Mechanisms of mechanically induced Osteoclastogenesis

in a novel *in vitro* model for bone implant loosening

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Linköping University Medical Dissertation No. 1696

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Linköping, Sweden 2019

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During the financial year 2015/16, Cornelia Bratengeier held the position of the Treasurer in DOMFIL, a post-grad section within Consensus which in turn is the student union organizing all students at the Faculty of Health Sciences at Linköping University.

Printed by LiU-Tryck, Linköping, Sweden, 2019

ISBN: 978-91-7685-014-5

ISSN: 0345-0082

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Abstract

Total joint arthroplasty is the primary intervention in the treatment of end-stage osteoarthritis. The majority of joint arthroplasties will last the whole lifetime. However, in some patients, the replacement will fail during their lifetime requiring a revision of the implant. These revisions are strenuous for the patient and costly for health care. To avoid a revision, therefore, is the uttermost goal of modern orthopedics. Joint replacement at a younger age, in combination with a more active lifestyle, increases the need for an early revision of the joint prosthesis. The main reason for revision surgeries is aseptic loosening, a condition where the prosthesis is loosening due to bone degradation at the peri-prosthetic interface in the absence of infections. The most well-established pathological mechanism for aseptic loosening is related to wear particles, generated from different parts of the prosthesis that will trigger bone degradation and bone loss. In addition, early micromotions of the prosthesis and resulting local pressurized fluid flow in the peri-prosthetic interface (supraphysiological loading) have also been identified as a cause for aseptic loosening. However, it remains unknown what cells are the primary responders to supraphysiological loading, and what underlying physical, cellular and molecular mechanism that triggers osteoclast differentiation and osteolysis.

The main goal of this thesis is to shed light on three currently unknown aspects of mechanical loading-induced peri-prosthetic osteolysis, leading to aseptic loosening of orthopedic prostheses: (1) Which cells are the primary responder to supraphysiological loading? (2) What characteristics of the mechanical stimulus induce an osteo-protective or osteo-destructive response? (3) Which cellular mechano-sensing mechanisms are involved in an osteo-destructive response?

The first study focused on the creation and validation of a novel *in vitro* model for mechanical loading-induced bone degradation. This model simulates the mechanical (over)loading conditions around a loosening prosthesis, as observed in the clinics, and in animal models for mechanical induced osteolysis. Using the MLO-Y4 osteocyte-like cell line, we verified that osteocytes exposed to supraphysiological mechanical loading in our model induce differentiation of progenitor cells into osteoclasts by direct cell-to-cell communication and through the release of soluble factors. This was accompanied by the release of an increased concentration of nitric oxide and higher expression of membrane-bound RANKL, while the concentration of soluble RANKL was not altered. These findings confirm the successful translation of mechanical-induced bone loss to an *in vitro* model for bone implant loosening.

The second study investigated if myeloid progenitor cells from the hematopoietic lineage respond to supraphysiological loading and their ability to induce osteoclast formation. Myeloid progenitor cells responded to the supraphysiological mechanical loading with a rapid increase of extracellular ATP within 2 minutes. Conditioned medium of myeloid progenitors exposed to this short stimulation period had osteolytic characteristics, i.e. it induced osteoclast formation. The release of osteoclast-modulating soluble factors by the stimulated myeloid progenitors was dependent on the ATP-gated P2X7 receptor. Taken together, cells in the hematopoietic lineage are sensitive to changes in the mechanical microenvironment and the ATP-P2X7 axis is crucial for the release of osteolytic factors in response to mechanical stimulation.

The third study intended to determine the physical parameters (velocity, intensity, and duration) of the mechanical stimulus that can induce osteoclast differentiation rather than inhibit osteoclast differentiation. Myeloid progenitor cells were exposed to loading profiles with a controlled variation of peak wall shear stress rates, amplitudes, and duty cycles. In the *in vitro* model, the release of ATP and the induction of osteoclast differentiation were depended on a combination of high loading amplitude ($3.0 \pm 0.2 \text{ Pa}$), prolonged duty cycle ($\geq 22\%$), and rapid alteration of minimum/maximum loading values (square wave). These findings suggest that cells in the hematopoietic lineage respond to loading in a viscoelastic manner, needing a combination of high amplitude, loading duration, and rapid alterations in loading values to induce an osteolytic response. In addition, we confirmed the potential involvement of Piezo1 in loading profiles that modulate osteoclast formation, while SERCA2 was exclusively present in loading profiles that prevents osteoclast formation. Piezo1 might thus act as a first line of response to the mechanical loading, while SERCA2 potentially regulates the cellular response in osteo-protective loading profiles.

The fourth study determined whether differentiation of *human* mesenchymal- and hematopoietic stem cells into the osteoblastic- and osteoclastic lineage affects their ability to induce osteoclast differentiation in response to supraphysiological mechanical loading. Supraphysiological mechanical loading of human mesenchymal stem cells uniformly resulted in a strong induction of osteoclast formation, while more differentiated osteoprogenitor cells and pre-osteoblasts showed donor-specific differences in the response to supraphysiological loading. Supraphysiological mechanical loading of monocytes also stimulated osteoclast formation, while pre-osteoclasts did not release osteoclast-modulating soluble factors upon mechanical loading. This suggests that mesenchymal stem cells and monocytes are strongly

responding to supraphysiological loading and that differentiation highly influences the response of stem cells and their lineage-committed progenitor cells to mechanical loading.

In conclusion, supraphysiological mechanical loading, mimicking the peri-prosthetic pressurized fluid flow around a loosening implant, has been successfully implemented in an *in vitro* model for bone implant loosening. Using this model, we uncovered the involvement of mesenchymal stem cells and myeloid progenitor cells (monocytes) in mechanical loading-induced peri-prosthetic osteolysis. We expect that the proposed new *in vitro* model is a helpful tool to further advance the knowledge in aseptic loosening, by uncovering the mechanoresponsive cellular mechanism to supraphysiological mechanical loading. The identification of the respondent cells in mechanical loading-induced prosthetic loosening gives the opportunity to deliver targeted treatment strategies. Furthermore, identifying the physical parameters that define the shift towards an osteo-destructive response emphasizes the importance of the prosthetic design and surgical technique to reduce mechanical loading-induced bone degradation around a prosthesis.

Populärvetenskaplig sammanfattning

Artros är en smärtsam, progressiv, degenerativ ledsjukdom som försämrar ledernas funktionalitet i till exempel höft och knä. Detta har en enorm inverkan på livet för både människor i arbetande ålder och för de äldre, då artros är rankad som den elfte ledande orsaken till handikapp i världen. För att återställa ett aktivt liv med ökad livskvalité krävs en total ledutbytesoperation vilket nuvarande är den enda tillgängliga medicinska behandlingen. Antalet ledutbytesoperationer ökar globalt och Sverige är bland de länder med högst incidens. En naturlig förklaring till detta är den ökade livslängden tillsammans med det faktum att patienter idag har högre krav på deras livskvalitet och därmed genomgår total ledutbytesoperation i en yngre ålder. Trots att ledartroplastik är en framgångsrik operation som kommer vara i flera år, händer det i några procent av fallen att ledprotesen blir lös. Eftersom det är så många patienter som genomför ledartroplastik blir ytterligare operationer vid misslyckat implantat ett stort problem både för patienten och för sjukvården. Den vanligaste orsaken till misslyckat implantat är aseptisk lossning – ett tillstånd när implantatet förlorar förbindningen med omgivande ben, utan några tecken på infektion. När ledutbytesoperationen misslyckas krävs en mer komplicerad och riskfylld procedur för att ersätta implantatet.

En av de många anledningarna till aseptisk lossning är instabilitet i implantatet. Om implantatet inte är korrekt integrerat med den omgivande benvävnaden, kommer det att röra sig när man går eller tränar. Benet reagerar på det rörliga implantat genom att bilda ett tunt lager av vävnad runt implantatet – vilket bildar en kapsel för att isolera implantatet ytterligare. I denna vävnad samlas lokala vätskor från benet. Vid promenad eller träning blir det snabba vätskeförflyttningar mellan ett instabilt implantat och benvävnaden som påverkar den mekaniska belastningen på benet, vilket förstör benet. Om vi vill förhindra bennedbrytning och därmed aseptisk lossning av implantat så behöver vi förstå denna process bättre.

I denna avhandling introducerar jag ett nytt verktyg till benforskningsområdet. Denna modell reflekterar den mekaniska situationen i gränssnittet mellan ben och implantat vid mekanisk instabilitet och hur det kan påverka bennedbrytning och aseptisk lossning. Fördelen med denna nya *in vitro*-modell för mekanisk inducerad bennedbrytning att man kan använda humana celler från kirurgirester och är därför inte helt beroende av försöksdjur. Vi kan använda dessa celler för att isolera en specifik cellpopulation från benet och studera deras respons (svar), på ett fokuserat och rent tillvägagångssätt. Genom att använda denna modell kunde vi öka vår kunskap gällande tre faktorer: (1) Mesenkymala stamceller och monocytter är mottagliga för

ändringar i mekanisk belastning och svarar med att öka antalet bennedbrytande osteoklaster. (2)Hög belastningsintensitet och förlängd belastningstid definierar övergången från en benbeskyddande stimulans till en benförstörande stimulans. (3)Den underliggande mekanismen till svaret på den mekaniska belastningen beror på interaktionen av adenosintrifosfat med dess receptor P2X7.

Denna nya kunskap kan hjälpa oss att fokusera behandlingen på valda cellpopulationer eller att ompröva den nuvarande designen av ledproteser för att minska effekten av bende destruktiv stimulans. Därmed kan patienter som genomgår en ledutbytesoperation njuta av en aktiv och smärtfri livsstil, med en stabil protes, under en längre period.

Populärwissenschaftliche Zusammenfassung

Arthrose ist eine schmerzhaft und fortschreitend, degenerative Gelenkserkrankung, welche die Funktionsfähigkeit von Gelenken, wie Hüfte oder Knie, beeinträchtigt. Das hat eine enorme Auswirkung auf das Leben von betroffenen Personen im erwerbsfähigen Alter und älteren Personen. Osteoarthritis belegt weltweit den elften Platz unter den Hauptursachen für Lebenseinschränkungen. Die derzeit einzig mögliche medizinische Behandlung, um einen aktiven und qualitativ hochwertigen Lebensstil wiederherzustellen, ist das Einsetzen einer künstlichen Gelenksporthese. Weltweit nimmt die Zahl der Operationen für künstliche Gelenksporthesen zu, und Schweden gehört zu den Ländern mit der höchsten Zahl. Mögliche Erklärungen für die zunehmende Zahl der Gelenksooperationen sind, dass die durchschnittliche Lebenserwartung höher ist und dass Patienten eine höhere Lebensqualität beanspruchen und dadurch in jüngeren Jahren eine künstliche Gelenksporthese erhalten. Obwohl das Einsetzen einer künstlichen Gelenksporthese eine sehr erfolgreiche Prozedur ist und das künstliche Gelenk für viele Jahre genutzt werden kann, gibt es einen geringen Prozentsatz an Personen wo die Prothese instabil wird und schmerzen verursacht. Da der Anteil an Personen, die eine künstliche Gelenksporthese erhalten kontinuierlich steigt, wird die Langzeitstabilität der Prothese und das Verhindern von Revisionseingriffen ein Hauptproblem für Patienten und die Gesundheitsfürsorge. Der häufigste Grund für einen Revisionseingriff ist eine aseptische Lockerung - ein Zustand, bei dem die Prothese die Verbindung zum umliegenden Knochen verliert, ohne Anzeichen einer Infektion. Wenn eine künstliche Gelenksporthese fehlschlägt, ist ein komplizierteres und riskanteres Verfahren zum Ersetzen der Prothese erforderlich – ein Revisionseingriff.

