Effects of the Lyme disease pathogen *Borrelia burgdorferi* on murine bone biology

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A thesis submitted in conformity with the requirements for the degree of
Master of Science

Faculty of Dentistry
University of Toronto

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Abstract

This thesis reports the effects of infection by the Lyme disease pathogen *Borrelia burgdorferi* on bone biology in mice. Bone mineral density, microarchitecture, and cellular morphology were analyzed. *B. burgdorferi* was found to directly invade bone. Infection induced osteopenia in long bones but not vertebrae. Trabecular bone was deteriorated by infection, but cortical bone was unaffected. Bone loss was not due to increases in osteoclastogenesis or bone resorption. Instead, it was associated with reduction in osteoblast numbers. Osteoid-producing and mineralization activities of existing osteoblasts were unaffected by infection. This suggested that osteopenia and disruption of trabecular bone in response to *B. burgdorferi* infection was likely caused by mechanisms which impaired osteoblastogenesis and/or induced osteoblast cell death. Together, these data represent the first evidence that *B. burgdorferi* infection can induce bone loss, and suggest that this phenotype is due to the effects of infection on osteoblastogenesis and/or osteoblast survival.
Preface

Author Contributions

My contributions to this volume include:

**Thesis:**

i). Writing of Literature Review and Thesis Discussion

**Manuscript** (submitted to *Infection & Immunity*):

i). Initial draft and revisions: all figures and tables, manuscript text

ii). Execution and analyses of all experiments except those shown in Fig. 2-1A-B, with training and assistance from L. Zhang and technical assistance from staff the Toronto Phenogenomics Centre. Project supervision was provided by M. Grynpas and principal supervisor T. Moriarty, who assisted with figure preparation, data analysis, and manuscript text

**Other contributions during graduate studies:**


Acknowledgements

I would like to first express my warmest, sincerest gratitude toward Dr. Tara Moriarty for being the most supportive, caring supervisor that any student would dream to have. Ever since my undergraduate years, her wise guidance and her contagious enthusiasm have been potent yeasts catalysts to the brewing of my scientific knowledge and passion. I would also like to thank Dr. Marc Grynpas for giving illuminating pointers when I was at a complete loss of directions, and for his generosity in setting up a collaboration. Lucia Zhang, I cannot thank you enough: you are most wonderful for training me patiently and putting up with my constant, panicked pestering; I can hardly imagine getting this far without you. I also cannot picture how this journey would have turned out without Nataliya Zlotnikov, my partner-in-crime, with whom I learned carefully, exercised carelessly, and philosophized copiously whilst bending over mangled murine innards at ungodly hours. And you, too, Nupur Gupta; what would these years have been like without our late night conversations questioning the meaning of life, occurring any time between 8p.m. and 4a.m.? I cannot forget, of course, all the other lovely Moriarty lab members for all the good laughs, ample dancing, occasional (?) drinking, and for simply being there when I needed them—Rhodaba Ebady, Anna Boczula, Alex Katz, Helena Pětrošová, and Azad Eshghi, thank you all for making this experience amazing.

In addition, I would like to extend my gratitude to Lily Morikawa and Yingchun Zhu of the Phenogenomics Centre; thank you so much for your patience and expertise in processing my countless histology samples. I would also be in deep, deep trouble if it were not for DCM staff Jeffrey Reid, Rhian Duke, and Amy Cao, who worked hard to keep my mice plump and happy. Many thanks also to Adele Changoor, Ana Viniegra-Urbina, Yongqiang Wang, Dr. Bernhard Ganss, and Yuichi Ikeda for lending me a hand in developing protocols for many interesting experiments.

Finally, I do not know what I would be today without the support of my loving parents and my valuable friend Melody Yan. Thank you so much for staying by my side, and for being the magic sponges that help absorb and/or destroy negative metabolite (?) by-products as I munch through the less-than-satisfactorily tasty portions of the gigantic cake called life.
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<td>Mineralization surface</td>
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<td>Mitogen-activated protein kinase</td>
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<td>Receptor activator of nuclear factor κB ligand</td>
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<td>Volume of interest</td>
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<td>Volumetric bone mineral density</td>
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<tr>
<td>Wingless-type mouse mammary tumour virus integration site</td>
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Chapter 1
Literature Review

1.1. Lyme Disease

1.1.1. Epidemiology

Since its initial discovery in the 1970s (Steere et al., 1977), Lyme disease (Lyme borreliosis), an Ixodes tick-borne infection, has emerged to become the most commonly reported vector-borne disease in the northern hemisphere (Wilske, 2005). In particular, the past two decades have seen a sharp rise in Lyme disease prominence. In the United States, reported incidence rates nearly tripled over the past fifteen years (Centers for Disease Control and Prevention, 2013). Additionally, after assessment of clinical records and laboratory reports, US Centers for Disease Control and Prevention (CDC) concluded incidences to be grossly underreported, and estimated a true annual incidence of 300,000 cases—ten times the number of reported cases (Kuehn, 2013).

In Canada, Lyme disease became first reportable in 2009, and the number of cases have since then more than quadrupled, from 144 in 2009 to 682 in 2013 (Public Health Agency of Canada, 2014). Climate warming has also been projected to cause spread of Ixodes scapularis tick habitats in Canada (Leighton et al., 2012), thus increasing probability of exposure to infected vectors. Leighton et al. estimated that by 2020, 80% of the Eastern Canadian population will be residing in tick-inhabited areas, more than quadrupling from 18% in 2010. Interestingly, statistics from the Public Health Agency of Canada (PHAC) showed that Lyme disease has a bi-modal population distribution. Children aged 5-14 and adults aged 55-74 are particularly vulnerable, with susceptibility peaking in middle-aged adults between 55-59 years of age—a rare phenomenon in infectious diseases (Public Health Agency of Canada, 2014). These epidemiological features are similar to the epidemiology of Lyme disease in the United States (Mead, 2015).

1.1.2. Borrelia burgdorferi

The causative agents of Lyme disease are members of the spirochetes Borrelia burgdorferi sensu lato species complex, which consists of more than 20 named and unnamed species
(Piesman and Schwan, 2010) that are spreading geographically (Ostfeld, 2009). Exact species causing pathogenesis may vary across global regions. In North America, Lyme disease is most frequently associated with *B. burgdorferi* sensu stricto (hereafter referred to as *B. burgdorferi*) (Bankhead et al., 2006; Mead, 2015). In Europe, however, Lyme disease (there more commonly referred to as Lyme borreliosis) is attributed to infection by three major species, listed in order of frequency: *B. afzelii*, *B. garinii*, and *B. burgdorferi* (Kurtenbach et al., 2006; Makhani et al., 2011). Much remains unknown about the exact mechanism of pathogenesis.

The *B. burgdorferi* cell envelope consists of a protoplasmic cylinder covered by an outer and an inner lipid membrane (Barbour and Hayes, 1986). Between the two membranes is the periplasmic space, which contains peptidoglycan and flagellar filaments (Kudryashev et al., 2009). A distinct feature of *B. burgdorferi* is that it lacks lipopolysaccharide (LPS) on the outer membrane, and has immunoreactive glycolipids in place instead (Meriläinen et al., 2015). The localization of *B. burgdorferi* flagella within the periplasmic space is also unusual, as other bacteria commonly have them outside the cell (Harman et al., 2013). Additionally, these flagella are not only responsible for bacterial movement, but also contribute to confining *B. burgdorferi* cell shape, akin to skeletal functions (Motaleb et al., 2000).

