with the increase from vibration failing to reach statistical significance (P = 0.135, Figure 56). Energy to failure of the LIPUS and vibration treatments exhibited increases relative to the control group with the LIPUS being significantly greater [74.4% (P = 0.001) and 43.9% respectively]. No differences were noted for bone-implant stiffness.

Table 11: Mechanical push-out test results including stiffness of bone implant interface, max load to failure and energy to failure. *=significantly different than control (P < 0.005), †=significantly different than LIPUS (P = 0.003).

<table>
<thead>
<tr>
<th></th>
<th>Stiffness (N/mm)</th>
<th>Max Load (N)</th>
<th>Energy to Failure (mJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4wk study)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1190±128%</td>
<td>248±53%</td>
<td>29.8±9.5%</td>
</tr>
<tr>
<td>Vibration</td>
<td>1211±92%</td>
<td>298±69%</td>
<td>42.9±17.3%</td>
</tr>
<tr>
<td>LIPUS</td>
<td>1263±60%</td>
<td>341±57% *</td>
<td>52.0±11.2% *</td>
</tr>
<tr>
<td><strong>Study B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8wk study)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1940±149%</td>
<td>423±52%</td>
<td>51.0±10.5%</td>
</tr>
<tr>
<td>Vibration</td>
<td>1944±362%</td>
<td>433±104%</td>
<td>55.7±21.9%</td>
</tr>
<tr>
<td>LIPUS</td>
<td>2120±268%</td>
<td>439±101%</td>
<td>57.7±25.3%</td>
</tr>
<tr>
<td>Combined</td>
<td>1657±411% †</td>
<td>437±32%</td>
<td>70.0±18.7%</td>
</tr>
</tbody>
</table>
No significant differences were noted for maximum pushout load of study B (Figure 57).

The energy to failure of the combine treatment group was 37.2% greater than the control of study B numerically but not statistically different. The combined treatment group stiffness was significantly lower than LIPUS (21.8%, P = 0.003).
Figure 57: Max load to failure of implants in study B.

Fluorescence Evaluation

No differences were observed in the labelled mineralizing area around the implant at two or three weeks (Table 12). Also, no statistical differences in bone remodeling were seen at the later time points of five and six weeks. The vibration, LIPUS and combined treatment groups displayed more labelled mineralizing area than the control (77.0%, 41.5% and 60.8% respectively) which didn’t reach statistical significance due to the high standard deviation at six weeks as seen in Figure 58.
Table 12: Histomorphometric results of % fluorochrome labeled within 250 µm of the implant.

<table>
<thead>
<tr>
<th></th>
<th>Study A (4wk study)</th>
<th>Study B (8wk study)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calcein Green</td>
<td>Alizarin Red</td>
</tr>
<tr>
<td>Control</td>
<td>6.4±1.5%</td>
<td>6.4±2.3%</td>
</tr>
<tr>
<td>Vibration</td>
<td>7.2±0.8%</td>
<td>4.1±1.7%</td>
</tr>
<tr>
<td>LIPUS</td>
<td>6.8±2.7%</td>
<td>4.4±2.1%</td>
</tr>
<tr>
<td>Combined</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Figure 58: New bone formation within 250 µm of implant revealed by fluorescence. 2 & 3 week results from study A and 5 & 6 week results from study B.
**Histological Staining**

BIC, calculated using histomorphometric analysis, was increased at four weeks (study A) in both LIPUS and vibration groups relative to the control group [41.8% (P = 0.004) and 55.9% (P = 0.018) respectively, Figure 59]. Differences were not detected between treatment groups in the BIC histomorphometric analysis at eight weeks (study B, Table 13). No significant differences were detected in BV/TV within 250 µm of implants between treatment groups for study A (Table 13). The BV/TV of the vibration group of study B was significantly higher than that of the other groups as seen in Figure 60, [38.9% relative to control (P < 0.001)].

![Graph showing BIC (%) for Control, Vibration, and LIPUS groups](image)

*Figure 59: % BIC per histological results for Study A.*
Table 13: Histomorphometric results of acid fuchsine stained specimens. * = significantly different than control, ‡ = significantly different than vibration.

<table>
<thead>
<tr>
<th></th>
<th>Study A (4wk study)</th>
<th>Study B (8wk study)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BV/TV (%)</td>
<td>BIC (%)</td>
</tr>
<tr>
<td>Control</td>
<td>32.7±4.1%</td>
<td>17.9±4.1%</td>
</tr>
<tr>
<td>Vibration</td>
<td>35.1±5.8%</td>
<td>25.4±4.5% *</td>
</tr>
<tr>
<td>LIPUS</td>
<td>37.9±4.4%</td>
<td>27.9±7.3% *</td>
</tr>
<tr>
<td>Combined</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Figure 60: BV/TV per acid fuchshine stained histological results of Study B.
Discussion:

LIPUS has been shown to upregulate mRNAs (alkaline phosphatase, osteocalcin, insulin-like growth factors and bone sialoprotein) involved in bone healing and act on a cellular level at each phase of bone healing. However, prior LIPUS studies have failed to demonstrate beneficial effects in intramedullary implant mechanical stability. This study demonstrates that LIPUS treatments result in accelerated bone healing and lead to improved early osseointegration with significantly increased axial load capabilities and energy to failure. These benefits are most likely a result of the increased bone surrounding the implant in the form of BV/TV seen through the µCT results and the BIC revealed by the histomorphometric evaluations of the acid fuchsine stained specimens. Interestingly, these benefits were absent at midterm osseointegration as the native biological bone healing process catches up. There appears to be an upper implant mechanical stability limit, which is reached earlier with the LIPUS treatments in the utilized implantation model demonstrating its use as a therapeutic tool for reducing amputees’ rehabilitation time. It was noted that the cortical thickness at the diaphysis was lower in the LIPUS treatment group relative to the control. However, this statistical difference isn’t thought to be meaningful with the mean thickness difference being lower than 10% and there were no other differences at the diaphysis between treatment groups.