Einer der vielen Gründe für eine aseptische Lockerung ist die Instabilität der Prothese. Wenn eine künstliche Gelenksporthese nicht ausreichend mit dem Knochen verbunden ist, bewegt es sich beim Gehen oder Trainieren. Durch diese Bewegungen bildet sich eine dünne Gewebeschicht um das Implantat, die eine Kapsel bildet und die Prothese weiter isoliert. Unter dieser dünnen Gewebeschicht lagern sich Flüssigkeiten aus dem Knochen ab. Während des Gehens oder des Trainings verändern diese eingelagerten Flüssigkeiten blitzartig die mechanische Belastung und dies führt zu einer Zerstörung des umliegenden Knochens. Wenn wir die aseptische Lockerung von Prothesen verhindern möchten, müssen wir diesen Prozess besser verstehen.

In dieser Doktorratsarbeit stellten wir ein neues *in vitro* Modell für die Knochenforschung vor. Dieses Modell spiegelt die mechanische Situation an der Grenzfläche zwischen Knochen und Prothese bei mechanischer Instabilität wider. Das neuartige *in vitro* Modell für mechanisch induzierten Knochenverlust hat den Vorteil, dass man menschliche Zellen aus chirurgischem Abfallmaterial verwenden kann und somit nicht vollständig von Versuchstieren abhängt. Mit diesen Abfallmaterial können wir eine spezielle Zellpopulation aus dem Knochen isolieren und ihre Reaktion in einem fokussierten und sauberen Ansatz untersuchen. Mit diesem Modell konnten wir unser Wissen auf drei Arten erweitern: (1) Mesenchymale Stammzellen und Monozyten reagieren auf die veränderte mechanische Belastung mit zunehmender Anzahl an knochenabbauenden Osteoklasten. (2) Eine Kombination von hoher Belastungsintensität und verlängerten Belastungsdauer resultiert in einen Reiz der Knochen zerstört. (3) Der Mechanismus, welcher der Reaktion auf die mechanische Belastung zugrunde liegt, hängt von der Wechselwirkung von Adenosintriphosphat mit dem Zellmembran Rezeptor P2X7 ab.

Dieses neue Wissen kann uns helfen, die Behandlung auf spezifisch ausgewählte Zellpopulationen zu konzentrieren oder das derzeitige Design von Gelenkprothesen zu überdenken, um die Wirkung des knochenzerstörenden Reizes zu verringern. Dadurch können Patienten, die eine künstliche Gelenkprothese erhalten, über einen längeren Zeitraum einen aktiven und schmerzfreien Lebensstil mit einer stabilen Prothese genießen.

List of Papers

- I. Fahlgren A, **Bratengeier C**, Semeins CM, Klein-Nulend J, Bakker AD.
Supraphysiological loading induces osteocyte-mediated osteoclastogenesis in a novel *in vitro* model for bone implant loosening.
J Orthop Res. 2018 May;36(5):1425-1434.
doi: 10.1002/jor.23780

- II. **Bratengeier C**, Bakker AD, Fahlgren A.
Mechanical loading releases osteoclastogenesis-modulating factors through stimulation of the P2X7 receptor in hematopoietic progenitor cells.
J Cell Physiol. 2019 Aug;234(8):13057-13067.
doi: 10.1002/jcp.27976

- III. **Bratengeier C**, Liszka A, Hoffman J, Bakker AD, Fahlgren A.
High shear stress amplitude in combination with prolonged stimulus duration determine induction of osteoclast formation by hematopoietic progenitor cell.
Manuscript, currently under revision (FASEB)

- IV. **Bratengeier C**, Liszka A, Schilcher J, Bakker AD, Fahlgren A.
Differentiation stage of human bone marrow stem cells determines their catabolic response to fluid shear stress
Manuscript

Acknowledgments

Many people have supported me during my time as a Ph.D. student and I am deeply grateful. I want to highlight a few people specifically, as they contributed significantly to my success:

Anna Fahlgren *Thank you for giving me the opportunity to become part of your research team and guiding me to become a creative, but also a critical researcher. I am grateful for the freedom you gave me throughout my education to develop my personal and professional skills.*

Per Aspenberg *Thank you for your help and support at the beginning of my education. Sadly, you were not able to see my full ended metamorphosis to a critical researcher. You will always be remembered.*

Mikael Sigvardsson *Thank you for stepping in as my co-supervisor and your support with human stem cell biology.*

Aneta Liszka *Thank you for being there and helping me; for all the endless hours in the cell culture labor with fluid flow experiments; for your (emotional) support when things went rough or got stressful; for always having a contagious happy attitude which just kept us going.*

Pernilla Eliasson *Thank you for always having an open door and taking your time for discussions.*

Malin Hammerman, Magnus Bernhardsson, Love Tætting, and Olof Sandberg *Thank you for all the help, fun discussions about research and the world outside research in the lunchroom.*

Franciele Dietrich Zagonel *Thank you for all your help and emotional support during my Ph.D.. You always had an open ear to hear out my concerns and help me to lift my problems. I also loved, that you tried to recreate some of my recipes.*

Astrid Bakker, Cor Semeins, and Jenneke Klein-Nulend *Thank you for taking me into your Laboratory at Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam (ACTA) and teaching me the ways of fluid flow simulation.*

All people, working at the **animal facility, core facility, former KEF, cell biology floor 9 and 10.** *Thank you for all the help during my education, discussions we had in the lunchroom, and after-work events.*

I want to thank my friends, for being at my side during these years to support me and help me to enjoy the small things in life outside research.

Anna-Maria Andersson *Your encouragements and support during long shopping days or strolls through nature always helped me to keep my aim in focus and not be discouraged by small drawbacks. Thank you for all the baking events and countless cakes we went through while discussing our Ph.D. experiences.*

Sofia Eleftheriadis *Thank you for always showing interest in my research and listing to endless stories about the life of a Ph.D. student. I really enjoyed our weekly sessions in the swimming pool where we could just talk about everything – I miss those times after you left Linköping. Thank you too, for all the baking events and countless cakes we ate.*

Héctor Rodríguez Déniz *Thank you for being such a great housemate during our stay in Lysingsgatan. It was always great fun during the movie nights and endless discussions about everything and anything – with or without cake.*

During my Ph.D., I had the opportunity to support students during their final project.

Teresa H. Jungwirth, Kathrina Radl, Linnea Andersson, and Lekha Pezhumkattil *Thank you all for letting me support you during your final projects. It was a pleasure to see you grow into a more independent and critical researcher over time, which also helped me to develop even further as a researcher.*

Einen ganz besonderen Dank möchte ich meiner Familie aussprechen. Ohne sie wäre es mir noch möglich gewesen meine Träume zu verwirklichen. Ich danke euch und hab euch lieb, von ganzem Herzen.

Meiner Mutter, **Christine Bratengeier**: *Danke Mama, für all deine Unterstützungen und Aufmunterungen! Ohne deine Unterstützung hätte ich es niemals so weit gebracht. Du hattest immer ein offenes Ohr, wenn die Situationen schwierig wurden und hast mich immer daran erinnert, wie stolz du auf mich bist.*

Meiner Großmutter, **Leopoldine Ulbricht**: *Danke Omili, für all deine Unterstützungen! Auch wenn uns die Distanz schwergefallen ist, war es jedes Mal ein großartiges Gefühl nach Hause zu kommen und einfach die gemeinsame Zeit zu genießen.*

Meinem Bruder, **Andreas Förster**, und seiner Familie **Ingrid, Georg und Clemens**: *Danke für euer Interesse an meiner Arbeit. Eure Unterstützungen während meines Ph.D's in Schweden haben mir immer neue Kraft gegeben.*

*“If we knew what it was, we were doing,
it would not be called research, would it?”*

-Albert Einstein

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Introduction

Bone and Osteoclast formation

Mechanical loading and structural adaption of the bone

Although bone appears to be stiff and rigid, it is a highly dynamic tissue. Through regular exercise, the bone experiences mechanical loading that induces a process, called remodeling. During mechanical loading, the skeletal structure adapts to the functional demands and thus bone becomes stronger. On the contrary, lack of mechanical loading on bone puts the remodeling on hold and the bone becomes less dense and weaker. These observations have already been formulated in the 19th century as Wolff's Law [1]. Later, the loading-induced adaption of bone through effector cells in the bone, namely osteocytes, osteoblasts, and osteoclasts, has been described by a practical description of Wolff's law, the Mechanostat theory [2]. The Mechanostat theory is based on two popular assumptions: (1) Mechanosensitive cells sense the elastic deformation of mineralized extracellular matrix and respond according to the intensity of the deformation [3, 4], or (2) mechanical strain results in microscopic damages in the mineralized extracellular matrix that modulates the cellular response by paracrine signals [5, 6].

Mechanosensitive cells in the bone

The process of bone remodeling is regulated by the complementary activities of osteoblasts, osteocytes, and osteoclasts.

Osteoblasts – bone-forming cells

Osteoblasts originate from immature mesenchymal stem cells and are responsible for bone formation. Bone formation occurs through the production of osteoid, a collagen type 1-rich precursor matrix. Mechanical loading on osteoblasts promotes proliferation through activation of the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway and the mineralization of the osteoid through upregulation of alkaline phosphatase activity (ALP) [7]. This supports the formation of mineralized bone matrix. During this process, some osteoblasts become trapped within the mineralizing bone matrix, transforming into osteocytes.

Osteocytes – maintaining bone homeostasis

Osteocytes connect with other osteocytes, with osteoblasts and bone-lining cells, or the local blood supply through the lacunar-canalicular network within the mineralized bone [8]. This fluid-filled network plays a critical role in bone metabolism, bone adaptation, and mechanotransduction [9]. During loading, the strain on the mineralized matrix compresses the fluid within the lacunar-canalicular network, amplifying the mechanical signal which is subsequently sensed by the osteocytes [4]. These amplified signals determine the action of the osteocytes, orchestrating the balance between bone-forming osteoblasts and bone-resorbing osteoclasts [10]. One such regulator, whose secretion is depended on mechanical loading, is Sclerostin [11], which inhibits canonical Wnt-signaling [12] and osteoblast differentiation.

Osteoclasts –bone-resorbing cells

Osteoclasts are specialized, multinucleated giant cells and responsible for bone resorption. They are formed after the fusion of multiple monocytic/macrophage precursors that originate from the hematopoietic stem cell lineage, circulating in the bone marrow and blood [13]. At the bone surface, osteoclasts form a ruffled border that seals a resorption pit, called Howship's lacunae. At the Howship's lacunae, the pH shifts into an acidic range, which dissolves the bone matrix and release minerals from the mineralized matrix [14]. Osteoclast precursor cells are known to be susceptible to mechanical loading. A combination of shear and compressive loading on monocytes induce the production of pro-inflammatory mediators, e.g. Interleukin (IL) -6, IL - 8, or Tumor necrosis factor (TNF) α [15], and promotes osteoclastogenesis [16]. Osteoclast formation is also enhanced in presences of compressive mechanical forces on macrophages, through an increased release of TNF- α and activation of the macrophage colony-stimulating factor receptor (c-Fms) -mediated signaling [17].