The *B. burgdorferi* genome is also highly unusual, and consists of a single linear chromosome and a collection of plasmids. However, despite such complexity, *B. burgdorferi* does not appear to encode classically-defined toxins or mechanisms of secreting toxins (Fraser et al., 1997). It has been postulated that this is because *B. burgdorferi* did not evolve to cause disease in mammals, and bacterial products required for bacterial survival induce pathology in mammals instead (Tilly et al., 2008). Some differentially-expressed outer-surface lipoproteins appear to be required for infectivity (e.g. VlsE, OspC) (Radolf et al., 2012); however, even genes that encode these proteins vary significantly (Glöckner et al., 2006). Such variability and the lack of defined virulence factors strongly suggest that Lyme disease pathology is mediated by mammalian host inflammatory response to the invading spirochetes (Norris et al., 2010).
1.1.3. *Ixodes* ticks

*Ixodes* ticks (e.g. *Ixodes scapularis*) serve as a reservoir and vector for North American *B. burgdorferi* species. For each stage of the enzootic life cycle they undergo—larva, nymph, and adult—they ingest one blood meal (Caimano et al., 2016). Larvae are uninfected when hatched, and feed on small reservoir hosts (i.e. squirrels and birds) (Kurtenbach et al., 2006; Radolf et al., 2012). They then moult into nymphs and transmit pathogens acquired during the initial blood meal. It is at this nymphaal stage where ticks primarily infect dead-end hosts such as humans (Radolf et al., 2012). Adult ticks are considered incompetent *B. burgdorferi* hosts because they feed only on larger animals such as deer, which are not a major mammalian reservoir for *B. burgdorferi* (Telford et al., 1988).

Transovarial transmission of *B. burgdorferi* is not observed in ticks (Brisson et al., 2012). Therefore, for *B. burgdorferi* to be continuously maintained in tick populations over successive years, the bacterium must be acquired during the lifetime of each tick. The primary vertebrate reservoir hosts for *B. burgdorferi* in North America are small, highly abundant rodents such as mice and squirrels (Kurtenbach et al., 2002; Piesman and Happ, 1997).
Diagram 1. Enzootic cycle of *Borrelia burgdorferi* transmission.
1.1.4. Clinical manifestations, vaccination, and treatment

*B. burgdorferi* is capable of infecting the bloodstream (spirochetemia) and widespread tissue dissemination from the vasculature. Therefore, Lyme disease symptoms progress in phases from the initial site of inoculation to areas of dissemination, where pathogens eventually establish foci of multiplication and survival. Early Lyme disease symptoms may be similar to cold or flu symptoms: they often include low-grade fever, headache, nausea, and migratory arthralgias and myalgias (Habegger, 2014), and may often be accompanied by the bull's-eye-shaped skin lesion, erythema migrans (EM). EM commonly appears at the tick bite site 1-2 weeks post-feeding; it is relatively asymptomatic—often persisting unnoticed by patients—and may spread overtime into either a single large lesion or multiple smaller lesions as a result of bacterial dissemination (Bockenstedt and Wormser, 2014). If no antibiotic treatment intervenes, *B. burgdorferi* disseminates further into a wide variety of tissues and organ systems, some common examples being joints, the cardiovascular system, and the nervous system (Bockenstedt and Wormser, 2014).

Of these resultant complications, migratory Lyme arthritis is the most common late-stage manifestation in North American patients, affecting as many as 60% of untreated patients months after infection (Steere et al., 1987). Neurologic manifestations may also develop months after infection, often in the form of cranial nerve palsy sometimes accompanied by meningitis and radiculoneuropathies (Bockenstedt and Wormser, 2014). Carditis is relatively rare, contributing to 1% of cases reported to the CDC (Bockenstedt and Wormser, 2014). The most frequent Lyme disease manifestations differ in European countries due to differing etiologic *Borrelia* species. For example, European patients often develop acrodermatitis chronica atrophicans (ACA) and lymphocytoma, but these manifestations are extremely rare in the United States (Busch et al., 1996; Mead, 2015).

There has been considerable debate and interest regarding the best Lyme disease treatment approach. Lyme disease manifestations are generally treated with antibiotics, but responses may differ in individual patients and for different *Borrelia* species or strains (Borchers et al., 2015; Preac Mursic et al., 1996). Efficacy of antibiotic treatment is also known to decline as the time between initial infection and treatment commencement increases (Oksi et al., 2007).
The optimal duration of treatment is another subject of debate. While some advocate more aggressive and longer treatment for patients with persistent symptoms (Cameron et al., 2004), other randomized control led trials have demonstrated no benefit in extending duration or intensity of antibiotic treatment in patients with EM (Kowalski et al., 2010; Oksi et al., 2007; Wormser et al., 2003). Some patients receiving more intense antibiotic treatment even developed adverse effects such as diarrhea (Wormser et al., 2003). These findings, of course, do not eliminate the possibility that some patients may still benefit from more aggressive therapy; they simply illustrate that treatment must be carefully administered for each individual patient.

In a number of patients, symptoms may persist even after adequate antibiotic treatment. Some may develop antibiotic-refractory Lyme arthritis, where synovitis persists for months or years after therapy (Steere and Glickstein, 2004). This has been postulated to be triggered by autoimmunity associated with *B. burgdorferi* infection (Steere and Glickstein, 2004). Another minority of patients may suffer from relapsing nonspecific symptoms, such as fatigue, musculoskeletal pain, and cognitive impairment. This constellation of symptoms is often referred to as post-Lyme disease syndrome, although there are no clear indications that this is in fact caused by *B. burgdorferi* infection (Marques, 2008).

### 1.1.5. Host immune response to *B. burgdorferi*

It is widely thought that the major symptoms of Lyme disease are mainly triggered by host inflammatory responses to *B. burgdorferi*, rather than by pathogenic activities of the bacterium (Tilly et al., 2008).

Innate immune response to inflammation begins with recognition of *B. burgdorferi* by receptors on dendritic cells and macrophages (Weis and Bockenstedt, 2010). The most common of these recognition pathways involve Toll-like receptors (TLRs), particularly TLR2 which recognizes lipoproteins; other receptors such as CD14, nucleotide-binding oligomerization domain (NOD)-like receptors, and scavenger receptors can also be involved (Weis and Bockenstedt, 2010). Upon TLR stimulation, a cascade of downstream pathways stimulate production of both pro- and anti-inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-10, tumour necrosis factor (TNF)-α, and type I interferons (IFN)
Adaptive immunity is also actively involved. Chemokines which recruit B and T cells can be detected in patient fluids such as blood and CSF (Bockenstedt and Wormser, 2014; Rupprecht et al., 2008). Recruitment of Th1, Th2, and Th17 cells are all relevant to host-specific factors, strain of intruding \textit{B. burgdorferi}, and duration of infection (Bockenstedt and Wormser, 2014). For example, an early Th1 response could be a vital step in limiting \textit{B. burgdorferi} burden (Strle et al., 2014), whereas an early Th17 response may be connected to lessening bacterial clearance (Montgomery et al., 2002). It is also important to note that the composition of adaptive immune cells can vary greatly depending on the type of infected tissue. For example, in response to infection, Th1 cells are predominantly recruited to the blood (Steere and Glickstein, 2004); B cells are predominantly recruited to the cerebrospinal fluid (CSF) (Henningsson et al., 2011); polymorphonuclear leukocytes (PMNs) and CD8$^+$ T cells are predominantly recruited to the spinal fluid (SF) (Shen et al., 2010).