LMHF vibration has been demonstrated as a successful treatment for fracture healing with several animal studies showing increased callus formations and higher mineral contents. The preliminary study—Osseointegration of Additively Manufactured and Threaded Implants in Rats through Whole Body Vibration—revealed an increase in BV/TV around osseointegrated implants with 45 Hz WBV at 0.6 g which is supported by several prior studies. However, our study failed to demonstrate significant improvements in mechanical
stability of the implant as shown by other studies\textsuperscript{46,122}. It was hypothesized that the transcutical model provided too much initial stability limiting the sensitivity of the study to osseointegration benefits. The dual limb local vibration stimulators of the current study were tuned to deliver similar LMHF vibration to the implant site as our optimal WBV determined in the preliminary study. An increase in BIC was seen at 4 weeks and BV/TV and BIC at 8 weeks with evaluation of the stained specimens and both μCT and evaluation of the stained specimens respectively. Interestingly, the numerical increase in max load to failure of the vibration group at 4 weeks failed to reach a statistical difference to the control group. At 8 weeks there was no difference in mechanical stability of the vibration group relative to the control group. These findings further confirm the marginal benefits to mechanical stability realized through vibration stimulation. Nevertheless, vibration may aid in preventing bone resorption during the rehabilitation period while the limb is not yet loaded.

Combined treatment of LIPUS and locally applied vibration failed to demonstrate an improvement in midterm osseointegration with no significant increases in bone morphology or mechanical stability. Contrary to expectations, combined treatment results in a decrease of the bone-implant stiffness. Studies have demonstrated that low bone-implant stiffness can lead to micromotions\textsuperscript{159,160} which have been shown to result in undesirable fibrous bone ingrowth\textsuperscript{30}. However, it is unclear if lower bone-implant stiffness would be desirable once full osseointegration is achieved with mature bone to distribute the energy of an impact load over a longer time reducing peak loading.

This study was not without limitations. Reaching the mechanical limits of the bone-implant interface was not anticipated in early osseointegration and in an attempt to reduce the number of animals used in this study, combined treatment effects were only investigated for
midterm osseointegration. Because the mechanism of bone healing through vibration and LIPUS isn’t completely understood, a cumulative benefit of early osseointegration with combined treatments of LIPUS and vibration may still be realized and should be investigated.

Also, no long term mechanical or bone density benefits of the increased BV/TV and BIC seen midterm from locally applied vibration treatments were investigated. It should be determined if these increases persist after treatment stops and what their long-term effects are on mechanical stability as the bone matures.

This study didn’t investigate the cumulative effects on the periosteum of combined LIPUS and vibration treatments for aiding osseointegration of the proximal part of the tibial component of a total knee replacement, dental implants or collared implants for maintaining distal loading of the residual bone. Another limitation of this study is that a larger variation than anticipated in the fluorescent labeled mineralized area may have prevented detecting a statistical increase resulting from LIPUS, vibration or combined treatments.

Finally, the AM implants were designed to fit a range of intramedullary canals without concern for the axial orientation of the implant during surgical placement to facilitate mass production and ease of surgery. A key benefit of using AM implants is that they can be customized to match patient specific anatomy. In a clinical environment, the implant would be designed to more closely interface with the endosteal surface, which may affect the bone healing response to treatments and the resulting mechanical stability as studies suggested vibration primarily affects cancellous bone and the endosteal surfaces by acting on osteoblasts or precursor cells.
Conclusion:

Although vibration demonstrated improved osseointegration of titanium implants in the bone canal with increased BIC; this study establishes LIPUS as superior for accelerating early osseointegration through increased axial force to fail the bone-implant interface. This demonstrates the potential benefit of LIPUS as a therapeutic tool for reducing amputees’ rehabilitation period. The benefits of LIPUS are no longer present during midterm osseointegration as the native biological ingrowth process appears to catch up. However, vibration therapy continues to increase the amount of bone around the implant without affecting mechanical properties of the bone-implant interface. Interestingly, combining the treatments resulted in decreased bone-implant stiffness while maintaining axial load to failure. A lower stiffness at the bone-implant interface may reduce peak impact loading by distributing the energy as the interface elastically flexes. A combination of LIPUS with locally applied vibration will be beneficial for reducing the incidence of accidental bone-implant overloading. Vibration has demonstrated its therapeutic benefits for increasing bone around an implant and can potentially decrease implant loosening preventing the need for revision artificial joint surgeries.

Acknowledgements:

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Additional Contributions: Thanks goes to Seth Bollenbecker for his countless hours of animal handling in the 2nd cohort; developing radiographs; collection and preparation of specimens for mechanical, $\mu$CT and histologic evaluation; and imaging and evaluation of histological slides.

We thank the Small Animal Imaging Facility at the UNC Biomedical Imaging Research Center for providing the $\mu$CT imaging service, and the imaging core is supported in part by an NCI cancer core grant, P30-CA016086-40.