Osteoclastogenesis

Osteoclastogenesis is a well-understood mechanism. The key regulator in terms of osteoclast formation and functions are macrophage-colony-stimulating factor (M-CSF) and receptor activator of NF- κ B ligand (RANKL) [18, 19]. RANKL occurs in the majority in its membrane-bound form and plays a major part in the RANKL/RANK/OPG signaling system [20]. However, RANKL can also occur in its soluble form, derived from the membrane as a result of either proteolytic cleavage or alternative splicing [21] by either matrix metalloproteases (MMP)

[22] or by a disintegrin and metalloproteinase (ADAM) [23]. In either way, the binding of RANKL to its receptor RANK on myeloid progenitor cells promotes the differentiation of osteoclasts, by induction of the transcription factors nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1). The activation of NF- κ B leads to the induction of the key regulator for osteoclastogenesis: nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) [24]. On the other hand, osteoblasts and osteocytes can release osteoprotegerin (OPG) as a negative regulator for osteoclast differentiation by acting as a decoy receptor for RANKL, preventing its binding to RANK [25].

In addition, a broad variety of co-stimulatory factors which further enhance osteoclast formation have been identified, including cytokines (e.g. IL-1, IL-6), inflammatory markers (e.g. TNF α , LPS), co-expressed surface receptors or proteins (e.g. OSCAR, Annexin II), soluble decoy receptors (e.g. DcR3), or proteins involved in Wnt-signaling (e.g. WNT5a, WNT5b) – just to name a few [26, 27].

Mechanically induced osteolysis as a new dogma in aseptic loosening

Primary total joint arthroplasty - the surgery of the last century

Introduced more than a hundred years ago, total joint arthroplasty completely transformed the treatment of arthritic joints. Osteoarthritis is a painful condition where joint cartilage and underlying bone is broken down. To restore a high-quality and active lifestyle for affected patients, total joint arthroplasty is considered the most successful medical intervention of the last century [28, 29]. During the last century, implants used in total joint arthroplasty have been undergoing drastic changes in material and design to support long term survival. In the early 1960s, the principle of low friction arthroplasty was introduced, and the principle is still preserved in modern prostheses [30]. Being superior to all other treatments modern healthcare has to offer, the average age of patients undergoing this procedure has decreased. But also, the number of young patients (<65years) undergoing joint arthroplasty has been increasing. These patients have a high risk to need revision surgeries (changing the old implant to a new) because of a longer life expectancy and a more active lifestyle [31]. For each year primary total joint arthroplasty can be delayed, the risk of loosening decreases by 1.8% [32]. However, in many cases, a delay is not possible. Taking global aging into consideration, these early interventions result in a continuously increasing number of primary total joint arthroplasty and cases of loosening at an earlier age that requires revision surgery worldwide [33-36]. The number of

revision surgeries of the hip is projected to increase with >300% until 2030 [37, 38], which in turn will put an enormous burden on the clinical and economic situation of the national health care systems [39-41]. To improve implant survival in these patients has become the challenge of our century.

Aseptic loosening in total joint arthroplasty

The main questions are: Why do implants get loose and how can we delay or prevent this process? In average, 3% to 11% of all primary total joint arthroplasties will fail. These numbers are highly dependent on technical aspects, such as implant material [42], fixation technique [43], and patient-related aspects, such as pre-diagnostic conditions [44], obesity [45], abuse of tobacco [46], and age [47]. Clinical observations have identified a variety of different reasons for the failure of total joint arthroplasty (Table 1), ranking aseptic loosening as one of the leading causes for revision surgeries. Aseptic loosening is often diagnosed on radiographs during routine follow-up, where small gaps between the bone and the implant can be seen [48]. These gaps are formed in the absence of infections. During different activities, (e.g. walking, standing up, walking stairs upwards, etc.) fluid in these gaps, at the peri-prosthetic interface, quickly accelerates which results in loading that is several orders of magnitude higher during gait cycles [49], compared to a well osseointegrated and stable implant [50]. This is associated with the formation of a “synovial-like” fibrous tissue membrane between implant and bone [51, 52] and led to the development of multiple theories about the underlying mechanisms of aseptic loosening [53].

Table 1. Categorical Reasons for revision surgery in Sweden, United Kingdom, Australia, and the United States of America

Most Common Reason for Revision Surgery						
	Annual Report	Location	Aseptic loosening	Dislocation	Infection	Periprosthetic fracture
Swedish Hip Arthroplasty Register [54]	2017	Hip	58%	14%	14%	9%
Swedish Knee Arthroplasty Register [55]	2018	Knee	35%	15%	28%	5%
National Joint Registry for England, Wales, Northern Ireland and the Isle of Man [56]	2018	Hip	49%	16%	4%	11%
		Knee	38%	4%	6%	4%
Australian Orthopaedic Association National Joint Replacement Registry [57]	2018	Hip	25%	21%	18%	20%
		Knee	25%	8%	23%	3%
American Joint Replacement Registry [58]	2018	Hip	12%	13%	8%	4%
		Knee	21%	8%	8%	n.a.

The previous dogma in aseptic loosening

The main theory for early failure of an implant has been suggested to be due to wear particle-associated adverse local tissue reactions. Wear particles induce severe inflammatory reactions of the peri-prosthetic soft tissues that result in tissue necrosis and thus aseptic loosening of the implant [59]. The use of new materials for articulating surfaces, such as combinations of metal-on-metal and ceramic-on-ceramic was an attempt to reduce the total amount of wear particles compared to cross-linked polyethylenes. However, a metal-on-metal implant might exceed the volume of metal wear debris by up to 100 times compared to polyethylene surfaces, due to smaller metal particle size [60, 61]. This increases the wear-related complications which further

increase the rates for revision surgeries for metal-on-metal implants [62], putting considerable strain on the health care costs [63].

A new dogma in aseptic loosening accepted: mechanically induced osteolysis

Recent clinical and experimental observation has introduced other theories for aseptic loosening of implants: early micromotions [64] and high fluid pressure [65]. Initial stability is a key factor of the clinical success and prosthetic micromotion is a predictive factor for joint replacement surgery. A total migration of the implant of 0.85mm after six months and more than 1.2mm after two years predicts an increased probability of revision surgery to up to 50% [66]. Also, higher intracapsular pressure is an indicator of an increased risk for revision surgery [48, 67, 68]. A rise in pressure up to 198mm Hg is associated with osteolytic areas, which could endanger the perfusion and the supply of oxygen of the bone [69]. The impact of mechanical factors to induce bone degradation has also been seen in post-mortem-retrieved total knee replacements. Trabecular bone, which was initially interlocked with the surrounding cement, was degraded resulting in small gaps in the peri-prosthetic interface [70]. These observations strengthen the role of mechanical factors as another factor for later prosthetic loosening (Figure 1).

Figure 1: Over a period of six years, micromotion induced mechanical loading resulted in osteolytic lesions which formed hollow areas of resorbed bone around the prosthesis, provided by Jörg Schilcher, LiU.

Mechanical induced peri-prosthetic osteolysis in animal models

In early attempts to understand the mechanism underlying mechanical loading-induced bone-implant loosening, animal models were introduced by Per Aspenberg [71, 72]. In these models, a dynamic pressurized fluid flow-induced bone loss in the absence of wear debris particles was seen. In the animal models, an implant was created that could induce a mechanical loading that would simulate what happen in the peri-prosthetic interface during instability. In these models, several physical parameters were tested such as constant loading [73], dynamic loading [71, 72], and impact pulse [74], which all were able to induce bone degradation. The more standardized animal model is the rat model for pressure-induced osteolysis. After proper osseointegration, a piston was loaded and created a pressurized fluid flow into the underlying bone, which induced osteoclast differentiation and bone loss was induced after 2 minutes loading twice daily for only 3 days. The pressure piston was simulating the pressurized fluid flow around a loosening implant in the clinics. [75, 76]. With the help of these models, it was possible to verify mechanical loading-induced osteoclast formation and bone resorption [72, 74, 77]. The rat model was used to determine the role of physical factors such as flow and pressure [74], determine osteoclast differentiation [72], or study the effect of anabolic and anti-catabolic treatments [78]. However, there were big limitations to use the animal model due to cost and time. Moreover, it was not possible to study the specific mechanism in mechanical induced osteolysis, e.g. what cells are the primary responders to supraphysiologic loading and to isolate physical and molecular mechanisms.

Aims and Hypotheses

The overall aim of this thesis was to close current gaps in knowledge of mechanical loading-induced peri-prosthetic osteolysis: (1)What cells are the primary responder to supraphysiological loading? (2)What characteristics of the mechanical stimulus induce an osteo-destructive response, rather than an osteo-protective response? (3)Which cellular mechano-sensing mechanisms are involved in an osteo-destructive response? This work resulted in four scientific papers.

- I. ***The aim*** was to set up and validate a novel *in vitro* model for mechanical overloading-induced bone-implant loosening.

The hypothesis was that supraphysiological loading of MLO-Y4 osteocytes enhances the secretion of soluble and membrane-bound factors that stimulate the formation of osteoclasts compared to physiological loading.

- II. ***The aim*** was to determine whether murine hematopoietic progenitor cells are responding to supraphysiological loading.

The hypothesis was that hematopoietic bone marrow progenitor cells are mechanoresponsive and can communicate through soluble factors, released via the ATP/P2X7-axis, with other progenitor cells in the bone marrow to increase or reduce osteoclast formation.

- III. ***The aim*** was to determine which physical parameters in the fluid flow stimulation defines an osteo-protective or osteo-destructive response in murine hematopoietic progenitor cells.

The hypothesis was that a high initial fluid displacement rate (peak wall shear stress rate) is the driving cause for the induction of an osteo-destructive response.

- IV. ***The aim*** was to determine how differentiation of human mesenchymal and hematopoietic stem cells into either the osteoblastic or osteoclastic lineage affects their response to supraphysiological loading.

The hypothesis was that cells at earlier stages of differentiation are more responsive to mechanical overloading in terms of induction of osteoclast formation.

Methodological considerations

Current gaps of knowledge in mechanically induced osteolysis

Although the currently available rat model for mechanically induced osteolysis reflects the clinical situation very well, it has some limitations. Due to these limitations, it has not been possible yet to fully understand several key aspects: (1) Which local cells respond to supraphysiologic loading, (2) What physical parameter determines the osteo-destructive response, and (3) What underlying cellular mechanisms induce the mechanical loading induced osteolysis?

In an animal model, it is difficult to isolate and evaluate the response of a specific cell type located in either the bone matrix or bone marrow due to the systemic response. Although the systemic response reflects the clinical situation, it is less helpful to investigate the response of a specific cell population. An *in vitro* model gives the possibility to follow cell-specific response to applied mechanical loading on an isolated and well-defined cell population. This opens the opportunity to identify cell types that are early respondents in mechanical loading-induced bone-implant loosening.

Although the controlled application of different variations on the mechanic loading in the rat model has been demonstrated [71-74], it was not possible to identify the nature of the mechanical stimulus that will induce osteoclast differentiation and results in peri-prosthetic osteolysis. This is crucial to understand which physical parameter of the mechanical loading determines the shift from an osteo-protective response to an osteo-destructive response. In an *in vitro* model, the application of the mechanical loading is automated and highly controlled, making changes in amplitude, loading duration, and shear stress rate easily applied.