1.1.6. Musculoskeletal effects of \textit{B. burgdorferi} infection

As previously described, musculoskeletal pain and Lyme arthritis are well-known late-stage manifestations of Lyme disease. \textit{B. burgdorferi} presence in affected synovial tissue has been confirmed as early as 1996 by DNA amplification (Jaulhac et al., 1996). Lyme arthritis is characterized by recurrent bouts of monoarticular or asymmetric oligoarticular swelling and pain which affects large joints, or migratory polyarthritis in large and small joints (Borchers et al., 2015; Steere et al., 1987). Interestingly, when Lyme disease was initially discovered, the temporomandibular joints were also frequently affected (Steere et al., 1987), but recently this has rarely been described. Lyme arthritis attacks eventually subside in many patients, but a minority may develop chronic synovitis, which may progress to erode and destroy joints similarly to rheumatoid arthritis (Johnston et al., 1985).
Even though Lyme arthritis and relevant immunology are of considerable interest, only a few clinical studies have recorded effects of *B. burgdorferi* infection on bone tissue outside of joints (Oksi et al., 1994), some of which will be discussed later.

### 1.2. Bone Biology

Bone is a constantly remodelled dynamic tissue. As an organ, it serves a few important roles: to support and attach muscles, to protect other vital organs (e.g. bone marrow and brain), and to act as a reserve for minerals (e.g. calcium) (Feng and McDonald, 2011). It is a heterogeneous composite of several phases. These include hydroxyapatite (Ca_{10}(PO_4)_6(OH)_2), a mineral phase, and an organic phase (approximately 90% type I collagen, 5% noncollagenous proteins, 2% lipids by weight) (Boskey, 2013).

All bones within the skeleton contain tissues with two types of structures: cortical and trabecular. Cortical bone is the protective outer portion of bone that provides mechanical support; trabecular bone is the inner portion that provides strength and also acts as the bone-remodelling site (Boskey, 2013).

Bone remodelling is a homeostatic physiological process involving balancing activities of osteoclasts (bone-resorbing cells) and osteoblasts (bone-forming cells). During bone remodelling, bone resorption and bone formation are coupled; old bone is resorbed by osteoclasts and replaced by new bone formed by osteoblasts (Feng and McDonald, 2011). Via complicated signalling mechanisms, the body regulates this process tightly to ensure bone mass and mechanical strength remain constant in a healthy organism. This process takes place within bone remodelling compartments (BRCs), which are comprised of individual units called basic multicellular units (BMUs) (Hauge et al., 2001; Parfitt, 2001). A full BMU contains osteoblasts, osteoclasts, osteocytes, and bone-lining cells (Feng and McDonald, 2011). All of these cell types will be discussed in detail except for bone-lining cells, which are quiescent, outlining cells of the BRC whose direct molecular influences on bone remodelling are still not as well understood as the other cells (Feng and McDonald, 2011).
1.2.1. Basic multicellular unit (BMU)

1.2.1.1. Osteoblasts

Osteoblasts are polarized, cuboidal bone-building cells that line the bone surface. They have three stages of differentiation: from mesenchymal progenitors to preosteoblasts to mature osteoblasts (Dudley and Spiro, 1961). Together with osteocytes and bone-lining cells, these cells are known as osteoblast lineage cells (Long, 2012).

While exact molecular marker profiles of mesenchymal osteoblast progenitors are unclear and debated, preosteoblasts—a transitional state between mesenchymal progenitors and mature osteoblasts—are generally accepted to be characterized by expression of the transcription factor RUNX2, as well as osterix (OSX) during more advanced stages of differentiation (Long, 2012). Preosteoblasts are also known to actively divide in vivo (Owen, 1963). Mature osteoblasts, on the other hand, are marked chiefly by their secretion of type I collagen in order to produce the osteoid (non-mineralized bone), as well as a variety of extracellular proteins such as osteocalcin and alkaline phosphatase (ALP) (Dudley and Spiro, 1961; Long, 2012). Osteoblasts trapped in their secreted matrix become osteocytes, which are mechanosensing major components of mature bone tissue (Bonewald, 2011; Long, 2012).

There are two distinctly different processes by which bone can be formed: intramembranous or endochondral ossification. While the processes of these two types of bone formation differ significantly, the skeletal end results of these two types of bone formation are structurally the same. They are, however responsible for forming different parts of the skeleton (Long, 2012; Olsen et al., 2000).

Intramembranous ossification is relatively rare, occurring only in flat bones such as parts of the skull and the clavicle; during this process, mesenchymal progenitors differentiate directly into preosteoblasts and mature osteoblasts (Long, 2012; Olsen et al., 2000). Endochondral ossification is responsible for forming a vast majority of vertebral long bones; during this process, progenitors first condense to form chondrocytes and perichondral cells that ensheath a cartilage primordium (Kronenberg, 2003; Long, 2012). Chondrocytes eventually undergo hypertrophy, and only afterwards is osteoblast formation from perichondral cells triggered...
New osteoblasts continue to form postnatally, although their origins are not well understood (Long, 2012).

β-catenin-dependent WNT signalling is the most important and most studied pathway shown to regulate postnatal bone accrual (Long, 2012). Mutations at various checkpoints in the pathway can induce bone loss and multiple bone diseases characterized by either bone loss or bone gain, such as osteoporosis pseudoglioma syndrome (juvenile-onset osteoporosis) (Gong et al., 2001), sclerosteosis, and Van Buchem disease (bone-thickening) (Balemans et al., 2001, 2002; Brunkow et al., 2001). Detailed mechanisms by which β-catenin-dependent WNT signalling affect bone health, however, have not been elucidated (Long, 2012). Other important signalling molecules and systemic hormones involved in osteoblast regulation include bone morphogenic proteins (BMPs), fibroblast growth factor (FGF) family proteins, leptin, parathyroid hormone (PTH), growth hormone, insulin growth factor (IGF) 1, insulin, and sex hormones including testosterone and estrogen (Long, 2012).

1.2.1.2. Osteoclasts
Osteoclasts are multinucleated, bone-resorbing cells of monocyte/macrophage origins rich in mitochondria (Novack and Teitelbaum, 2008). Tartrate-resistant acid phosphatase (TRAP) is used as a marker for osteoclast visualization in histology (Novack and Teitelbaum, 2008). Differentiation of osteoclasts require contact with osteoblast-lineage cells or their stromal precursors (Novack and Teitelbaum, 2008; Takahashi et al., 1988). These osteoblast-lineage cells produce receptor activator of NFκB ligand (RANKL), which binds to its receptor RANK on surfaces of osteoclast precursors and triggers osteoclastogenesis (Lacey et al., 1998; Simonet et al., 1997; Yasuda et al., 1998). This osteoclastogenic process is inhibited by a circulating decoy RANKL receptor, osteoprotegerin (OPG) (Novack and Teitelbaum, 2008; Simonet et al., 1997), which binds and neutralizes RANKL (Kassem et al., 2015; Simonet et al., 1997). The RANKL/OPG ratio (or RANKL/OPG axis) in osteoblasts is an important variable that dictates effectiveness of osteoclastogenesis, and is often affected in inflammatory conditions (Kassem et al., 2015).
Another cytokine required for osteoclast differentiation in vitro is macrophage colony stimulating factor (M-CSF). A supply of solely RANKL and M-CSF are sufficient to generate functional osteoclasts in vitro (Novack and Teitelbaum, 2008). M-CSF is produced by a variety of cell types. It binds to the tyrosine kinase receptor c-Fms, initiating critical downstream signals involving a variety of pathways such as PI3K/Akt and MAPK (Novack and Teitelbaum, 2008). Expression of M-CSF is stimulated by the common cytokine TNF-α, which also acts synergistically to RANKL (Fuller et al., 2002; Novack and Teitelbaum, 2008). An additional signalling mechanism that serves important roles in osteoclast differentiation involves the immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors DAP12 and Fcγ (Koga et al., 2004; Mócsai et al., 2004). They are costimulators activated by cell-to-cell contact with ligands expressed on osteoclast precursors and osteoblasts (Koga et al., 2004; Mócsai et al., 2004).