Recognition is also due to Stephen Kallianos for his many hours of animal handling in the first cohort. Appreciation is also extended to Tyler Scheviak for developing Matlab code to calculate cortical thickness. Gratitude also goes to Bioventus for loaning the Exogen LIPUS units and donating the necessary ultrasound gel for the study. Appreciation for instruction and use of the EXAKT grinding and cutting system goes to the Histology Lab in the Department of Population Health and Pathobiology, College of Veterinary Medicine, NCSU.
Chapter 6: Conclusion and Future.

Summary of Treatments:
This research focused on shortening the rehabilitation time for transdermal osseointegrated prosthetics while also increasing the bone density of the amputated limb. The results from this study pave the path towards greatly improving the post-surgical rehabilitation of a wide variety of traditional osseointegrated implants.

Other studies have shown that LIPUS accelerates bone healing by upregulating mRNAs\textsuperscript{14,15} and act on a cellular level at each phase of bone healing\textsuperscript{53}. Prior studies investigating the effects of LIPUS on improvement in osseointegration have been focused on transcortical implantation targeting dental implants leaving it unclear as to whether the stimuli can penetrate the intramedullary canal to improve osseointegration\textsuperscript{16,59-61}. These prior LIPUS studies have also failed to demonstrate a benefit in implant mechanical stability. However, this study has clearly demonstrated that the stimuli of LIPUS treatments does in fact create a beneficial effect resulting in improved early osseointegration with significantly increased bone formation and mechanical stability. Interestingly, the beneficial gains of LIPUS at the early healing time point are absent at midterm osseointegration as the native biological bone healing process catches up. There appears to be an upper implant mechanical stability limit which is reached earlier with the LIPUS treatments demonstrating its use as a therapeutic tool for reducing amputees’ rehabilitation time.

LMHF ground-based vibration has been shown to increase bone mass or volume in normal animals\textsuperscript{7,41-44} and increase bending strength and bone formation in animal models\textsuperscript{8,45} through upregulating gene expression related to chondrogenesis and osteogenesis\textsuperscript{6}. While several studies have investigated various treatment frequencies of WBV, the optimal vibration amplitude
for accelerating bone formation hadn’t been previously identified\textsuperscript{7,8}. This study revealed that BV/TV around osseointegrated implants is influenced by 45 Hz WBV with maximal bone formation occurring at 0.6 g. Impaired bone formation was expected at high vibration levels (> 1 g) due to excessive micromotion. Unexpectedly, BV/TV was not impaired at the highest vibration amplitude of 1.2 g but was increased relative to the control group.

Past vibration studies have reported increased bone ingrowth into porous coated titanium alloy implants in transcortical turkey and intramedullary rat models\textsuperscript{46,122}. Osseointegration studies have shown potential in promoting early ingrowth through LMHF vibration treatment; however, have failed to establish an improved mechanical stability\textsuperscript{10,11}. The transcortical rat model of our preliminary study, while revealing an increased BV/TV around an osseointegrated implant, did not produce significant improvements or impairments in bone ongrowth or mechanical stability with LMHF vibration at early or late time points. The transcortical model provides good initial stability, which may limit the sensitivity to mechanical osseointegration enhancements from LMHF vibration.

Previous studies have demonstrated LMHF vibration as a successful treatment for fracture healing with increased callus formations\textsuperscript{34,35} and higher mineral contents\textsuperscript{13,33,36}. While a couple of studies have demonstrated improvements in bone formation through locally applied vibration, no studies have investigated the effects of locally applied vibration on the mechanical stability of osseointegrated implants\textsuperscript{13,33}. A local vibration stimulator would be beneficial in the clinical application of enhancing osseointegration of percutaneous implants. This study validated an improvement in bone formation around an osseointegrated implant at both early and mid-term rehabilitation time points with locally applied LMHF vibration tuned to deliver comparable amplitudes at the implant as delivered with the optimal WBV amplitude discovered in the
preliminary study. Interestingly, the numerical increase in max load to failure of the vibration group at the early time point failed to reach a statistical difference to the control group. At mid-term rehabilitation, there was no difference in mechanical stability of the vibration group relative to the control group.

The marginal benefits to mechanical stability realized through locally applied vibration corroborates the lack of stability benefits generated from whole body vibration. Nevertheless, vibration treatment throughout the study increases bone formation around osseointegrated implants. While vibration does not improve bone-implant integration, an optimized LMHF vibration amplitude may aid in preventing bone resorption during the rehabilitation period while the limb is not yet loaded.

Contrary to the expected cumulative benefits through combined treatments of LIPUS and locally applied vibration, this study failed to demonstrate an improvement in midterm osseointegration with no significant increases in bone morphology or mechanical stability. Although LIPUS was demonstrated as superior to vibration for accelerating early osseointegration through increased mechanical and bone formation, it may still be beneficial to clinically treat patients receiving transcutaneous osseointegrated implants with both LIPUS and LMHF vibration due to bone resorption during the non-weight bearing period of the rehabilitation.

**Summary of Implant Designs:**

The preliminary study revealed through both mechanical and histomorphometric evaluations that EBM implants provide superior connection to bone compared to machine-threaded implants. Torsional implant failure and bone-implant stiffness were both significantly increased with the
use of EBM implants relative to threaded implants. Histomorphometric measurements also
demonstrated increased early bone formation around the implant through an unknown
mechanism. This discovery is supported by other research that has revealed that EBM threaded
implants osseointegrate more securely than machine threaded implants\textsuperscript{103}. This improved
mechanical stability highlights the benefits of using EBM not only to produce custom designs,
but also custom surfaces for osseointegration.