It is equally important to translate findings in animal models and *in vitro* models for the application in human medicine. Thus, *in vitro* models can be easily used to apply loading on cells isolated from human biopsies thereby validating finding from the cells of animals to human applications. In addition, the *in vitro* model can be further used for the approach of personalized medicine by screening potential treatment in the presence of mechanical loading.

In vitro models have proven to be important tools to advance knowledge in a broad field of clinical research [79-83]. Thanks to the currently available animal models the knowledge in mechanically induced peri-prosthetic osteolysis has been continuously increasing over the last decades [84]. However, we still lack crucial knowledge about responded cells, the nature of the

osteo-destructive mechanical stimulus and involved mechanism to apply treatment strategies to delay or prevent prosthetic loosening. This highlights the necessity of an *in vitro* model to simulate the mechanical loading situation around a loosening implant to close the current gaps of knowledge in mechanical loading-induced bone-implant loosening.

The novel *in vitro* model for bone implant loosening

The *in vitro* model for bone implant loosening is adapted from the pulsating fluid flow model introduced by Jenneke Klein-Nulend and colleagues [85]. It uses commercially available cell culture medium for the application of the fluid flow loading. The medium is collected in an open reservoir, which is infused with 5% carbon dioxide in air blend and used to sample the conditioned medium. Different loading profiles are addressed to a pump which flows the medium through the system in a highly controlled manner. Before and after the parallel-plate flow chamber, in-line flow sensors record the flow profiles to estimate the stimulation applied to the cells in the parallel-plate flow chamber. The cells mounted into the parallel-plate flow chamber are seeded on a pre-coated glass slide, to support attachment (Figure 2)

Figure 2. Schematic overview of the novel *in vitro* model for bone implant loosening

Loading profiles in vitro mimic the loading situation in the peri-prosthetic interface

Three major loading profiles were used in the novel *in vitro* model for bone implant loosening to mimic different loading situations: (1) physiological loading in intact bone, (2) stress shielding that occurs around the inserted implant due to lack of loading, and (3) supraphysiological loading due to pressurized fluid flow in the peri-prosthetic interface (Figure 3).

Figure 3. Overview of the three major loading profiles applied in the novel *in vitro* model for bone implant loosening. The dotted black lines represent the input signal to address the pump, while blue and orange lines represent the recorded loading profile in physiological loading and supraphysiological loading, respectively.

Physiological loading

Physiological loading occurs in the bone during physical activity. Therefore, a validated pulsating fluid flow *in vitro* model, which has been introduced by Jenneke Klein-Nulend and used for decades to investigate the mechanisms in fluid flow-induced bone formation [83, 85–89], was used. The simulation of a physiological load in the *in vitro* model was based on sine waveform with a low amplitude (0.7 ± 0.3 Pa) – a similar loading profile as observed during gait cycles – at a frequency of 5 Hz, which lies in-between 1–3 Hz for walking cycles and reaching 8–9 Hz for running cycles [50, 90]

Stress Shielding

Stress shielding is a phenomenon that occurs when metal implants, plates or screws are used in total joint replacement arthroplasty or to support bone healing. Although the higher stiffness of the material allows early weight-bearing and patient mobility, it reduces the physiologic loading on the bone [91, 92]. The simulation of a stress shielding in the *in vitro* model was based on the absence of any active mechanical load, resulting in an unloaded condition.

Supraphysiological loading

Supraphysiological loading is the novel loading profile, which simulates the pressurized fluid flow upon loading of a loosening hip implant as a result of early micromotions. The analysis of the loading profile in the clinical situations [48] and in the rat model [74] identified that a square wave as the critical waveform to stimulate osteoclast formation (Figure 4). A square wave is a periodic wave where the amplitude alternates at a steady frequency between two fixed values - a minimum and maximum. By default, the duration spent at the minimum and the maximum value is equally long. To mimic the pressurized fluid flow around a loosening implant, supraphysiological loading profile was introduced that was simulated by a high amplitude square waveform ($3.0 \pm 0.2 \text{ Pa}$) at a frequency of 1 Hz [93].

Figure 4. Loads during cycles of stimulation in clinical observation, an animal model for osteolysis and in the novel *in vitro* model for bone implant loosening. From clinical observations, the loading profile was translated into the rat model for pressure induced osteolysis. From the rat model, the loading profile was translated into the *in vitro* model for bone implant loosening, reflecting the loading situation in the animal model and in the clinics.

Strengths

Possibilities to analyze the response to mechanical loading in the *in vitro* model

The response of a selected cell population to mechanical loading in the *in vitro* model can be analyzed in many different ways (Table 2). Conditioned medium after the application of the

different loading profiles can be used to analyze soluble factors which may act in an autocrine, paracrine or endocrine manner. Also, intracellular changes in cells exposed to the different mechanical loading profiles can be further analyzed. Different loading regimes may cause the release of different combinations of soluble factors or results in a different change in the intracellular environment, resulting in the activation of different surrounding cells or intracellular pathways.

Table 2. Overview of the possibilities to analyze the response of a selected cell population to mechanical loading by the *in vitro* model for bone implant loosening used (or attempted to use) in this thesis.

Manipulation on cell population prior to stimulation	<ul style="list-style-type: none"> • Sorting (e.g. cellular characteristics, Magnetic-activated cell sorting) • Blocking of pathways (e.g. Antagonist) • Activation of pathways (e.g. Agonist)
Conditioned medium	<ul style="list-style-type: none"> • Danger associated signaling molecules (e.g. Adenosine triphosphate, Lactate dehydrogenase, Nitric oxide) • Time-point analysis (e.g. accumulation of soluble factors over time) • Targeted protein detection (e.g. Enzyme-linked immunosorbent assay) • Protein screening (e.g. Mass spectrometry) • Analysis of soluble factors (e.g. Osteoclast differentiation, Osteoblast mineralization)
Stimulated cells	<ul style="list-style-type: none"> • Targeted protein expression (e.g. Immunocytochemistry) • Protein screening (e.g. Mass spectrometry) • Targeted gene expression (e.g. Real-time polymerase chain reaction) • Analysis of cell-to-cell communication (e.g. Co-culture) • Opening of pores (e.g. Cellular uptake assay)

Inter-species analysis of shared pathways

In an *in vitro* model, it is possible to stimulate a well-defined population of cells and analyze their specific pattern of communication (e.g. DAMPs, soluble factors, membrane-bound

factors, etc.). These cells can originate either from animal tissue or human biopsies, which gives the opportunity to compare the observed response directly between different species. Comparison between species can help to isolate general disease processes and molecular mechanism. This is possible by targeted interventions, such as knocking-out or knocking-in genes, blocking specific parts of pathways, or investigate the response to different drugs/treatments. These interventions are easily and cost-effectively applied in *in vitro* models.

Time and cost-efficient

Research that includes experimental animals is a long and costly process. Studies using experimental animals can take months or years to perform and analyze results. The previous animal model for mechanical induced bone loss included two surgeries, with a five weeks period for osseointegration. For all histological analysis, at least four weeks were needed for decalcification. The investigation of substances (e.g. treatment therapies) is highly limited per study. The rat model for mechanically induced osteolysis required rats that were relatively big (300-500g) to fit the orthopedic implant [74]. In the case of a pharmacological study, the weight of the animal determines the amount of an active substance needed, which further increases the costs [94]. Taking the fast amount of substances currently in the queue for future commercially available treatments worldwide, the inefficiency and high costs render the use of experimental animals difficult [95]. In contrast, many cell-based *in vitro* applications can be adapted to a cost and time-efficient “high throughput” screening method [96], compared to animal models.

Application of the 3Rs and improved animal welfare

In research, the welfare of experimental animals is very important. If animals are suffering stress or pain, the effects on the experimental outcome are unpredictable and might render the conducted study inconclusive. There is growing displeasure on the inferior outcome of animal models in contribution to the development of human clinical interventions or their inconsistency with clinical outcomes [97]. The introduction of the three Rs principle intended to provide ethical guidelines for experimental animals, which is in line with using an *in vitro* model. Instead of having animals undergo anesthesia, pain killer treatment and surgery, cells can be isolated from sacrificed animals (Refine). This also lowers the number of experimental animals needed, as cells can be expanded and used for several studies (Reduce). In addition, cells from the human origin (e.g. surgical waste material) can be isolated to be used in an *in vitro* model (Replace).

Limitations

Cell culture supplements alter cellular response.

The bone possesses a specific structure that allows the transfer of nutrients by the blood vessels to bone cells. When extracting cells from their naïve tissue and culturing them *in vitro*, they are still high in demand for the supply of nutrients. Animal sera, traditionally Fetal Bovine Serum (FBS), provide essential components such as hormones, vitamins, proteins for attachment, and growth factors [98]. Although essential, the supplementation of *in vitro* cultures with FBS has several disadvantages: (1)The production of FBS is a by-product of the food industry, which makes it to an ill-defined mixture of components, prone to geographical and seasonal batch-to-batch variations [99]. (2)The geographical and seasonal changes in FBS affects the proliferation and differentiation of cells *in vitro*. In addition, potential contamination by mycoplasma, endotoxins, and viruses is of growing concern [100]. (3)The composition of FBS can interfere with experimental outcomes [101].

The effect of FBS on hematopoietic progenitor cells was something experienced during this thesis, rendering sometimes the application of mechanical loading dysfunctional. The selection of the correct supplement is crucial for valid results (Table 3, Figure 5). But the main questions are: Which FBS shows correct and valid results? Is the visible increase or decrease in osteoclast formation only an artifact caused by the FBS?

We backed up the selection of the “correct” FBS by two commonly accepted facts: (1)Physiological loading (e.g. through physical activity) results in gain of bone mass and drives the adaptation of the bone structure, which is preserved for a long period even after the regular physical activity has stopped [102-105]. In several *in vitro* models (e.g. chewing (vibrational) load, low-magnitude high-frequency vibration (LMHFV), intermitted compressive force, etc.), mechanical loading in a physiological range led to the reduction of osteoclast formation or osteoclast resorbing activity [106-108]. (2)Unloading-driven alterations in the mechanical loading show a significant reduction of bone mass in weight-bearing bones [109]. This is due to an increased formation of osteoclasts [110, 111]. The alternative to the application of FBS in culture medium is the use of a specialized serum-free alternative medium. Although better defined than the addition of FBS, specifically added growth factors or supportive substances might still influence the mechanoresponse of cultured cells.