1.2.1.2.1. Osteoclast polarization and cytoskeleton organization

Osteoclasts are polarized when attached to bone. The nuclei are localized towards the non-resorptive basolateral domain, while the resorptive surface consists of a convoluted plasma membrane called ruffled border surrounded by an actin ring sealing zone (Novack and Teitelbaum, 2008). The αvβ3 integrin is the primarily responsible for osteoclast-to-bone attachment, and is expressed in osteoclast precursors (McHugh et al., 2000). αvβ3 integrin recognizes the Arg-Gly-Asp (RGD) domains in bone matrix proteins—such as fibronectin, vitronectin, and osteopontin—and in turn triggers a number of downstream signalling pathways (Novack and Teitelbaum, 2008). Prior to triggering signalling pathways, however, integrins must mediate matrix contact and form focal adhesions and stress fibres (Novack and Teitelbaum, 2008). Mammalian osteoclasts are special in that their actin is organized into sealing zones rather than stress fibres, and podosomes are formed in lieu of focal adhesions (Faccio et al., 2003). These podosomes are made up of a core of F-actin surrounded by αvβ3 and other cytoskeletal proteins such as α-actinin; they form as osteoclasts attach to bone, and are thought to fuse to form a sealing zone (a ring of actin surrounded by αvβ3) (Novack and Teitelbaum, 2008). Bone resorption involves repeated attachment of osteoclasts to bone, degradation of matrix by osteoclasts, and subsequent detachment;
presence of integrins mediating attachment is critical for this cyclic process to optimally function.

The most important signalling molecule associated with the β3 cytoplasmic domain of αvβ3 is c-Src, a proto-oncogene which, when genetically disrupted in mice, results in severe osteoporosis and complete loss of ruffled membranes and actin rings (Boyce et al., 1992; Soriano et al., 1991). During regulation of osteoclast activity, c-Src acts both as a kinase and an adaptor (Miyazaki et al., 2004; Schwartzberg et al., 1997). In its inactive state, c-Src interacts with the β3 cytoplasmic domain only; when αvβ3 is activated, Syk, a tyrosine kinase, is then recruited to the β3 tail to be phosphorylated by the now activated c-Src (Zou et al., 2007). This recruitment of Syk also depends on Syk's association with DAP12 and FcRγ (Zou et al., 2007), which are aforementioned molecules important for osteoclast differentiation.

1.2.1.2. Mechanisms of bone resorption by osteoclasts
The principle of osteoclast resorption centres around intimate contact between the bone surface and the osteoclast ruffled membrane, which contains proton pumps that acidify the resorption space and secreted enzymes that degrade bone (Baron et al., 1985). Acidification of the environment begins with intracellular carbonic anhydrase (CA) (Riihonen et al., 2007), which generates H\(^+\) ions and bicarbonate ions by catalyzing a reaction between water and carbon dioxide. These H\(^+\) ions are pumped into the resorptive space via H\(^+\) ATPases, which are proton pumps transported to bone-opposed plasma membrane by acidified vesicles (Abu-Amer et al., 1997; Blair et al., 1989; Mattsson et al., 1994), forming the ruffled membrane. To balance out the proton transportation, osteoclasts mediate intracellular pH and electroneutrality by energy-independent Cl\(^-\)/HCO\(_3^+\) exchangers and charge-coupled Cl\(^-\) channels respectively (Schlesinger et al., 1997; Teti et al., 1989). The voltage-gated Cl\(^-\)/H\(^+\) exchanger ClC-7 is also a vital electroneutrality mediator. It is abundantly located on the ruffled membrane, and mutations in the CLCN7 gene lead to osteopetrosis in both mice and humans (Kornak et al., 2001). The end result of the cooperation between exchangers, passive channels, and H\(^+\) ATPases is the accumulation of H\(^+\) and Cl\(^-\) ions within the resorptive
space. The entire resorptive microenvironment acidification process, therefore, is akin to the production of hydrochloric acid (HCl) within that space.

Diagram 2. Schematic of osteoclast resorption mechanisms.

Once osteoclast attachment to bone surface occurs and an acidic pH is achieved in the resorptive space, the mineral phase begins to be dissolved. Lysosomal enzymes, such as cathepsin K, are also secreted and polarized out through the ruffled border membrane in order to degrade the bone matrix proteins (Mulari et al., 2003; Saftig et al., 1998).
1.2.1.3. Osteocytes
Osteocytes are terminally differentiated, mechanosensing cells distributed throughout mineralized bone matrix (Santos et al., 2009), with approximate equal distance between each other (Dallas et al., 2013). They are the most abundant cells in the bone, and they are terminally differentiated from osteoblasts as osteoid production slows (Franz-Odendaal et al., 2006; Palumbo et al., 1990), though the exact mechanisms and criteria of differentiation are still not well understood (Dallas and Bonewald, 2010; Dallas et al., 2013; Franz-Odendaal et al., 2006). Identifying osteocyte differentiation markers have been challenging due to a lack of osteocyte cell lines and difficulty in osteocyte isolation. Nevertheless, several markers have been identified to date. These include matrix extracellular phosphoglycoprotein, sclerostin, dentin matrix protein-1, and phosphate regulating endopeptidase homolog, X-linked (PHEX) protein (Santos et al., 2009).

1.2.1.3.1. Osteocyte structure, cytoskeleton, and mechanotransduction
Osteocytes have a characteristic dendritic cell shape and processes that emanate multidirectionally from the cell body through the canaliculi of the bone (Santos et al., 2009). These processes form an intricate intercellular network known as the lacunocanalicular system (Santos et al., 2009; Vatsa et al., 2007). All osteocytes are completely embedded within a mineralized extracellular matrix, which theoretically makes it difficult for them to access nutrients and communicate with other cells. The lacunocanalicular system is, however, specifically adapted to solve this problem (Dallas et al., 2013). To form this system, structures of osteocytes change from plump, polarized polygons characteristic of osteoblasts into stellate, dendritic shapes, losing up to 70% cell volume and reducing cellular organelles such as endoplasmic reticulum and Golgi apparatus (Dudley and Spiro, 1961; Palumbo, 1986). Osteocytes then connect with each other using their dendritic processes, allowing for in-between passage of nutrients and biochemical signals through the gap junctions on dendrite tips (Dallas et al., 2013; Doty, 1981). The dendrites have also been shown to have abilities to extend and retract, and to have the potential to not only communicate with osteoblasts, but also to make and break contact with other cells in the bone marrow space (Dallas et al., 2013).
Osteocytes are among cell types most sensitive to mechanical loading (Dallas et al., 2013). Compared to osteoblasts and fibroblasts, they are more sensitive to fluid shear stress rather than substrate stretching (Kleinnulend et al., 1995; Klein-Nulend et al., 1995). The fluid flow hypothesis is one of the most widely accepted hypotheses which offers an explanation on how osteocyte perform mechanosensing. It states that osteocytes are able to sense mechanical load on the bone via interstitial canalicular fluid in the lacunocanalicular system (Santos et al., 2009). Upon bone deformation, the canalicular fluid flows from higher pressure regions to lower pressure regions; in turn, upon sensing fluid shear stress, osteocytes produce signalling molecules which regulate osteoclast and osteoblast activity (Cowin et al., 1995; You et al., 2000). There has been considerable debate over exactly which portion of the osteocyte senses the loading. Some have suggested that mechanosensing occurs only through the dendritic processes (Adachi et al., 2009; Han et al., 2004), while others have shown that the cell body (Nicolella et al., 2008) or the primary cilia (Malone et al., 2007; Xiao et al., 2006) also have roles. Evidence has been shown for all three proposals, and it is unclear whether these locations within the osteocyte work separately or in conjunction to sense mechanical loading (Dallas et al., 2013).