Surface texture of an implant has been shown to greatly affect osseointegration. The
coarse textured surface design used in both phases of the pilot study yielded superior fixation
properties compared to the fine textured counterpart demonstrating an increased interlocking
area between the bone and implant for a given implant diameter. Other studies have shown that
altering the surface of an implant improves its mechanical stability, increasing implant resistance
to removal. Medium-particle (75 µm) blasting resulted in improved torque resistance compared
to fine (25 µm) and coarse (250 µm) particle blasting, confirming that a moderate surface texture
results in better mechanical stability\textsuperscript{68,69}. AM manufactured osseointegrated implants inherently
have a moderate surface texture with a roughness of 35 ±12 µm\textsuperscript{64} and can be manufactured to
match an infinite range of residual bone geometries making them a logical consideration for
osseointegrated implants.

Modification of AM implant surface roughness doesn’t seem to affect BV/TV or BIC.
This finding supports previous observations that bone-implant contact is unrelated to surface
roughness\textsuperscript{68}. Furthermore, BV/TV and BIC were not found to not influence fixation strength of
AM implants in the pilot study. These results are supported by another study demonstrating a
significant increase in max push-out force with no histomorphologic differences in BV/TV or
BIC\textsuperscript{99}.  

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The improved mechanical properties don’t result from an increase in bone contact area. Instead, the different mechanical properties appear to be a reflection of how the surface profile interacts with the ongrown bone under torsional loading. Interstices in the AM design provide increased surface area normal to the shear force at the bone-implant interface. Both the EBM and the coarse DMLS designs provide superior interlocking mechanism between the bone and implant through larger interspersed areas normal to the shear load relative to the compared implants. This emphasizes that patient specific implants with high geometric detail can be fabricated while maintaining a surface roughness for superior osseoincorporation through DMLS due to its superior spatial resolution.

Cortical bone may provide superior mechanical stability in osseointegrated implants compared to trabecular bone. Matching the implant to the residual bone would greatly increase the contact with the cortical shell and therefore should also enhance mechanical stability of osseointegration highlighting the need for patient specific implants.

**Future Research:**

There were several limitations to this study upon which many additional studies should be built in order to fully understand the impact of treatments prior to moving to a clinical study. One such limitation was that the mechanical limits of osseointegration in the “NSF Study” were apparently reached by eight weeks as the native biological bone healing process caught up in the control group demonstrated by the minimal increase in pushout resistance of the LIPUS group between the 4 and 8-week time points. A cumulative effect of the treatments may still be seen at an early rehabilitation time point since the mechanism of bone healing through vibration and LIPUS isn’t completely understood. A study could be undertaken comparing the superior early
osseointegration benefits of LIPUS to the combined effects of local vibration and LIPUS at four weeks relative to a control group. This study could be carried out in conjunction with one of the proposed implant studies below.

Another limitation to this study was the lack of differentiation in tissues targeted during the histological staining. While toluidine blue stains the tissues high in DNA and RNA such as the cartilage\textsuperscript{62,161}, we found it to be unsatisfactory for producing substantial contrast between the bone and interstitial space in the “Preliminary Study” for determining BV/TV and BIC. Acid fuchsin acts on the cytoplasm and highlights the collagen matrix of the bone and produced satisfactory contrast for bone evaluation\textsuperscript{131,162}. Therefore, acid fuchsin was used throughout the study for histomorphological evaluation of BV/TV and BIC. The technique in this study did not offer a means of differentiating newly formed bone and mature mineralized bone. Von Kossa stain has been demonstrated to target the calcium mineral deposits in bone indicating bone maturity\textsuperscript{163}. The increased BV/TV and BIC in the vibration group of the midterm “NSF Study” didn’t result in any additional mechanical stability benefits. It would be beneficial to examine the specimen slides from the midterm groups using von Kossa stain to determine the stage of bone development in the treated groups relative to the control. Determining the extent of mineralization would give a better understanding of the bone maturity at eight weeks as a result of the treatments.

Furthermore, an additional study should be conducted to look at the long term mechanical and bone density benefits of the increased BV/TV and BIC seen from locally applied vibration treatments in the intramedullary rodent model. This study fails to answer the question as to whether the increases in BV/TV and BIC persist after treatment stops and what their long-term effects are on mechanical stability as the bone matures. This proposed study should also
investigate whether long-term vibration treatment will retard bone mineralization resulting in lower bone-implant stiffness. Studies have established that low bone-implant stiffness leads to micromotions\textsuperscript{159,160} which have been shown to result in undesirable fibrous bone ingrowth\textsuperscript{30}.