Table 3. Description of FBS or serum-free alternative culture media screened in the *in vitro* model for bone implant loosening according to the supplier

Company	Product/Lot number	Serum specification provided by the supplier
GE Healthcare Life Sciences	SV30160.03HI RB35939	HyClone FBS, EU approved, Heat Inactivated, South American
Merck Millipore	ES-009-B VP1804110	US Origin, EmbryoMax® ES Cell Qualified FBS, sterile-filtered, suitable for stem cell culture
Merck (Sigma Aldrich)	F9665 BCBW5069	Heat Inactivated, non-USA origin, sterile-filtered, suitable for cell culture
Merck (Sigma Aldrich)	F6765 16M289	USA origin, Charcoal Stripped, sterile-filtered, suitable for cell culture
Merck (Sigma Aldrich)	F4135 17H165	USA origin, Heat Inactivated, sterile-filtered, suitable for cell culture, suitable for insect cell culture, suitable for hybridoma
Merck (Sigma Aldrich)	F0392 16M280-A	USA origin, dialyzed by ultrafiltration against 0.15 M NaCl, sterile-filtered, suitable for cell culture
GE Healthcare Life Sciences	SV30160.03HI RB35944	HyClone FBS, EU approved, Heat Inactivated, South American
Thermo Fischer Scientific	10500064 08Q3066K	Fetal Bovine Serum, qualified, heat-inactivated, Brazil
Thermo Fischer Scientific	16140071 1956855	Fetal Bovine Serum, qualified, heat-inactivated, United States
Biowest	S1810 S14344	Fetal Bovine Serum (FBS) South America origin
GE Healthcare Life Sciences	SH30071.03 AXF41913	HyClone FBS Characterized Heat Inactivated, US Origin
Irvine Scientific	91206 RD170602	PRIME-XV Mouse Hematopoietic Cell Basal Medium; Serum-free medium that supports self-renewal of murine hematopoietic cells while maintaining multipotency.

Figure 5. Fetal Bovine Serum (FBS) has a tremendous effect on the mechanoreponse of hematopoietic progenitor cells to fluid flow shear stress.

The design of the current model provides limited through-put of samples

The current design of the *in vitro* model for bone implant loosening has a major influence on the number of samples that can be stimulated per day. Depending on stimulation duration, treatment of cells prior to mechanical loading, and harvesting procedure of cells and conditioned medium post-mechanical loading, the number of samples per day can be as little as six. In addition, the glass slide used as seeding surface for the cells is either 4.8 or 15cm² big, which requires between 3×10^4 - 1.5×10^6 cells for each sample depending on cell size and size of the chamber. This proves difficult when working with small amounts of human biopsies where

the number of cells in low passage numbers is limited. Thus, the adaption of the current design is highly needed to provide the simultaneous stimulation of multiple samples, generating a high-throughput model.

The lack of depth in a two-dimensional (2D) model

Although 2D monolayer models are cost-effective, well established and provide easy access for cell analysis, these types of cultures do in general not well reflect the conditions *in vivo*. Especially in a complex connective tissue like bone, the constant interaction with different cells and extracellular matrix is essential [112].

Currently, the focus has shifted into organoids such as spheroid cultures, as they present a simpler approach on three-dimensional (3D) cell culture allowing cellular self-organization and cell-to-cell communication [113]. This would provide an acceptable 3D model for cells of the osteoblastic lineage as they often communicate directly via gap-junctions to control proliferation, differentiation, and survival [114, 115]. Membrane-bound factors on mesenchymal stem/progenitor cells (MSC), as well as cells of the osteoblast lineage, are also fundamental regulators of hematopoiesis and differentiation of hematopoietic stem cells (HSC) [116]. However, these spheroids are non-adherent and thus the application of mechanical loading proves difficult. Other approaches to create artificial bone are by the cultivation of bone cells on scaffolds. These scaffolds can be made from different material with alternating properties, which reflects a specific purpose or research question. In general, these applications are used in terms of cell colonization and migration [117]. However, scaffolds are rarely uniform and variation in pore sizes are high which affect cell migration and attachment [118] and will render the controlled application of the mechanical loading difficult.

However, when mimicking the mechanical loading induced osteolysis around a prosthesis, a 2D model resembles the clinical situation better than a complex 3D model. One reason is the thickness of the interface between the bone and the implant. The synovial-like tissue is a very thin layer (20-40µm) [119] that is similar to the thickness of the parallel-flow chamber used in our *in vitro* model. In addition, having cells present in a monolayer helps to distribute the fluid shear stress equally. In 3D scaffolds, commonly used in bone grafts, characteristics such as lower porosity, wettability, and roughness of the material, or length affect the linear fluid flow [120].

Results

Study I, Supraphysiological Loading Induces Osteocyte-Mediated Osteoclastogenesis in a Novel *In Vitro* Model for Bone Implant Loosening.

Exposure of MLO-Y4 to either physiological loading, supraphysiologic loading or unloading (stress shielding) for one hour did not affect morphological characteristics (cell area, cell perimeter, cell feret's diameter), viability and proliferation (DNA content, Ki-67), inflammatory markers (COX-2, IL-6, PGE₂), osteoclast-modulating genes (RANKL, OPG), and osteogenic markers (RUNX-2, SP-7, ALP, OPN, SPARC).

During this loading duration, a cumulative increase of releases nitric oxide (NO) was detected in both, physiological loading and supraphysiologic loading, but not stress shielding. The concentration of NO was higher in supraphysiological loading compared to physiologic loading at all time points measured.

Mechanically stimulated MLO-Y4 showed osteoclast-modulating properties via direct cell-to-cell communication in a co-culture with bone marrow progenitor cells. Supraphysiologic loading increased the number of multinucleated, TRAP⁺ osteoclasts compared to physiological loading, while stress shielding showed more osteoclast formation compared to supraphysiologic loading. This was in line with increased detection of membrane-bound RANKL after supraphysiologic loading.

Soluble factors in the conditioned medium after one-hour loading duration also showed osteoclast-modulating properties. Supraphysiologic loading increased the number of multinucleated, TRAP⁺ osteoclasts to a similar extent as unloading, compared to physiological loading. While the concentration of the soluble form of RANKL was not altered by the different mechanical loading regimes, OPG was increased only in supraphysiologic loading.

Study II, Mechanical loading releases osteoclastogenesis-modulating factors through stimulation of the P2X7 receptor in hematopoietic progenitor cells.

Incubation of bone marrow from male C57bl/6J wild-type mice for two days in the presence of M-CSF resulted in a mixed population of monocytes, macrophages and megakaryocytes.

During a one-hour loading duration on these myeloid progenitor cells, a cumulative increase of released adenosine triphosphate (ATP) was detected in both, physiological loading and

supraphysiologic loading, but not stress shielding. The concentration of extracellular ATP plateaued after two minutes of mechanical loading and steadily declined after ten to fifteen minutes. The concentration of ATP was higher in supraphysiological loading compared to physiologic loading at all time points measured. The release of ATP was loading-dependent, as consistently low levels of lactate dehydrogenase (LDH) confirmed an intact cellular membrane.

Soluble factors in the conditioned medium after one-hour and two-minutes loading duration also showed osteoclast-modulating properties: Supraphysiologic loading increased the number of multinucleated, TRAP⁺ osteoclasts, compared to the osteoclast assay positive control, while physiologic loading decreased the number of multinucleated, TRAP⁺ osteoclasts. Osteoclast-inducing soluble co-factors such as IL-6 and TNF- α were not detected in the conditioned medium (unpublished data).

After two minutes of loading, the release of soluble factors upon supraphysiological and physiologic loading was dependent on the ATP-gated P2X₇ receptor. Inhibiting the interaction between mechanically-induced extracellular ATP and the P2X₇ receptor with the selective antagonist Brilliant Blue G (BBG) completely abolished observed osteoclast-modulation. On the contrary, stimulation of the P2X₇ receptor with its agonist BzATP, in absence of mechanical loading, induced the release of osteoclastic soluble factors, increasing osteoclast formation to a similar extent as supraphysiological loading. This depended on the formation of non-selective pores by P2X₇, as confirmed by cellular uptake of YO-PRO-1 iodide upon supraphysiological loading.

Gene expression analysis after one-hour loading confirmed the upregulation of mechanoresponsive genes (c-Jun, c-Fos, NF κ B, COX-2), while inflammatory markers (IL-1 β , IL-6, TNF- α , PTGES2), osteogenic markers (BMP2, NOG, SP-7, CTNNB1, OPG) and osteoclastic markers (RANKL, NFATc1) remained unchanged.

Study III, High shear stress amplitude in combination with prolonged stimulus duration determine induction of osteoclast formation by hematopoietic progenitor cells.

Hematopoietic progenitor cells were exposed for two minutes to dynamic fluid flow with a controlled variation of peak wall shear stress rates, amplitude and duty cycle.

In contrary to our hypothesis, peak wall shear stress rate was not the driving factor for the release of osteoclast-inducing soluble factors by hematopoietic progenitor cells. The cellular

level of Piezo1, the concentration of extracellular ATP and the release of osteoclast-inducing soluble factors weakly correlated with peak wall shear stress rate.

On the other hand, loading amplitude and active loading duration per cycle explain much better the release of extracellular ATP and osteoclast-inducing soluble factors. A high amplitude (3.0 ± 0.2 Pa) results in an increased release of extracellular ATP and osteoclast-induced soluble factors compared to a lower amplitude (0.7 ± 0.3 Pa). At a constant high amplitude, the active loading duration per cycle (duty cycle) showed in continuously increasing the formation of osteoclast between 22% and 50%. However, the fact that a high amplitude with a short duty cycle (<22%) does not induce osteoclast formation, suggests that a combination of a higher amplitude with a longer active loading duration promotes an osteo-destructive response. The resulting Area under the Curve (AuC = amplitude*loading duration) showed a strong correlation with osteoclast formation.

The analysis of the cellular level of Piezo1 showed that Piezo1 might be involved in loading profiles that are capable to modulate osteoclast formation, while it was less detectable in loading profiles that did not alter osteoclast formation. On the contrary, cellular levels of SERCA2, a potential inhibitor of Piezo1, were only detectable in loading profiles that reduced osteoclast formation.

Study IV, Differentiation stage of human bone marrow stem cells determines their catabolic response to fluid shear stress.

Isolation of human bone marrow-derived plastic-adherent mesenchymal stromal cells and CD34+ hematopoietic stem cells from the resected femoral neck of six patients undergoing primary total hip arthroplasty resulted in cells with stem cell characteristics. The quality after the expansion of mesenchymal stem cells was verified by multi-lineage differentiation assay into chondrocytes, adipocytes, and osteoblasts. The self-renewal capacities of CD34+ hematopoietic stem cells were verified by cellular expansion after exposure to a combination of SCF/c-kit, TPO, and Flt-3 ligand protein.

In the *in vitro* model for bone implant loosening, cells of human origin responded similarly to physiological and supraphysiological loading compared to cells of murine origin. One hour of physiological loading on mesenchymal stem cells (day 0) and their lineage-committed cells, osteoprogenitor cells (day 4) and pre-osteoblastic cells (day 7), showed a decrease of osteoclast

formation. Similar was observed in monocytes (day 4), but not in pre-osteoclasts (day 7). Supraphysiological loading, on the other hand, showed donor-specific responses depending on the stage of differentiation.

Differentiation of mesenchymal/hematopoietic stem cells into the osteoblastic/osteoclastic lineage altered the osteoclast-inducing response in osteoprogenitor cells (day 4), pre-osteoblastic cells (day 7), and pre-osteoclasts (day 7). The stage of differentiation was verified by gene expression analysis. While all donors showed an increase of osteoclasts formation when mesenchymal stem cells were stimulated for one hour with supraphysiological loading, two out of six donors did not show an increased osteoclasts formation in osteoprogenitor cells (day 4) and pre-osteoblastic cells (day 7). In the hematopoietic lineage, one hour of supraphysiological loading and stress shielding increased osteoclast formation, while pre-osteoclasts (day 7) did not release osteoclast-modulating soluble factors.