There are a number of other signalling molecules identified to date which are relevant to osteocytes' conversion of mechanical strain signals into a cellular response. First, upon sensing fluid shear stress, intracellular calcium increases rapidly; then, within seconds to minutes, molecules relevant to bone anabolism—such as nitric oxide (NO), adenosine triphosphate (ATP), and prostaglandin—are released (Dallas et al., 2013). NO inhibits bone resorption, promotes bone formation, and reduces osteocyte apoptosis (Kulkarni et al., 2010). ATP has been shown to be released through Cx43 hemichannels opened by mechanical loading (Cherian et al., 2003, 2005), and may control bone anabolic response via the P2X7 nucleotide receptor, which is an ATP-gated ion channel whose deletion in mice abolished osteocyte prostaglandin release \textit{ex vivo} and reduced bone anabolic response by 70\% (Li et al., 2005). Prostaglandins are key effectors of bone anabolic response to mechanical stress; treatment with prostaglandins can enhance bone formation, and inhibition of prostaglandins can block effects of anabolic loading (Pead and Lanyon, 1989). Another relevant signalling pathways activated by mechanical loading is the Wnt/β-catenin pathway, which is important
for osteoblast differentiation. Osteocytes express negative regulators of the Wnt/β-catenin pathway, such as Dickkopf1-related protein 1 (DKK1) and sclerostin (Poole et al., 2005); these proteins must be down-regulated by an unknown mechanism to activate the Wnt/β-catenin pathway in order to build an osteogenic response to stress (Kramer et al., 2010; Tu et al., 2011). In addition, osteocytes have been shown to enhance bone formation at fracture sites by recruiting MSCs via secretion of osteopontin (Raheja et al., 2008). Both avian osteocytes (Vezeridis et al., 2006) and MLO-Y4 cells (Heino et al., 2004) have also been shown to secrete factors that increase osteoblast differentiation and ALP activity.

Osteocytes can also regulate bone resorption by affecting osteoclast activity. Both MLO-Y4 cells (Kartsogiannis et al., 1999) and primary chick osteocytes (Tanaka et al., 1995) have been shown to enhance osteoclast differentiation and activation in vitro. Expression of the important osteoclastogenic factors RANKL and M-CSF has also been observed in osteocytes in vivo and in vitro (Ikeda et al., 2001; Kartsogiannis et al., 1999; Kulkarni et al., 2010; Kurata et al., 2006; Zhao et al., 2002). In addition, osteocyte death via apoptosis can recruit osteoclasts to the site of damage (Dallas et al., 2013). Since living osteocytes have been hypothesized to secrete unknown molecules that inhibit osteoclast activity, it is suggested that osteocyte death may trigger osteoclast recruitment due to loss of these inhibition factors (Gu et al., 2005). One study supporting this hypothesis shows that targeted in vivo killing of osteocytes using the diphtheria toxin receptor mouse model induced osteoclast formation and bone loss (Tatsumi et al., 2007). Osteocyte-derived apoptotic bodies can also induce osteoclastogenesis in vivo and in vitro, while osteoblast-derived apoptotic bodies had no effect (Kogianni et al., 2008).

1.2.2. Bone remodelling

1.2.2.1. Steps of bone remodelling

Bone is a dynamic tissue. Throughout an organism's lifespan, osteoclasts are constantly resorbing old bone while osteoblasts lay down new bone; this process is termed bone remodelling, and it is necessary to maintain skeletal structural integrity. The balance between these constant bone-building and bone-resorbing activities is central to skeletal homeostasis.
Bone turnover takes place in a temporary anatomic compartment called the bone remodelling compartment (BRC), which contains functional basic multicellular units (BMU) characterized by the presence of osteoblasts, osteoclasts, osteocytes, and bone-lining cells (Feng and McDonald, 2011).

The bone remodelling process can be roughly divided into the following phases: initiation/activation, resorption, reversal, formation, and termination (Raggatt and Partridge, 2010).

During initiation, in the case of daily wear and tear on bone, signals from mechanosensing osteocytes trigger osteoclast precursor recruitment from the bone marrow (these precursors directly cross the bone-lining cell monolayer) or from capillaries that penetrate into the BRC (Parfitt, 2002). In the case of bone damage, osteocyte apoptosis can directly increase osteoclastogenesis (Aguirre et al., 2006). This is because under resting condition, osteocytes secrete transforming growth factor β (TGF-β), which is an osteoclastogenesis inhibitor; damage and apoptosis of osteocytes, therefore, removes the inhibitory signal and allows osteoclastogenesis to proceed (Raggatt and Partridge, 2010).

The next phase is the resorption phase. In this phase, recruited osteoclast precursors attach to the bone matrix and differentiate into osteoclasts under the influence of osteoblast-produced monocyte chemoattractant protein-1 (MCP-1) (Li et al., 2007). Osteoblasts and osteocytes express M-CSF, RANKL, to promote osteoclastogenesis; while OPG production is reduced (Ma et al., 2001). Once mature osteoclasts are formed, the resorption phase begins, and old or damaged bone is degraded via mechanisms involving sealing zone formation and acidification with H+ ions, as described earlier.

While resorption and reversal occur, recruitment of mesenchymal stem cells (MSCs) and osteoprogenitors from similar origins (bone marrow and capillaries) is also initiated (Feng and McDonald, 2011). MSCs are able to differentiate into preosteoblasts and mature osteoblasts (Feng and McDonald, 2011). Once enough osteoblasts form, bone formation phase begins to overshadow bone resorption. This is the phase that is sustained for the longest while bone resorption continues (Feng and McDonald, 2011). During this phase, osteoid synthesis is also mediated by MSCs, which, upon recruitment to the resorption lacunae, begin secreting bone matrix proteins such as collagen type I, proteoglycans, glycosylated proteins (i.e. tissue-nonspecific alkaline phosphatase (TNAP)), small integrin-binding ligand (SIBLING) proteins, Gla-containing proteins, and lipids (Raggatt and Partridge, 2010). These molecules compile to form the osteoid. Hydroxyapatite is then incorporated into the osteoid for bone to assume its final form (Feng and McDonald, 2011). The extracellular matrix then undergoes a series of modifications to mature further and become competent for mineralization, involving a number of non-collagenous proteins such
as TNAP, nucleotide pyrophosphatase diesterase, and progressive ankylosis (ANK) protein which generate an ideal extracellular concentration of inorganic phosphate suitable for mineralization (Feng and McDonald, 2011).

Signals that coordinate the transition between bone resorption and bone formation phases are still not well understood. The earliest proposals hypothesize that coupling molecules such as insulin-like growth factors (IGF) and TGF-β are released from the bone matrix during bone resorption (Tang et al., 2009); however, studies have also shown that in both mice and humans with functionally defective osteoclasts, bone formation remains preserved (Martin and Sims, 2004). These observations then led to the surmise that osteoclasts, rather than matrix, produce the coupling factors instead (Martin and Sims, 2004). Candidate coupling mechanisms include: sphingosine 1-phosphate (S1P, secreted by osteoclasts; promotes osteoblast precursor recruitment and mature osteoblast survival) (Pederson et al., 2008); and the EphB4-ephrin-B2 bidirectional signalling complex.

Finally, when an equal amount of resorbed bone has been replaced with mineralized new bone, the bone remodelling cycle concludes (Raggatt and Partridge, 2010). The exact termination signals are unclear, although there has been increasing evidence showing that osteocytes are involved (Raggatt and Partridge, 2010). Once mineralization is complete, mature osteoblasts can also undergo apoptosis. They can also revert to bone-lining cells or become embedded in the matrix and eventually differentiate into osteocytes (Raggatt and Partridge, 2010). This reestablishes the resting bone environment until the next cycle of bone remodelling begins (Raggatt and Partridge, 2010).