Concerns have been raised by reviews of submitted manuscripts that this work doesn’t use an ovarectomized (OVX) rodent model as many implant osseointegration studies have done\textsuperscript{7,122,148}. An OVX model simulates an estrogen deficiency experienced with postmenopausal osteoporosis\textsuperscript{164}. An estrogen deficient OVX subject would experience a higher rate of bone loss relative to healthy subjects on account that estrogen has been shown to inhibit osteoclasts which are responsible for bone resorption\textsuperscript{165}. Contrary to the reviewers’ concern that not using an OVX model lacks the required challenge to bone healing to assess treatment benefits, many other implant osseointegration studies have chosen to use intact animals\textsuperscript{62,147,149,166,167}. While an OVX model may be appropriate for addressing long term fixation of osseointegrated implants as patients age\textsuperscript{168}; the primary target for percutaneous osseointegrated implants are healthy and active trauma amputees\textsuperscript{4,169}. Bone resorption and diminished bone-mineral density are seen in the residual bone of an amputee\textsuperscript{170}. The loss of muscle and bone in amputees resulting from the reduced muscle function is not predominantly due to an estrogen deficiency or the associated lack of osteoclast inhibition, but the upregulation of receptor activator for nuclear factor kappa-B ligand (RANKL) and its concomitant increase incell differentiation into osteoclasts\textsuperscript{171}. A treatment that addresses the RANKL upregulation rather than the estrogen deficiency would be a more clinically accurate model. Botulinum toxin (BoTox) has been used to induce localized muscle paralysis which resulted in rapid bone degradation due to an upregulation in RANKL\textsuperscript{172-174}. Future animal studies investigating acceleration and/or enhancements in implant osseointegration would be greatly reinforced through the incorporation of a BoTox induced
paralysis of the muscles originating and inserting into the bone associated with the implant. This BoTox challenge would better represent the muscle loss and associated bone degradation experience by an amputee than the OVX model.

While the intramedullary rodent model acts as a suitable simulation of early implant osseointegration prior to the application of external loads, it fails to provide a mechanism to investigate mid and long-term healing when the external loading is increased to the point of functional loading. A weight bearing rodent model for evaluating osseointegration as external rehabilitation loads are increased would provide additional factors effecting osseointegration such as bone remodeling, bone mineralization, fibrous ingrowth, etc. The implant should be left unloaded during the early healing period by treating the animal with BoTox to induce muscle paralysis (discussed in the prior paragraph) to mimic the clinical approach. Once initial implant stability is achieved the limb can be gradually loaded through reduction of the BoTox injections.

To surgically place the weight bearing implant, a lateral incision is made exposing the muscles of the upper hind limb. The vastus lateralis and biceps femoris are divided to gain access to the caudal-lateral aspect of the femur. The bone is then divided using a reciprocating bone saw to remove a small section of the diaphysis. The AM implant is fabricated as two mating components (distal and proximal, see Figure 61). The two halves of the femur are offset from their longitudinal axis for reaming and insertion of the proximal and distal implant halves. The limb is slightly stretched to allow assembly of the implant which snaps together locking the two halves from future separation. The mating features of the implant supports torsional loading preventing unintended relative rotation of the implant halves. The implant is fabricated with a collar on both the distal and proximal halves to osseointegrate with the residual bone$^{141}$. Both collars extend away from the bone with an octagonal cross-section for future mechanical
evaluation through torsional loading. Fluorochrome labeling and in-vivo µCT can be utilized to assess early and mid-term osseointegration. A larger rodent model, such as a rabbit, may better facilitate the development of a weight bearing implant osseointegration model for investigation of long term treatment effects.

Figure 61: Weight bearing implant concept. A. Proximal implant, B. Distal implant.
Arthrofibrosis—painful joint restriction as a result of excessive scar tissue—is often an outcome of joint surgery, trauma and immobilization. Therapies are being investigated to reverse joint contracture. Studies have investigated therapeutic vibration to treat osteoarthritis with a resulting improvement in balance and decreased pain. However, only limited case studies have been performed demonstrating decreased pain associated arthrofibrosis with no studies evaluating the therapeutic effects of vibration on injured tissue. Also, LIPUS has been shown to reduce the inflammation in the synovial membrane of the joint in animals with rheumatoid arthritis. A cumulative benefit would be expected in treating diseased joints since LIPUS and vibration act on the associated tissues through different biological mechanisms. The four week “NSF Study” provided a cursory look at the individual effects of locally applied vibration and LIPUS. Prior to isolating the femurs, the knee joint was preserved and all surrounding muscle tissue removed. A subsequent leg extension test was executed as described by Efird et al. (Figure 62). The LIPUS group responded with a 2.65 deg (Figure 63, Appendix D) increase in extension relative to the control. The vibration group experienced a 3.73 deg (P = 0.013) increase in leg extension relative to the control. These results demonstrate that there may be a clinically relevant benefit to using locally applied vibration and LIPUS as a therapeutic tool for reducing arthrofibrosis. However, it must be noted that this study was not designed to appropriately investigate the benefits of treatment on contracture. Locally applied vibration, LIPUS and combined treatments should be properly investigated in a contracture model as describe by Efird et al. to determine the individual and cumulative benefits of the treatments.
Figure 62: Leg extension setup per Efird et al.

Amputee prosthetic devices are moving towards percutaneous osseointegrated implants as an alternative to the traditional socket prosthetics. Additive manufacturing provides a means of producing osseointegrated implants that can be easily customized for patient specific anatomy. Various surface textures can also be produced through the additive manufacturing process affecting implant stability. This study proved that coarse textured surfaces provide superior bone-implant fixation compared to fine textured surfaces for titanium alloy implants. However, the specific Ra value which will result in the optimal fixation of titanium
osseointegrated implants was not identified. A future study needs to be conducted to determine the optimal roughness for implant fixation to the endosteal surface by examining Ra values above and below that of the coarse textured implant of the “NSF Study” in an intramedullary rodent model.