Discussion

Validation of the new *in vitro* model for bone implant loosening

Supraphysiological loading does not harm stimulated cells

The application of supraphysiological loading can potentially harm stimulated cells due to the rapid alterations in minimum and maximum values in the square wave. This can lead to the detachment of the cells during supraphysiological loading. As a first control, we performed visual inspections of the cells on the glass slides after loading and could see that cells were still attached. Further, the harsh conditions applied with supraphysiological loading could potentially cause membrane damage or induce apoptosis. In osteoblasts, reversible membrane damage upon mechanical loading is a regulatory mechanism to induce calcium signaling [121] while in osteocytes, apoptosis is a known mechanism to recruit osteoclast precursors and induce bone degradation [122]. During the early stages of apoptosis, the cells appear smaller in size [123]. However, morphological analysis of MLO-Y4 osteocyte-like cells [Study I] and murine myeloid progenitor cells [Study II, III] stimulated with supraphysiological loading did not show changes in the cell area, cell perimeter, and cell feret's diameter compared to physiological loading or unloading.

Another way to investigate cellular damage is through lactate dehydrogenase (LDH). LDH is an intracellular enzyme that leaks into the extracellular space in high concentrations upon cell plasma membrane damage. Thus, makes it an easily applied method to investigate membrane rupture [124] due to supraphysiological loading in the conditioned medium. Over a period of sixty minutes [Study II], murine myeloid progenitor cells released a very low concentration of LDH that was similar to the unloaded control. Therefore, we concluded that the cellular membrane remained intact when supraphysiological loading was applied.

In addition, another marker for apoptosis - caspase 3/7- remained unchanged in MLO-Y4 osteocyte-like cells [Study I]. However, when it comes to the analysis of enzymatic markers of apoptosis, timing is critical. Apoptosis is a complex process with an underlying signaling cascade that can take several hours or days until full in action [125]. Although we have investigated signs for apoptosis within one-hour after mechanical loading, we are confident that cells are not harmed. We were able to successfully induce osteoclast formation in a co-culture with bone marrow progenitor cells that were seeded on top of supraphysiologically stimulated, viable (no detachment of cells) MLO-Y4 osteocyte-like cells for six days [Study I].

Taken together, we have shown with visual controls, the analysis of morphological characteristics, extracellular LDH concentrations, investigating caspase 3/7 activity, and long-term co-culture of viable cells after stimulation that supraphysiological loading does not harm stimulated cells. This indicates that the response observed is directly related to the application of the mechanical loading and not due to second messaging through cellular membrane damage or apoptosis.

Cells and their extracellular environment

Lack of extracellular matrix in 2D culture and used adhesion surfaces

In comparison to bone tissue, cells in 2D culture greatly lack the molecular structure within the extracellular matrix (ECM). In bone, the ECM is a well-defined dynamic network and provides most of the structural integrity and strength. It consists mainly of calcium-phosphate in the form of hydroxyapatite and type I collagen [126] but several other proteins have been identified [127]. Fibronectin (FN) is one of them, which is incorporated into the ECM by osteoblasts during proliferation and differentiation [128, 129]. The current design of our *in vitro* model requires the seeding of the cells on a glass slide. Since glass has inferior attachment properties for cells, soluble polymers or extracellular matrix components were used to support attachment. In our studies, we used either Poly-L-lysine (PLL) [Study I] or FN [Study II-IV] to support the attachment of osteocytes or bone marrow stem/progenitor cells, respectively.

PLL is a water-soluble polymer that enhances attachment through electrostatic interactions rather than ligand-integrin interaction. Negatively charged ions of the cell membrane interact with positively charged surface ions of the PLL. However, PLL has been reported to induce a low Ca^{2+} flux in spermatocytes [127]. FN, on the other side, interacts with integrins on the cellular surface [130] and plays a key role in promoting cell adhesion of monocytes and macrophages. However, this activates integrin signaling which further induces changes in gene expression [131]. Considering the high prevalence of type I collagen in the bone ECM, it would have resembled the extracellular matrix on the glass surface. However, it was shown that type I collagen and fibronectin have similar binding capacities for osteoblasts under low and high dynamic strain [132]. Further, the deformational strain between 4-9% on monocytes/macrophages results in increased of PU.1 (a marker for early and late stages of myeloid differentiation[133]) and *c-Jun* gene expression, demonstrating mechanoresponsive characteristics when attached to fibronectin-coated surfaces [134].

Taken together, the attachment of cells onto PLL or FN can trigger Ca^{2+} flux or changes in cellular gene expression, altering cell behavior. However, both agents have been widely used in studies where mechanical loading is applied to cells in a 2D model. We could see that the observed osteoclast-modulation effect complies with previously reported observations. Thus, we are confident that the observed effects of the cellular behavior originated from the application of mechanical loading rather from the agents used to support the attachment of the cells.

MLO-Y4 cells are different from osteocytes in vivo but respond to mechanical loading

More than 20 years ago, the MLO-Y4 osteocyte-like cell line (MLO-Y4) has been introduced [135, 136]. Since then, MLO-Y4 have gained a reputation as a gold-standard to study the effects of mechanical stress and their communication with other bone cells. Thus, we used MLO-Y4 for the validation of the *in vitro* model for bone implant loosening that applies fluid shear stress on the cells [Study I].

Physiological fluid shear stress, a similar stimulus as *in vivo* in the lacunar-canalicular network during loading [137], on MLO-Y4 result in the release of nitric oxide (NO) and prostaglandin E_2 (PGE_2) [83], in an upregulation of COX2, and downregulation of the RANKL/OPG-ratio [138] – promoting osteoblast activity and bone formation. Although we have seen that MLO-Y4 induced an increased release of NO after mechanical loading in our *in vitro* model, we failed to see reported changes in COX2 gene expression, PGE_2 release, or RANKL/OPG-ratio [Study II]. Despite the lack of change in these factors, we clearly saw a reduction of osteoclast formation through soluble factors and via direct cell-to-cell communication of physiologically stimulated MLO-Y4. However, recent studies have started to question if MLO-Y4 truly represent the characteristics of matured osteocytes. Interestingly, they seem to have more in common with osteoblasts than with mature osteocytes [139].

In contrast to osteocytes *in vivo*, MLO-Y4 lack the expression of the osteocyte-specific markers SOST and FGF23 [140]. SOST/Sclerostin is a direct negative regulator of bone formation by inhibition of the canonical WNT signaling pathway [141]. Osteocytes regulate phosphate levels in plasma through reduced reabsorption of phosphate in the kidney by secretion of the endocrine factor FGF23 [142]. Thus, the search for a new cell model to study osteocyte mechano-regulation has been conducted, which resulted in the new OCY454 cell line. Like MLO-Y4,

OCY454 originates from mouse long bone, is capable of secreting soluble sclerostin and dentin matrix acidic phosphoprotein1 (DMP1) and displays changes in gene expression in SOST, DMP1, and OPG [140]. In addition, stimulating OCY454 in parallel-flow chambers with the oscillatory fluid flow for two hours at a peak shear stress of 1.2Pa results in flow-induced changes of intracellular Ca^{2+} , and release of ATP and sclerostin. In addition, released soluble factors in the conditioned medium affected ALP activity in MC3T3-E1 pre-osteoblasts but failed to affect osteoclast formation on RAW264.7 osteoclast precursors.

Taken together, there are differences between MLO-Y4 and osteocytes *in vivo* and finding “the” perfect cellular model is almost impossible. However, MLO-Y4 are useful to investigate the response of mechanical loading. Although we have reported an increase of NO after mechanical loading, we failed to see expected changes in COX-2, PGE₂, and RANKL/OPG-ratio after physiological loading. Nevertheless, we confirmed osteoclast-modulation upon mechanical loading by soluble factors and direct cell-to-cell communication, properties associated with osteocytes. Thus, choosing MLO-Y4 for the validation of the novel *in vitro* model for bone implant loosening was valid as the osteoclast-modulating response induced by the mechanical loading can be directly compared to previous studies.

Do osteocytes play a role in mechanical loading-induced osteolysis?

Even though osteocytes respond to mechanical loading, their involvement in mechanical loading-induced implant loosening is highly questionable. In the lacunar-canalicular network, osteocytes can respond to, or forward, signals over long distances [143]. However, the majority of the osteocytes are trapped deep within the mineralized matrix of the bone, making them hardly accessible [144] and only a few, very early embedded osteocytes can establish a connection to the bone marrow space with their thin canaliculi [145]. This might limit the sensing of the local pressurized fluid flow in the bone marrow cavity by osteocytes which diminishes their influence on mechanical loading-induced implant loosening. In joint arthroplasty, the implant is inserted in the bone marrow cavity, close to the endosteal surface in the bone marrow. There, endosteal and sub-endosteal regions compose the endosteal bone marrow stem cell niche [146], which provides a unique environment for hematopoietic and mesenchymal stem cells and their lineage-committed progenitor cells [147, 148] [Study II-IV].

Taken together, stem cells in the endosteal bone marrow niche, directly located next to the inserted orthopedic implant, could sense changes in the local mechanical microenvironment.

Thus, stem cells and their lineage-committed progenitor cells are potentially key players that respond to the shifts in the mechanical loading.

Direct osteoclastic effect of supraphysiological loading on stimulated cells

The direct effect of physiological loading on bone cells has been demonstrated in patients [149] and in several *in vitro* studies, as described below. However, little is known about the direct induction of osteoclast formation on precursor cells due to supraphysiological loading. This was mainly due to the lack of proper *in vitro* models to isolate the direct effect of the supraphysiological loading on cells.

Loading in a physiological range on cells of the mesenchymal lineage (stromal cells, osteoblasts) indirectly inhibit osteoclast formation through reduced expression of the osteoclastic genes RANKL [150, 151]. However, the direct effect of mechanical loading on osteoclast formation and activity have also been shown: In murine monocyte/macrophage cell line RAW264.7, a 10% elongation at a frequency of 0.5 Hz for 48h resulted in a decrease in osteoclasts due to a decrease in osteoclast-specific genes (TRAP, MMP-9, CathK, and CTR) [152]. Another study on RAW264.7 cells showed that stimulation for one hour with mechanical vibration (20 μ m displacement at a frequency of 4 Hz) for three consecutive days inhibited osteoclast formation due to down-regulated DC-STAMP, preventing the fusion process of osteoclast precursors [106]. Bone marrow cells from male C57Bl/6 mice subjected to 5% elongation at a frequency of 0.167 Hz during early phases of osteoclast formation (day 2-4 in a 7-day culture) reduce the number of multinucleated, TRAP+ osteoclasts [153].

Besides our newly introduced *in vitro* model, there is currently only one *in vitro* loading system available that produces supraphysiological fluid shear stress. There RAW264.7 cells were loaded with amplitudes between 0.5-17Pa for one hour at a frequency of 1Hz. The application of oscillatory supraphysiological shear stress resulted in an increased osteoclastic number and activity [154]. Although the design of this specific multi-well fluid loading (MFL) system has the advantage to stimulate several samples simultaneously, the inconsistent application of the fluid shear stress within one well makes it difficult to control the mechanical loading. Furthermore, this model uses dextran, a complex branched polysaccharide made from glucose molecules, to raise the viscosity of the medium to achieve fluid shear stress that reflects supraphysiological loading. However, dextran has been shown to increase resorption activity, but not the number, of osteoclasts *in vitro* [155].

Taken together, we have shown that myeloid progenitor cells from the bone marrow modulate osteoclast formation through soluble factors [Study II-IV]. However, the direct induction of osteoclast formation on supraphysiologically stimulated myeloid progenitor cells in our *in vitro* model still need to be investigated.