1.2.2.2. Osteoblast-osteoclast coupling
While the respective bone-forming and bone-resorbing functions of osteoblasts and osteoclasts seem to counteract each other, these cells are in fact also known to enhance each other's differentiation and function. It is well established that osteoblast lineage cells and their stromal precursors are necessary for osteoclast differentiation, as they actively produce RANKL (Novack and Teitelbaum, 2008). Endocrine signalling can also affect osteoclast activity through osteoblast lineage cells. Most notably, the parathyroid hormone (PTH)
recruits osteoblasts and induces their production of RANKL (Takayanagi, 2012). Other osteoblast-seeking factors such as vitamin D₃ and prostaglandin E₂ (PGE₂) can also induce osteoclastogenesis in vitro during co-culturing of osteoclast precursors with osteoblasts (Teti, 2013). Of these two factors, vitamin D₃ is known to directly target osteoblast lineage cells and induce RANKL expression via activating its cytosolic receptor (Carlberg and Campbell, 2013; Lee et al., 2002). PGE₂ also induces RANKL expression via a pathway converging with that of PTH on cyclic AMP (cAMP) through their cell surface receptors (Blackwell et al., 2009).

Inflammatory cytokines can also contribute to osteoblast and osteoclast cooperation in either direction. Osteoblasts are known to produce many pro-osteoclastogenic cytokines, including RANKL, M-CSF, interleukin (IL)-1β, IL-6, IL-11, leukemia inhibitory factor, oncostatin M, etc. They also produce anti-osteoclastogenic factors such as OPG, granulocyte-macrophage-stimulating factor (GM-CSF), IL-3, IL-12, and IL-18 (Redlich and Smolen, 2012; Teti, 2013). This implies that osteoblasts are able to control their pro-osteoclastogenic functions and maintain a balanced response during physiological or pathologic bone remodelling.

One of the most notable cytokines involved in osteoclast modulation of osteoblast activity is TGF-β, which is a potent stimulator of bone formation activated by isolated osteoclasts (Teti, 2013). It has been proposed that TGF-β is a key coupling factor which stimulates formation at previous sites of resorption during bone remodelling (Teti, 2013); in fact, TGF-β follows the same SMAD/MAPK signalling pathway with bone morphogenetic proteins (BMPs), which are key therapeutic proteins involved in bone formation and tissue repair (Teti, 2013). Osteoclasts have also been proposed to secrete clastokines which function specifically as osteoblast anabolic factors. Examples of these clastokines include type 5 tartrate-resistant acid phosphatase (TRAcP), S1P, BMP6, Wnt10b, platelet-derived growth factor-BB (PDGF-BB), hepatocyte growth factor (HGF), and collagen triple helix repeat containing 1 (CTHRC1) (Teti, 2013). Of these clastokines, BMP6 and Wnt10b are already known as mediators of bone anabolism during regular bone formation. Recent studies, however, are highlighting their secretion by osteoclasts as part of a "tool kit" that modulates bone formation (Teti, 2013).
Osteoclasts and osteoblasts can also communicate bidirectionally through direct cell-to-cell contact via joining of the EphrinB2 ligand (expressed by osteoclasts) and the EphB4 receptor (expressed by osteoblasts) (Irie et al., 2009; Matsuo and Irie, 2008). This binding triggers downregulation of transcription factors such as c-Fos and NFATc1 in osteoclasts, suppressing osteoclast formation as a result (Teti, 2013). As this occurs in osteoclasts, EphB4 receptors on the osteoblasts also induce their intracellular pathways, resulting in a reduction of RhoA and enhancing osteoblast differentiation (Irie et al., 2009). EphrinB2/EphB4 bidirectional signalling is counterbalanced by the EphrinA2/EphA2 pathway, which regulates bone remodelling during the initiation phase (Kang and Kumanogoh, 2013). EphrinA2 and EphA2 are both expressed by osteoclast lineage cells to enhance differentiation. EphA2 only is expressed by osteoblast precursors to suppress bone formation (Kang and Kumanogoh, 2013).

The semaphorin/plexin pathway, originally identified as a means of axon guidance, is another pathway often involved in bidirectional signalling in the bone (Teti, 2013). Semaphorin 3A is produced by osteoblast lineage cells, and has been found to interact with osteoclast membrane protein neuropilin-1 (Hayashi et al., 2012). This interaction has separate roles in osteoclasts and osteoblasts. In osteoclasts, this interaction represses RANKL-stimulated osteoclastogenesis by inhibiting RhoA and ITAM signalling, decreasing osteoclastogenesis; in osteoblasts, this interaction activates the Wnt/β-catenin signal and increases bone formation (Hayashi et al., 2012).

Although osteoclast-osteoblast cross-talk is well-documented and extremely intricate, external interference with the function of one cell type often does not affect the function of the other. In other words, when the function of one cell type is compromised, the other cell type cannot respond and compensate. For example, treatment with denosumab, which inhibits osteoclast formation, does not affect osteoblast differentiation and activity (Guerrini et al., 2008; Sobacchi et al., 2007).
1.2.2.4. Bone remodelling disorders and other bone disorders

A number of disorders can arise due to imbalance in formation and resorption activities during bone remodelling. The most common bone disorder is osteoporosis, characterized by low BMD and high bone resorption. There are two types of osteoporosis: postmenopausal (type I) and age-related/senile (type-II) (Riggs et al., 1982). Postmenopausal osteoporosis is caused by estrogen deficiency, which leads to increased presence of osteoclasts due to loss of estrogen inhibitors (Feng and McDonald, 2011). A number of drugs that target osteoclasts, such as bisphosphonates, have been developed in recent decades as treatment (Feng and McDonald, 2011). Age-related osteoporosis develops through a completely different mechanism, and affects both women and men at ages as early as the twenties (Khosla and Riggs, 2005; Raisz, 2005). In this disease form, reactive oxygen species (ROS) build up within cells due exposure to extracellular signals (e.g. cytokines, radiation) throughout aging (Balaban et al., 2005), and cause either cellular damage/death or trigger activation of cellular signalling pathways (Feng and McDonald, 2011). The effect is more universal and difficult to target as it occurs in multiple cell types; nevertheless, bisphosphonates is administered as treatment as well (Feng and McDonald, 2011). Osteoporosis can also be induced by glucocorticoids (GC) or immobilization. GC-induced osteoporosis is often seen as a side effect to GC treatment, which is harmful to osteoblast differentiation; immobilization-induced osteoporosis does not have a clear elucidated mechanism, but mechanosensing osteocytes have been proposed to play an important role (Feng and McDonald, 2011).