This study has answered several questions. However, numerous questions have arisen from this research. It is now clear that vibration can be applied locally to increase BV/TV. Nevertheless, further studies need to be carried out to determine long term effect of vibration. LIPUS accelerates osseointegration and should be investigated further in a clinical setting. AM osseointegrated implants provide superior mechanical stability. Yet the optimal roughness of the bone-implant interface has yet to be determined. The research undoubtedly points to a clear path for accelerating post-surgical rehabilitation and mechanical stability of transcutaneous osseointegrated implants as well as a wide variety of traditional osseointegrated implants.
References:


47. Rubin CT, Sommerfeldt DW, Judex S, Qin Y. Inhibition of osteopenia by low magnitude, high-frequency mechanical stimuli. *Drug Discov Today*. 2001;6(16):848-858.


69. Wennerberg A, Albrektsson T, Andersson B. Bone tissue response to commercially pure
titanium implants blasted with fine and coarse particles of aluminum oxide. Int J Oral Maxillofac
Implants. 1996;11(1).

70. Klokkevold PR, Nishimura RD, Adachi M, Caputo A. Osseointegration enhanced by
chemical etching of the titanium surface. A torque removal study in the rabbit. Clin Oral

71. Wong M, Eulenberger J, Schenk R, Hunziker E. Effect of surface topology on the

72. D'Lima DD, Lemperle SM, Chen PC, Holmes RE, Colwell CW. Bone response to implant

73. Iwaya Y, Machigashira M, Kanbara K, Miyamoto M, Noguchi K, Izumi Y. Surface

74. Ban S, Iwaya Y, Kono H, Sato H. Surface modification of titanium by etching in

75. Jeyapalina S, Beck JP, Bachus KN, Williams DL, Bloebaum RD. Efficacy of a porous-
structured titanium subdermal barrier for preventing infection in percutaneous osseointegrated


93. Allvac nd. *Titanium Ti6Al4V (grade 5) STA.*


96. Hosseini S. *Fatigue of ti-6Al-4V.* INTECH Open Access Publisher; 2012.


169. Usufzy P. Rare procedure in US promises amputee quality of life. 


Appendix A: Evaluation of mid-shaft cortical thickness.

Steps to Use “cbt.m” to Determine the Average Cortical Bone Thickness

1. In mimics create a new mask using the bone thresholding values and name it “entire bone”
2. Use “region grow” and select the cortical bone region of the mask to separate this part of the mask, name this new mask “cortical bone”
3. In mimics, make sure the “cortical bone” mask is showing, and only this mask
4. Hide all other masks
5. Click on a mask other than the “cortical bone” mask, to make the mask opaque
6. Measure the distance on the axial slice using “Measure”->”Distance” on the horizontal and vertical axes from endpoint to endpoint
7. Store the heights in the first column and widths in the second column of a spreadsheet named “heightwidths.xlsx”, corresponding to the ascending numeric order of the scan number
8. Save this excel file in the MATLAB folder on your computer
9. Go to file-> Export -> “BMP/JPEG...”
10. Make sure “Axial” and “Current” are selected
11. On the dropdown choose “bmp” as the file type
12. Choose the folder for the image to be exported to
   a. Put in the “MATLAB” folder
13. Click “apply”
14. Open matlab
15. Type “open cbtall”
16. Check line 6 and make sure the file name inside fileparts(which()) is one of the desired images to be analyzed

17. Run the file by pressing “f5”

18. The figure that pops up shows the steps the program took to analyze the bone thickness (for cbt.m only)

19. Close out of the figure, and in matlab look in the Command Window to see a statement stating the average cortical thickness of the scan (for cbt.m only)

NOTES

* name the .bmp files by just the number for the scan

* scans with the number less than 10 should have a 0 in front, for ex scan 7 is ‘07.bmp’

* to be safe, sort the matlab folder in ascending order in windows explorer before executing code

* make sure that the # of rows in “heightwidths.xlsx” is equal to the # of *.bmp in folder

MATLAB code for cbtall.m:

```matlab
clear
clc
close all

%% Get file
folder = fileparts(which('01.bmp')); % Determine where CT folder is
files = dir(fullfile(folder,'*.bmp')); % gets all .bmp files
```
 [~, index] = sort({files.name}); \% sorts the files folder in ascending order

imageFiles = files(index); \% creates imageFiles based off of the sorted array

\%\% Determine Size of Image

image = []; \% creates empty matrix

for k = 1:length(imageFiles);
    image{k} = imread(imageFiles(k).name); \% creates an image array to contain all images in folder
end

\%\% read in excel sheet and get dimensions

hw = xlsread('heightwidths.xlsx'); \% reads in excel sheet

heights = hw(:,1); \% mm, measured from top left corner to bottom left corner in mimics

widths = hw(:,2); \% mm, measured on mimics from bottom left corner to bottom right corner

sizeimage = [];

sizeim = [];

pixelheight = [];

pixelwidth = [];

mmperpixel = [];

for c = 1:numel(imageFiles);
    sizeimage(c) = size(image{c}); \% creates an array containing the size coordinates
sizeim = sizeimage{c}; % size of current image
pixelheight(c) = sizeim(1); % height of whole image in pixels
pixelwidth(c) = sizeim(2); % width of whole image in pixels
mmperpixel(c) = (heights(c)./pixelheight(c) +
widths(c)./pixelwidth(c))./2; % takes the average of two mm/pixel conversions to get the final conversion value
end

%% Convert Image to BW (binary)

BW = [];
for b = 1:numel(image);