Does osteoclast number reflect resorption activity?

We evaluated the response of MLO-Y4, murine myeloid progenitors and human stem/progenitors in the mesenchymal/hematopoietic lineage by comparing manually counted numbers of multinucleated, TRAP+ osteoclasts between the different loading profiles [Study I-IV]. However, the number of mature osteoclasts does not necessarily reflect their activity to resorb bone. This theory was first observed in trabecular bone from human iliac crest bone biopsies of postmenopausal osteoporotic women. There, quantitative histological determination of osteoblast and osteoclast number and activity suggested that osteoclasts, although lower in number than osteoblasts, have a much higher activity [156]. This suggests that a low number of osteoclasts can have a higher resorption activity compared to a high number of osteoclasts. However, there is also evidence that resorption activity and osteoclast numbers are directly related. In an IL-6 knockout (IL-6^{-/-}) murine model, increased trabecular resorption was directly associated with an increased number of TRAP+ osteoclasts. Furthermore, an increased concentration of intracellular TRAP was found in osteoclasts derived from neonatal rabbit bone cells, which showed a high resorption activity on dentin discs [157].

Taken together, these findings suggest that the evaluation of TRAP+ osteoclast number is a valid approach. However, it needs to be considered that in order to investigate resorption activity upon supraphysiological loading other methods need to be applied. One such method is the resorption pit assay where osteoclast precursor cells are seed onto bone slides in the presence of conditioned medium collected after supraphysiological loading. The osteoclast bone resorption activity can be evaluated by measuring the resorbed “pit”-area in correlation to osteoclast number [158].

Evaluation of mechanotransduction in stimulated cells

How to evaluate mechanical loading without directly investigating Ca²⁺ flux?

Calcium (Ca²⁺) flux is a direct result of mechanical loading in a variety of tissues. In the bone, the intensity of the mechanical loading in the physiological range determines the number of

responding osteocytes. Although an increased number of responding osteocytes will increase the level of Ca^{2+} signaling, the Ca^{2+} flux within each individual cell remains constant [159]. A similar observation was reported in Osteoblasts. There the fraction of responding cells that induced Ca^{2+} flux was lower when exposed to oscillation fluid flow compared to pulsatile or steady fluid flow. This resulted in the theory that bone cells engage in an “all-or-none response” and that loading intensity correlated with the number of responding cells [137]. The method to investigate Ca^{2+} flux in living cells is through a fluorescent dye, namely Fura-2. However, numerous limitations have been pointed out [160] in the application of Fura-2, for example, but not limited to: (1) Anion transporters actively pump Fura-2 out of the cells which results in dye leakage and substantially dye loss. (2) Illumination bleaches fluorescent dyes, which could result in a bad signal-to-noise ratio that potentially making the Fura-2 signal unreadable.

Taken together, the analysis of Ca^{2+} signaling is a helpful tool to investigate mechanical loading on cells. However, limitations in the method and inconsistent responses within a cell population makes it difficult to apply. Furthermore, the release of nitric oxide [83, 161] and ATP [162] are equally representative to investigate the response to mechanical loading and easily applied *[Study I-III]*.

Numerous mechanoresponsive receptors can release ATP

Extracellular adenosine triphosphate (ATP) is a potent signaling molecule that triggers the increase in intracellular Ca^{2+} of neighboring cells but also can act over long distances [163]. Low levels of ATP might promote the proliferation of osteoblasts, while excessive concentrations of ATP ($>1\mu\text{M}$) prevents bone mineralization [164]. The release of ATP in response to mechanical loading in cell culture has been widely studied [162] and the mechanical loading-induced release of ATP was observed by myeloid progenitor cells in our *in vitro* model. We could see that supraphysiological loading release a higher concentration of ATP compared to physiology loading *[Study II, III]*. ATP is the primary source for energy transfer within the cells and its release is therefore highly regulated, e.g. through Piezo1 [165-167]. Piezo1 is a trimeric three-bladed, propeller-shaped structure with a central pore-forming channel, located at the membrane bilayer [168] and mechanically activated through stretch, fluid shear stress, compression or osmotic stress on the cell [169-172]. Although we were able to detect Piezo1 on mechanically stimulated murine myeloid progenitor cells *[Study III]*, several other membrane proteins have been identified to release ATP:

(1) The pannexon channel is built by functional oligomers of pannexins, a class of membrane channels that highly resembles gap-junction proteins [173]. One subfamily of pannexins, Panx1, forms membrane channels permeable to ATP and other small signaling molecules (<1kDa) [174]. The mechanosensitivity of Panx1, including a loading-induced release of ATP, and has been demonstrated on oocytes [175], in neurons [176], and cancer metastasis [177]. Furthermore, osteocytes, that induce RANKL production by neighboring viable osteocytes through apoptosis, release ATP through Panx1 to activate P2X7 receptors [178]. During inflammation and damages tissue, ATP is released by infiltrating monocytes and macrophages through Panx1 to further recruit inflammatory effector cells [179].

(2) The connexon is a membrane hemichannel that is formed by six oligomerized connexins, and are categorized as the gap-junctions to establish cell-to-cell connections [180]. In bone, connexin 43 (Cx43) is the most abundant form [181]. The role of Cx43 in the release of ATP has been demonstrated in brain tissue [182], vascular endothelial cells [183], and macrophages [184]. Further, the fluid-flow induced release of ATP in MLO-Y4 osteocyte-like cells has been demonstrated to act through Cx43. Oscillating fluid flow on MLO-Y4, but not the osteoblastic cell line MC3T3-E1, prompted the uptake of Lucifer Yellow – a small fluorophore capable of passing through Cx43 hemichannel -, thereby demonstrating the permeability of Cx43 for ATP [185]. In addition, *in vivo* studies in Cx43-deficient mice (conditional knockout in osteocytes/osteoblasts) showed increased bone formation upon mechanical loading [186] and protection against the bone-resorbing effects of unloading [187, 188]. If depletion of Cx43 would also protect from mechanical overloading is unknown and is worth further investigations. Especially, considering the donor-specific response [*Study IV*] to supraphysiological loading, but not physiological loading, in osteogenic progenitors and pre-osteoblasts.

(3) The exocytosis of ATP-enriched vesicles is another possibility for cells to rapidly release ATP into the extracellular environment. This has been demonstrated in shear stress-induced releases of ATP-enriched vesicles of endothelial cells [189], and human lung carcinoma cell line [190]. In MLO-Y4, it was shown that the mechanically induced Ca^{2+} oscillations also influence the release of extracellular vesicles (EV). Analysis of the content of these vesicle-like structures revealed presents of sclerostin, RANKL, OPG [191], and ATP [192]. Inflammatory cells, like monocytes and macrophages, also mediate the release of ATP through vesicular exocytosis [193, 194].

Taken together, the release of ATP is highly regulated, and cells have a variety of different mechanisms to release ATP into the extracellular environment upon mechanical loading or

other stimulations (e.g. infection). However, without appropriate control experiments, similar as performed when identifying the ATP/P2X7-axis in the mechanically induced release of osteoclast modulating factors [Study II], we can only speculate about the potential involvement of the mechanoreceptor Piezo1 in the release of ATP. The application of the selective inhibitor GsMTx4 [195] might reveal if Piezo1 is a key player in the release of ATP upon mechanical loading on hematopoietic progenitor cells, or if other channels are involved.

What mechanical stimulus defines an osteo-destructive response in bone?

The isolation and identification of the “one” parameter in the mechanical stimulus, that defines an osteo-protective or osteo-destructive response is still an open question. Observations in the rat model for mechanical loading induced osteolysis suggested that the initial fluid displacement rate is a potential factor to trigger the induction of osteoclast formation [74]. In addition, the pulsating fluid flow *in vitro* model [90, 196] showed that the loading induced release of NO by osteoblasts was depended on the initial rapid alteration of shear stress (stress-kick). Both, observations, *in vivo* and *in vitro*, suggests that the peak wall shear stress rate is potentially relevant on how cells respond to loading in bone or around a loosening prosthesis.

However, exposing hematopoietic progenitor cells in our *in vitro* model to variations of peak wall shear stress rates, it seems to play only a minor role in the observed mechanical loading-induced osteoclast formation. We demonstrated that a combination of high loading amplitude (3.0 ± 0.2 Pa) and prolonged active loading duration per cycle (duty cycle) between 22%-50% provided a better explanation [Study III]. It was even possible to further increase the number of formed osteoclasts when the duty cycle was increased to 78%. However, a continuous stimulation at a high amplitude (duty cycle 100%) completely abolished the observed induction of osteoclast formation, indicating the necessity of an oscillating stimulation pattern (unpublished data). The lower and upper threshold in the duty cycle indicates that hematopoietic progenitor cells respond to an osteo-destructive stimulus in accordance with viscoelastic properties. However, this needs to be confirmed by analyzing hematopoietic progenitor cells by atomic force microscopy, one of the prime technologies for investigating such cellular properties [197].

Dynamic deformation of cells and viscoelastic properties are depending on the cellular cytoskeleton. Actin filaments, microtubules, and intermediate filaments are creating an

adjustable network to resist deformation to protect the intracellular compartments [198]. This results in a stiffening of the cytoskeletal network and an increased elastic modulus [199, 200].

Another mechanism is the stiffening of the cytoskeleton during differentiation of stem cells. In undifferentiated human MSCs, the cytoskeleton shows long, parallel, thin F-actin stress fibers, while differentiation into osteoblasts changes the cytoskeleton into robust F-actin stress fibers that show an interwoven pattern. These changes in the cytoskeleton resulted in a higher Young's Modulus after Atomic force Microscopy, resulting in a higher stiffness due to osteogenic differentiation [201, 202]. This could also explain the observed differences in the response to supraphysiological loading on later differentiation stages into the osteogenic/osteoclastic lineage in *Study IV*. The cytoskeletal stiffening has a direct effect on mechanical properties of the lipid bilayer [203, 204], which affects mechanosensitive channels [205].

Taken together, the viscoelastic properties of cells might define their response to mechanical loading, requesting certain thresholds in the duty cycle at a high loading amplitude to induce an osteo-destructive response.

Translation of findings for human applications

Similarities and Differences between mouse and human in bone biology

Experimental animals, especially mice, have been successfully used to model aspects of many human diseases. However, genetic and physiologic differences between mice and human make it necessary to reflect on limitations regarding the translation of findings. In research, inbred strains are commonly used, and one of them is the C57BL/6. The essence of an inbred strain is that all individuals are genetically identical. Although this is helpful in experimental setups during research, it displays a limitation as human show a high genetic diversity and thus individual responses are to be expected. This limits the translation of findings from genetically inbred mice [206] to human applications. In terms of skeletal physiology, mouse strains achieve peak bone mass long after sexual maturation, between 4-6 months of age, while human bone growth end after puberty. In mice, the prolonged bone growth is achieved through a continuous building of new cartilage at the epiphyseal side of the growth plate, while at the metaphyseal side the cartilage is replaced with bone [207]. In terms of remodeling in mice, uneven “cement lines” are formed that indicates that the remodeling process is currently happening [208]. Although bone remodeling occurs in both, mouse and human, there is one big difference: murine skeleton lacks osteons in the cortical bone. Although blood vessels are in the murine

bone, the remodeling occurs on different sides. In human skeleton, remodeling occurs around a blood vessel by osteoclasts and osteoblasts where precursor cells are recruited through the blood supply [209]. In terms of age-related bone loss of cancellous and cortical bone, C57BL/6 resembles the loss of bone mineral density closely compared to human [210]. In cancellous bone, pathophysiological characteristics such as a decline in wall thickness while in cortical bone, increased occurrences of pores that increase in size over time is visible [211]. In terms of mechanical loading, rats and mice have been widely used. An increase in bone mass and structure has been observed in rats and mice after regular exercise on a treadmill or through axial loading [212]. Mechanical unloading results in a decrease in bone mass and weaker bone structure [213, 214].