Apart from osteoporosis, which typically occurs later in life, many other bone remodelling diseases can be caused by a variety of factors, including genetic mutation. Disease-causing mutations can disrupt the bone remodelling balance by impairing functions of a range of bone components or surroundings, such as bone cells (e.g. osteopetrosis, pycnodysostosis, cystic angiomatosis, van Buchem disease, sclerosteosis, osteopoikilosis, Hajdu-Cheney syndrome), bone matrix proteins (e.g. osteogenesis imperfecta, hypophosphatasia), bone microenvironment regulators (e.g. hyperphosphatasia), and calcitropic hormonal activity (e.g. parathyroidism) (Beighton et al., 1976; Canalis and Zanotti, 2014; van Dijk et al., 2011; Farronato et al., 2014; Lee and Partridge, 2009; van Lierop et al., 2013; Malik et al., 2008; Sanyal and Stuart, 2010; Silve, 1994; Sobacchi et al., 2013).
Bone remodelling can also be disrupted by non-genetic factors in adulthood, resulting in disease. Nutrient deficiency, in particular of vitamin D, calcium, and phosphorus, can lead to weak, poorly mineralized bone. Vitamin D deficiency, for example, can cause rickets in children and osteomalacia in adults. Hormonal disorders—possibly resulting from other pathological complications—can also affect the skeleton. For example, abnormal parathyroid hormone (PTH) levels caused by chronic kidney disease can not only lead to abnormal bone remodelling, but also result in renal osteodystrophy, a complication linked to increased cardiovascular calcification (Feng and McDonald, 2011). Other unknown environmental factors can also induce disease; Paget's disease, for example, is a multifaceted condition hypothesized to be influenced by both genetic and environmental factors (i.e. viral infection); under the influence of this condition, both osteoblasts and osteoclasts are highly active and produce abnormal "woven" trabecular bone (Singer, 2009). Finally, a large number of bone disorders are local and affect a small region of the skeleton, often caused by inflammation and resorbing factors secreted by inflammatory white cells. Inflammation as a response to bacterial infections is a common cause of bone loss. Effects of severe inflammation can spread to induce bone loss in adjacent areas in cases such as periodontal disease and rheumatoid arthritis. Inflammation can also be directly induced by pathogens invading the bone, causing osteomyelitis, which will be discussed later.

This thesis will focus mainly on inflammation-related bone pathologies and discuss them in detail, particularly those associated with bacterial infections.

1.3. Osteoimmunology

It has been long known that chronic inflammation can disrupt the balance between bone resorption and bone formation, resulting in local and systemic bone loss. There have been many observations detailing mechanisms by which immune cells and inflammatory cytokines induce bone catabolism. Evidence has also been emerging recently for the role of the immune system in bone anabolism.
1.3.1. Osteoimmunology and bone catabolism

Inflammatory cytokines can directly affect bone catabolism by interfering with differentiation of the bone-resorbing osteoclasts. Apart from M-CSF and the RANKL/OPG axis, which have been previously discussed, many common cytokines are also well-established stimulators of osteoclastogenesis. Examples of such cytokines are IL-1, IL-6, IL-7, IL-11, IL-17, IL-18, interferon (IFN)-γ, and TNF-α (D’Amico and Roato, 2012). Most of these cytokines function by upregulating RANKL, which, again, is a key osteoclastogenic factor. Some of these cytokines can also work with each other or even amplify their own effects. For example, IL-6, the most abundant cytokine in the circulation (Naka et al., 2002), is induced by TNF and IL-1 (Devlin et al., 1998; Dinarello et al., 1986; Ikejima et al., 1990), and can bind with its own soluble receptor (IL-6R) to form a complex that amplifies its own activity (Murakami et al., 1993; Redlich and Smolen, 2012). IL-6 has been suggested to be the most potent cytokine in inducing inflammatory bone loss, as it is most abundant and can become involved in a number of physiological processes such as activation of hypothalamic-pituitary-adrenal axis as well as estrogen deficiency (Redlich and Smolen, 2012). IL-17 has also been particularly studied and noteworthy. Not only is it known as one of the strongest stimulators of RANKL (Redlich and Smolen, 2012; Wong et al., 2006), its potentially controversial role in inflammatory bone loss is also of great interest. On one hand, IL-17 upregulates granulocyte colony-stimulating factor (G-CSF) and help recruit neutrophils to fight infections (Schwarzenberger and Kolls, 2002); on the other hand, excess IL-17 has been shown to be detrimental to the bone (Chabaud et al., 2001; Lubberts et al., 2002; Yu et al., 2007). The anti-inflammatory and osteoclastogenesis-enhancing natures of IL-17 and other cytokines particularly complicate cases of inflammatory bone loss, such as rheumatoid arthritis or periodontal disease.

Apart from influencing inflammatory cytokine activity, osteoclasts are also known to be involved in cross-talk with immune cells. RANKL can modulate immunity by enhancing dendritic cells' (DCs) survival and increasing the ability of DCs to stimulate naïve T-cell proliferation (D’Amico and Roato, 2012). T-cells are also known to express RANKL (Greenblatt and Shim, 2013) as well as release IL-7 (D’Amico and Roato, 2012; Roato et al., 2006), which can in turn upregulate RANKL. Additionally, recent studies have identified
more direct cell-to-cell interactions between osteoclasts and T-cells have been identified. Osteoclasts have been found to present antigens to T-cells and activate immune responses (Kiesel et al., 2009).

Under inflammatory conditions, T-cells can also differentiate into Th17 cells, which can induce RANKL expression as well as secrete IL-17, which also upregulates osteoclastogenesis (D’Amico and Roato, 2012). Other helper T cells, specifically Th1 and Th2, also have implications in promoting bone resorption, though their roles tend to be context-dependent (Gao et al., 2007; Sato et al., 2006).

Apart from enhancing bone resorption, inflammatory mechanisms can also contribute to bone catabolism via suppression of bone formation. In a subcutaneous implant model of bone formation, basal presence of T cells was able to inhibit bone formation by its production of IFN-γ and TNF (Greenblatt and Shim, 2013; Liu et al., 2011). IFN-γ directly stalls osteoblastogenesis by inhibiting induction of RUNX2, and also synergizes with TNF to apoptose implanted MSCs (Liu et al., 2011). In addition, T cells are known to affect bone formation through hormonal axes. In a model of hyperparathyroidism, T cells can promote PTH's ability to mediate bone loss via expression of CD40L, which stimulates RANKL production and suppresses OPG production (Gao et al., 2008).

1.3.2. Osteoimmunology and bone anabolism

New evidence has emerged suggesting that activation of both the innate and adaptive immune systems can promote bone formation. Lipopolysaccharide (LPS) or bacterial lipoprotein injection has been shown to stimulate osteoclast differentiation in vitro (Sato et al., 2004); however, counterintuitively, while mice lacking in MyD88 (LPS response adaptor) display reduced osteoclast numbers, they also have overall reduced bone mass (Sato et al., 2004). This apparent negative effect of MyD88 deficiency on bone formation suggests that some form of immune stimulation is needed to maintain basal bone turnover (Greenblatt and Shim, 2013). It is still uncertain which stimulatory ligand activates MyD88-dependent enhancement of bone formation (Sjögren et al., 2012).
Additionally, T cells can also modulate bone formation by secreting growth factors. Cell-to-cell interaction can also occur between T cells (in particular regulatory T cell and CD8+ T cells with regulatory phenotypes) (D’Amico and Roato, 2012) and osteoclasts through CD137/CD137L interactions; CD137 is a member of the TNF receptor induced by T-cell receptor activation, while CD137L is the ligand expressed in DCs and osteoclast precursors (D’Amico and Roato, 2012). In vitro ligation of CD137L has been shown to suppress osteoclastogenesis by inhibiting multinucleation (Senthilkumar and Lee, 2009).

Overall, it is evident that many immunomodulatory mechanisms work in concert to maintain bone mass in case of averse inflammatory stimuli.

1.4. Osteomyelitis

Bacterial infections, by triggering inflammatory responses, can also affect bone biology. Direct infection, inflammation, and resultant destruction of the bone by bacterial agents constitute a condition termed osteomyelitis. Pathogenesis of osteomyelitis generally occurs by hematogenous spread, direct inoculation of microorganisms (e.g. surgical), and contiguous spread by direct contact (Calhoun et al., 2009). Patients with hematogenous osteomyelitis usually experience nonspecific pain and occasional acute symptoms such as fever and chills (Calhoun et al., 2009). Patients with osteomyelitis due to contiguous spread tend to experience more localized bone pain, erythema, swelling, and drainage around the area of trauma; fever and chills may occur during the acute but not chronic phase (Calhoun et al., 2009).