    BW{b} = im2bw(image{b}); % makes binary image array
end

%% Segment out cortical bone

L = [];
num = [];
Ltemp = [];
pixperobj = [];
ind = [];
cortical = [];
for p = 1:numel(BW);

    [L{p}, num{p}] = bwlabel(BW{p}, 8); % determines different white regions using 8-point connectivity

    Ltemp = L{p};
    pixperobj{p} = sum(bsxfun(@eq,Ltemp(:),1:num{p}));
 [~, ind{p}] = max(pixperobj{p});

cortical{p} = (L{p}==ind{p}); % creates an array of just the cortical bone images
end

%%% fill in holes
for g = 1:numel(cortical);
    filled{g} = imfill(cortical{g}, 'holes'); % Fill all holes using imfill
    holes{g} = filled{g} & ~cortical{g}; % Identify the hole pixels using logical operators
    bigholes{g} = bwareaopen(holes{g}, 200); % Use bwareaopen on the |holes| image to eliminate small holes
    smallholes{g} = holes{g} & ~bigholes{g}; % Use logical operators to identify small holes
    corticalfilled{g} = cortical{g} | smallholes{g}; % Use a logical operator to fill in the small holes in the original image:
end

%%% determine center of mass
for y = 1:numel(corticalfilled)
    s{y} = regionprops(corticalfilled{y}, 'centroid'); % gives matrix location of centroid
    centroids{y} = cat(1, s{y}.Centroid); % gives location of centroid in image
coordinates = centroids(y); %gets centroid of each image
xcenter(y) = coordinates(1); %x coordinate of centroid
ycenter(y) = coordinates(2); %y coordinate of centroid
end

%% Calculate Average Cortical Thickness
for r = 1:numel(corticalfilled);
    outer = [];
    inner = [];
    boundaries{r} = bwboundaries(corticalfilled{r}); %creates boundaries for inner and outer shell
    %for outer points
    boundary = boundaries{r}; %?
    outer = boundary{1}; %points on the outer boundary
    xouter{r} = outer(:,2); %x values of outer boundary points
    youter{r} = outer(:,1); %y values of outer boundary points
    distances{r} = sqrt((xouter{r} - xcenter(r)).^2+(youter{r} - ycenter(r)).^2); %euclidean distance of each outer boundary point to centroid
    avg(r) = mean(distances{r}); %avg pixel distance to outer boundary
    avgmm(r) = mmperpixel(r)*avg(r); %average distance in mm to outer boundary
    %for inner points
    inner = boundary{2}; %points on the inner boundary
xinner(r) = inner(:,2); %x values of inner boundary points
yinner(r) = inner(:,1); %y values of inner boundary points
distances2{r} = sqrt((xinner(r)-xcenter(r)).^2+(yinner(r)-ycenter(r)).^2); %euclidean distance of each inner boundary point to centroid
avg2(r) = mean(distances2{r}); %average pixel distance from centroid to outer boundary
avgmm2(r) = mmperpixel(r)*avg2(r); %average mm distance from centroid to outer boundary
avgthickness(r) = abs(avgmm(r)-avgmm2(r)); %computes average thickness for each scan
end

%% Export Excel Sheet
filename = 'corticalthickness.xlsx'; %creates the desired file name of the excel spreadsheet
xlswrite(filename, avgthickness'); %created the excel spreadsheet with average thickness data in it

for t = 1:numel(avgthickness)
    fprintf('The average cortical thickness of %s is %f mm \n', imageFiles(t).name, avgthickness(t)); %outputs average thickness for each scan
end
Appendix B: Evaluation of in-vivo double fluorochrome labeling.

In ZEN Blue

- Activate Channel DsRed and “save as w/ options” as xxx_r.tif.
- Activate Channel EGFP and “save as w/ options as” xxx_g.tif.
- Turn Range Indicator on and select channel which best highlights implant and “save as w/ options” as xxx_i.tif.

In Microsoft Windows Paint

- Open xxx_i.tif.
- Manually isolate implant from surrounding negative space.
- Fill implant with red.
- Save as xxx_i2.tif.

In Adobe Photoshop CS5

- Open xxx_i2.tif.
- Right click on layer and “duplicate layer”.
- Select lower layer: Select→All; Image→Adjustments→Desaturate;
  Select→ColorRange; click on implant and set “Selection Preview”=”White Matte”; OK;
  Select upper layer; Overlay as shown below.

- Turn off layer visibility of lower layer.
- Re-save xxx_i2.tif (set “layer compression”=”discard layers”).
In ImageJ 1.51j

- Open xxx_r.tif, xxx_r.tiff and xxx_i2.tif.
- Determine length of implant in pixels “W”
- Set the scale through Analyze->Set Scale-->
- Enter “Distance in pixels: (“W”/20)”, “Know distance: 1”, “Pixel aspect ratio: 1” and “Unit of length: mm”. Repeat for all three images.
- Get at proper zoom to see entire implant in xxx_i2.tif.
- Make sure cursor is centered at desired implant so zoom focuses in on the desired region.
- Goto “ Analyze/Tools/ROI Manager/”.
- Using wand: select implant in xxx_i2.tif.
- Hit “t” to add this line to ROI manager list and rename “Implant”.
- Using rectangle tool: create rectangle that covers length (from end of boss to proximal end) and proximal height of implant excluding the enlarged distal end (approximately 1.5mm long).
- Increase height of rectangle by 0.5mm.
- Next with the rectangular ROI still selected, select “Edit/Selection/Rotate/” and specify angle to rotate the rectangular ROI so that its width axis is parallel with the implant interface. You may have to do several rotations to get it as close to parallel as possible.
- Next, by clicking within the rectangular ROI and dragging center rectangle on implant and hit “t” to add to ROI manager list and rename “Box”.