Taken together, mice show in some aspects of the bone physiology differences to humans. However, in the response to mechanical loading rats and mice resemble the human response well. Nevertheless, it always needs to be considered that findings performed with mouse cells might not necessary be able to be directly translated to human applications. Thus, it is critical to validate the response to supraphysiological loading in our *in vitro* model on human cells [Study IV].

We evaluated how mesenchymal and hematopoietic stem cells and their lineage-committed progenitor cells from the femoral neck of donors, undergoing primary hip arthroplasty, respond to supraphysiological loading. Differentiation was induced as a tool to mimic the different stages of differentiation located in the endosteal bone marrow niche around an inserted implant. We evaluated the changes in the mechanoresponsive in either committed-progenitor cells from the early osteoblastic (mesenchymal stromal cells, osteoprogenitor cells, pre-osteoblasts) and osteoclastic lineage (monocytes, and pre-osteoclasts) to supraphysiological loading depending on their stage of differentiation. We confirmed that cells of human origin in early differentiation stages respond supraphysiologic loading similar as compared to murine cells, while donor-specific differences could be observed in later differentiation stages. Taken together, we have confirmed that our *in vitro* model for bone implant loosening can be used for cells of murine and human origin.

Conclusion

In conclusion, we successfully developed an *in vitro* model for mechanical loading-induced bone-implant loosening. Supraphysiological loading successfully mimics the loading-induced formation of bone-resorbing osteoclasts as previously observed in the rat model for loading-induced peri-prosthetic osteolysis and in clinical observations. Using this *in vitro* model, we were able to answer the following questions to close current gaps of knowledge in loading-induced peri-prosthetic osteolysis:

What cells are the primary responder to supraphysiological loading?

We have demonstrated that local stem cells and their early lineage-committed progenitors, as well as a cellular model representing mechanoresponsive osteocytes, are the primary respondent cells to supraphysiological loading and can induce osteoclastogenesis via soluble factors or direct cell-to-cell communication. Besides the MLO-Y4 osteocyte-like cell line, we demonstrated that murine myeloid progenitor cells, as well as human mesenchymal stem cells and human monocytes are strong responder to supraphysiological loading that can induced osteoclastogenesis. While the response to supraphysiological loading in early human osteoblastic-committed progenitor cells (osteo-progenitors and pre-osteoblasts) showed a patient-specific response, human pre-osteoclasts did not release osteoclast-modulating soluble factors after supraphysiological loading. This indicates, that cells in the local bone marrow niche, in both the mesenchymal and hematopoietic lineage, are primary responders to supraphysiological loading and could play a role in loading-induced peri-prosthetic osteolysis. Furthermore, we have validated our *in vitro* model for bone implant loosening for the application on cells of human origin.

What characteristics of the mechanical stimulus induce an osteo-destructive response, rather than an osteo-protective response?

We have demonstrated in murine myeloid progenitor cells that a combination of high loading amplitude ($\sim 3.0 \pm 0.2$ Pa) with a prolonged active loading period per cycle (duty cycle between 22% and 78%) and a fast alteration between the maximum/minimum loading values (square wave) defines an osteo-destructive response. The observed threshold in the duty cycle indicates that murine myeloid progenitor cells respond to supraphysiological loading in accordance with

the viscoelastic properties of the cells. In addition, we can assume that human cells respond also in accordance with viscoelastic properties as induction of differentiation into the osteoblastic or osteoclastic lineage, which often goes along with cytoskeletal rearrangements and thus changes in the cellular viscoelasticity, altered the osteoclast-inducing response to supraphysiological loading. In addition, we have seen in murine myeloid progenitor cells that SERCA2 might potentially be involved in a mechanism to distinguish between osteo-protective and osteo-destructive response, as SERCA2 was only detectable in cells stimulated by osteo-protective loading profiles. However, the involvement of SERA2 is just an early observation and needs further investigations to fully understand its role. Taken together, a combination of high loading amplitude and prolonged duty cycle defines the osteo-destructive response in loading-induced peri-prosthetic osteolysis.

Which cellular mechano-sensing mechanisms are involved in an osteo-destructive response?

Although the MLO-Y4 osteocyte-like cell line released of NO as the first line of response to supraphysiological loading, NO was not released by murine myeloid progenitor cells. Instead, we detected a loading-dependend release of ATP as fast as two minutes after initiation of the mechanical loading. We investigate the involvement of Piezo1, a membrane channel possessing the capacity to secrete ATP, in the response to supraphysiological loading. Although detection of Piezo1 indicates a potential role in the loading-induced release of ATP, further investigations are necessary to evaluate its involvement in the observed ATP release. Nevertheless, the interaction of loading-induced extracellular ATP with the P2X7 receptor was identified as a crucial axis in the release of osteoclast-modulating soluble factors in murine myeloid progenitor cells. Although we have identified the ATP/P2X7-axis as a crucial mechanism in the release of osteoclast-inducing soluble factors in murine myeloid progenitor cells, further investigation is necessary to fully understand the underlying mechanisms in loading-induced peri-prosthetic osteolysis.

The observations described in this thesis are just the early beginning to reveal key mechanobiological mechanisms that control the osteo-destructive response in loading-induced peri-prosthetic osteolysis. With the proposed *in vitro* model for bone implant loosening, a new tool has been introduced to advance the knowledge in aseptic loosening, which will help to develop novel treatment strategies to delay or prevent implant loosening.

Remarks for the Future

Is there an unknown soluble factor involved in mechanical loading-induced osteolysis?

The induction of osteoclast formation is a well-understood mechanism with their key regulators, M-CSF and RANKL. However, supraphysiological loading on MLO-Y4 did not increase the level of soluble RANKL but it showed an increase in OPG protein levels which could act as a potential regulatory mechanism by the stimulated osteocytes. Despite the higher levels of OPG and unchanged levels of soluble RANKL, conditioned medium of supraphysiologically stimulated MLO-Y4 osteocyte-like cells still induced osteoclastogenesis by soluble factors to a greater extent compared to conditioned medium from physiological loading [Study I]. As pointed out earlier in this thesis, osteoclast formation is complex and numerous co-stimulatory factors have been identified, including but not limited to IL-6 and TNF- α . However, targeted protein analysis performed in the conditioned medium after supraphysiological loading on murine myeloid progenitor cells failed to detect IL-6 and TNF- α (unpublished data). Our findings indicate, that supraphysiological loading might trigger the release of co-stimulatory soluble factor (or factors) which would explain the observed increase in osteoclast formation. RANKL – although undoubtedly being a key factor in osteoclast formation – might not be the leading cause for mechanically-induced osteolysis. This clearly indicates that further analysis is required to identify potential unknown soluble factors involved osteoclast formation.

We intended to shed light on this/these mysterious soluble factor(s) by comparing the secreted protein profile by liquid chromatography-mass spectrometry (LC/MS). The advantage with this combined technique is proteins are physically separated according to their size prior entering the MS where the peptides are vaporizing into charged particles (ions) to detect their mass-to-charge ratio (m/z). Since MS analysis is a highly sensitive method, contamination through regularly used cell culture supplements, e.g. FBS and antibiotics, rendered the obtained trial analysis of the peptide profiles by MS invalid. Sadly, we were not able to solve these problems yet despite that we have eliminated FBS in the conditioned medium. In addition, recently performed experiments proofed a similar response of murine myeloid progenitor cells independent on present antibiotics. Hopefully, in the near future, the next steps can be taken to isolate novel soluble factors involved in mechanical loading-induced osteolysis that will open new treatment strategies to delay or prevent implant failure.

Drug screening in the presence of mechanical loading is critical to prevent mechanical loading-induced osteolysis

There are no pharmaceuticals available to stop ongoing osteolysis and aseptic loosening. The only option is joint arthroplasty or revision. It is difficult with drug development for pharmaceuticals that should work for the skeletal tissue since the bone cells are constantly exposed to mechanical loading. There are currently no valid drug discovery models for mechanical induced bone loss that can combine drug screening with mechanical loading.

In addition, currently available drugs have been tested for their efficacy to improve bone metabolism in osteoporosis. Common beliefs are that aseptic prosthetic loosening could be prevented if implants have improved osseointegration [215]. These drugs are in general classified in two major categories: (1) anabolic, which targets osteoblast activity or number to improve bone mass [216], or (2) anti-catabolic, targeting osteoclast formation and activity [217]. A recent review reflected the effect of anabolic or anti-catabolic agents to improve prosthetic osseointegration and thus could prevent aseptic loosening. Although in animals models these agents showed a beneficial effect on prosthetic osseointegration, in clinical trials the results on prevent aseptic loosening are contradictory [218].

These contradictory observations of used drugs could be explained by the current ways of screening methods. The majority of the drugs have been developed in the absence of mechanical loading which might jeopardize their efficacy [219]. One major cause for this high failure rate might be the changing dynamic in mechanically active target tissues (e.g. muscle, bone) compared to the static ones. That influences the pharmacokinetics of systemically or locally delivered drugs. Increased cellular uptake of therapeutics on skeletal muscle cells or nasal epithelial cells has been reported, when the strain was applied [220, 221]. This situation could also apply to bone cells, highlighting the importance of drug screening during applied mechanical loading. However, in order to use our *in vitro* model for pharmacological studies, an adaption of the current design is necessary. To perform a screening application on a high-throughput level, multiple samples must be stimulated simultaneously instead of the current one-at-a-time design. This can be realized by the application of microfluidics, which would not only reduce the number of cells needed to be stimulated but also tremendously reduce costs as very low amounts of newly develops drugs/pharmaceuticals are needed.

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Papers

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THESIS AT A GLANCE

Study	Question	Methods	Answer
I	Can we simulate supraphysiological loading in an <i>in vitro</i> model?	Fluid Flow loading Direct and indirect osteoclast assay Gene expression Immunocytochemistry ELISA / NO assay	Yes
II	Do myeloid progenitor cells induce osteoclastogenesis upon supraphysiological loading? How are osteoclast-modulating soluble factors secreted?	Fluid Flow loading Indirect osteoclast assay ATP/LDH Blocking (BBG) and activation (BzATP) of P2X7 Gene Expression	Yes Through the ATP/P2X7-axis
III	What physical stimulus drives the osteo-destructive response in our <i>in vitro</i> model?	Fluid Flow loading Fluid Flow recording and analysis Indirect osteoclast assay ATP/LDH Immunocytochemistry	High Loading Amplitude Prolonged active loading duration per cycle
IV	Do (stem) cells in the human bone marrow respond to supraphysiological loading?	Fluid Flow loading Isolation of viable human MSC and HSC Induction of osteogenic and osteoclastic differentiation Indirect osteoclast assay Gene expression Immunocytochemistry	Yes, depending on the stage of differentiation