![Image showing rectangular ROI and lines](image-url)
Select “Implant” in ROI manager; Edit → Selection → Make Inverse; hit “t” and rename “Implant^1”

Select “Box” & “Implant^1” in ROI manager; right click and chose “and”; hit “t” and rename “ROI”

Select all ROI’s in ROI manager, right click and save as zip file.

Save as xxx_i2.tif

Activate window with xxx_r.tif.

Save as xxx_r2.tif

Image → Adjust → Color Threshold → select “Dark background”, set: Hue 0-255; Saturation 0-255; Brightness 15-255.

Image → Type → 8bit.

Image → Adjust → Threshold (85-85).

Select “ROI” in ROI manager.

Goto “Analyze/Set Measurement/” and make sure “area”, “area fraction” and “add to overlay” are selected.

With the area desired to be measured highlighted in the ROI manager list, click on the “Measure” button and the area of each region should appear in the Results window.

Send results to spreadsheet and label appropriately (remember to delete any unintended areas from the spreadsheet).

Repeat for window with xxx_g.tif.
Appendix C: Evaluation of ex-vivo acid fuchsine stained specimens.

In ImageJ 1.51j

- Open xxx.tif.
  - Image → Adjust → Color Threshold → select “Dark background”, set: Hue 0-255;
    Saturation 0-255; Brightness 0-15.
  - Image → Type → 8bit
  - Image → Adjust → Threshold (85-85)
  - Save as xxx_1.tif

In Microsoft Windows Paint

- Open xxx_1.tif.
- Fill implant with red.
- Save as xxx_2.tif.

In Adobe Photoshop CS5

- Open xxx_2.tif.
- Right click on layer and “duplicate layer”.
- Select lower layer: Select → All; Image → Adjustments → Desaturate;
  Select → ColorRange; click on implant and set “Selection Preview” = “White Matte”;
  OK; Select upper layer; Overlay as shown below.

- Turn off layer visibility of lower layer.
o Save as xxx_3.tif (set “layer compression”=“discard layers”).

In ImageJ 1.51j

o Open xxx_3.tif.

o Determine length of implant in pixels “W”

o Set the scale through Analyze--->Set Scale-->

o Enter “Distance in pixels: (“W”/20)”, “Know distance: 1”, “Pixel aspect ratio: 1” and “Unit of length: mm”. Repeat for all three images.

o Image→Type→8bit.

o Image→Adjust → Threshold (100-100).

o Process→Binary→Options: Iterations=2; Count=1; EDM Output=Overwrite; Do=Dilate.

o Get at proper zoom to see entire implant.

o Make sure cursor is centered at desired implant so zoom focuses in on the desired region.

o Goto “ Analyze/Tools/ROI Manager”.

o Using wand: select implant.

o Hit “t” to add this line to ROI manager list and rename “Implant”.

o Using rectangle tool: create rectangle that covers length (from end of boss to proximal end) and proximal height of implant excluding the enlarged distal end (approximately 1.5mm long).

o Increase height of rectangle by 0.5mm.
Next with the rectangular ROI still selected, select “Edit/Selection/Rotate/” and specify angle to rotate the rectangular ROI so that its width axis is parallel with the implant interface. You may have to do several rotations to get it as close to parallel as possible.

Next, by clicking within the rectangular ROI and dragging center rectangle on implant and hit “t” to add to ROI manager list and rename “Box”.

Select “Implant” in ROI manager; Edit→Selection→Make Inverse; hit “t” and rename “Implant^-1”.

Select “Box” & “Implant^-1” in ROI manager; right click and chose “and”; hit “t” and rename “ROI”.

Make sure no ROI is selected; Process→Binary→Options: Iterations=24; Count=1; EDM Output=Overwrite; Do=Dilate (be patient!).

Using wand: select dilated implant.

Hit “t” to add this line to ROI manager list and rename “Implant+25um”.

Select “Implant+25 um” & “ROI” in ROI manager; right click and chose “and”; hit “t” and rename “BIC”.

Select all ROI’s in ROI manager, right click and save as zip file.

Reopen xxx.tif.

Save as xxx_4.tif.

Set the scale through Analyze--->Set Scale-->.
o Enter “Distance in pixels: (“W”/20)”, “Know distance: 1”, “Pixel aspect ratio: 1” and “Unit of length: mm”.

o Image→ Adjust → Color Threshold → select “Dark background”, set: Hue 180-255; Saturation 0-250; Brightness 25-255.

o Image→ Type→ 8bit.

o Image→ Adjust → Threshold (85-85).

o Select “ROI & BIC” in ROI manager.

o Goto “Analyze/Set Measurement/” and make sure “area”, “area fraction” and “add to overlay” are selected.

o With the areas desired to be measured highlighted in the ROI manager list (ROI & BIC), click on the “Measure” button and the area of each region should appear in the Results window.

o Send results to spreadsheet and label appropriately (remember to delete any unintended areas from the spreadsheet).
Appendix D: Leg extension test for locally applied vibration and LIPUS treatments at 4 weeks.

Figure 63: Leg extension of tibia beyond 90 degrees of femur.