1.4.1. Classification

Osteomyelitis can be clinically classified in a number of ways including by duration (acute/chronic), by pathogenesis, and by site (Calhoun et al., 2009). Two clinical classification systems are most popular: the Waldvogel system (Waldvogel et al., 1970) and the Cierny-Mader system (Cierny et al., 2003). The Waldvogel system classifies osteomyelitis according to: duration (acute/chronic), source of infection (hematogenous/contiguous), and vascular insufficiency, in this order (Waldvogel et al., 1970).
This system does not account for pathogenesis by direct bacterial inoculation (e.g. via trauma or surgery); it is therefore seen to be more limited in clinical value in comparison to the Cierny-Mader system (Cierny et al., 2003). The Cierny-Mader system characterizes osteomyelitis into four stages based on a number of anatomical and clinical features. The first stage is the medullary stage, where detrimental effects occur in the trabecular bone only. The second stage is the superficial stage, where only cortical bone is affected. The third stage is the localized stage, where both cortical and trabecular bones are affected; however, the bone remains stable and the infection does not encircle the entire bone diameter. The fourth and final stage is the diffuse stage, where the infection spreads to the entire thickness of the bone and bone stability is lost (Calhoun et al., 2009; Cierny et al., 2003).

1.4.2. Pathology

During acute osteomyelitis, infection is often accompanied by edema, vascular congestion, and small vessel thrombosis (Calhoun et al., 2009). This leads to bone death due to compromise of medullary and periosteal blood supplies (Emslie et al., 1983). At this early stage, osteomyelitis can still be abolished by antibiotics and surgery before the dead bone (sequestra) further develops (Calhoun et al., 2009). Once the disease evolves into the chronic stage, fibrous tissue and inflammatory cells begin to form around the dead bone, and once the infection is contained, vascular supply to the area becomes even more limited (Calhoun et al., 2009). This leads to ineffective host inflammatory response, eventually resulting in bone necrosis. Treatment for both acute and chronic osteomyelitis usually involves a combination of antimicrobial therapy and debridement surgery (Calhoun et al., 2009).

Another factor associated with the refractory nature of chronic osteomyelitis is the persistence of bacteria within biofilms which adhere to the bone (Anwar et al., 1990; Gristina et al., 1985). Biofilms are cells embedded in hydrated polysaccharide matrix containing nucleic acids and proteins; killing bacteria within biofilms has been reported to require more than 50 to 1000 times the concentration of antimicrobial agents necessary to kill free-floating cells (Calhoun et al., 2009).
1.4.3. Staphylococcus aureus and osteoblast invasion

*Staphylococcus aureus* is the most common organism isolated in hematogenous osteomyelitis in the long bones and vertebrae (Berbari et al., 2015; Lew and Waldvogel, 1997). A number of animal studies have revealed that bone infection by *S. aureus* can increase levels of many cytokines, including: IL-1, IL-1β, IL-6, and TNF-α (Wright and Nair, 2010). All of these cytokines are known to enhance osteoclastogenesis and inhibit differentiation of MSCs into osteoblast-like cells (Wright and Nair, 2010).

There is another more direct, interesting, and unique mechanism, however, by which *S. aureus* contributes to net bone loss. *S. aureus* is known to invade, persist in, and potentially drive apoptosis of different cell types such as epithelial cells and endothelial cells (Garzoni and Kelley, 2009). In bones, the cell type affected is of the osteoblast lineage. This has been observed both *in vitro*, *in vivo*, and by microscopy in the fibula *ex vivo* (Bosse et al., 2005; Ellington et al., 1999; Reilly et al., 2000). The uptake of *S. aureus* requires expression of fibronectin-binding proteins (FnBPs) and many other MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Ahmed et al., 2001; Sinha et al., 1999). These FnBPs and other MSCRAMMs bind with extracellular matrix proteins such as fibronectin, and subsequently induce integrin α5β1-mediated uptake by target osteoblasts (Sinha et al., 1999).

1.5. Bacterial Infections and Bone Diseases

Apart from *S. aureus*, a number of other pathogens are also known to directly cause osteomyelitis, including *Streptococcus agalactiae, Streptococcus pyogenes, Haemophilus influenzae*, and even *Escherichia coli* in infants (Calhoun et al., 2009; Song and Sloboda, 2001). Interestingly, apart from long bones, short bones, and vertebrae, which are common infection targets, osteomyelitis has also been described in the jaw, though the exact etiology remains unknown (Patel et al., 2010). Unlike osteomyelitis in the long bones, osteomyelitis in the jaw is rarely induced by hematogenous spreading; rather, it is often caused by contact with adjacent odontogenic infections (e.g. periodontitis) (Patel et al., 2010).
Porphyromonas gingivalis, a Gram-negative bacteria prominently associated with periodontitis (Holt and Ebersole, 2005), has been recently found to induce bone resorption by enhancing RANKL activity through TLR2 activation (Kassem et al., 2015). Treponema denticola, a spirochete, is another periodontal pathogen known to induce alveolar bone resorption via unknown mechanisms (Bakthavatchalu et al., 2010). Another spirochete of the same species, the syphilis-causing Treponema pallidum, is also known to have high affinity for bone tissue (Fabricius et al., 2013). There have been documented clinical cases of bone lesion as a result of early and secondary syphilis (Dismukes et al., 1976; Ollé-Goig et al., 1988; Rosen and Solomon, 1976).

<table>
<thead>
<tr>
<th>Clinical association</th>
<th>Microorganism (s)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Osteomyelitis-related</td>
</tr>
<tr>
<td>Frequently isolated in any type</td>
<td>Staphylococcus aureus, Haemophilus influenzae (in children)</td>
</tr>
<tr>
<td>Foreign-body-associated infection</td>
<td>Coagulase-negative staphylococci, Propionibacterium spp</td>
</tr>
<tr>
<td>Nosocomial infections</td>
<td>Enterobacteriaceae, Pseudomonas aeruginosa, Candida spp</td>
</tr>
<tr>
<td>Bites, diabetic foot lesions, decubitus ulcers</td>
<td>Streptococci and/or anaerobic bacteria</td>
</tr>
<tr>
<td>Sickle-cell disease</td>
<td>Salmonella spp, Streptococcus pneumoniae</td>
</tr>
<tr>
<td>HIV infection</td>
<td>Bartonella henselae, B quintana</td>
</tr>
<tr>
<td>Human or animal bites</td>
<td>Pasteurella multocida, Eikenella corrodens</td>
</tr>
<tr>
<td>Immunocompromised patients</td>
<td>Aspergillus spp, Candida albicans, Mycobacteria spp</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>Others</td>
<td>Brucella spp, Coxiella burnetti, certain fungi</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Other disorders-related</th>
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</thead>
<tbody>
<tr>
<td>Periodontitis</td>
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<tr>
<td>Syphilis</td>
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**Table 1-1. List of microorganisms known to infect the bone** (Calhoun et al., 2009; Lew and Waldvogel, 2004; Zubery et al., 1998)
It is unknown whether the *B. burgdorferi*, a spirochete like *T. denticola* and *T. pallidum*, may induce similar bone pathology. However, despite profound interest in Lyme arthritis in the Lyme disease field, and despite evidence of direct *B. burgdorferi* infection of joints (Jaulhac et al., 1996), there have been very few studies documenting *B. burgdorferi* infection impact on bone. Migratory bone pain, bone erosion at articular surfaces, multifocal osteomyelitis, and other bone involvement have been reported in *B. burgdorferi*-infected rodents and humans (Houtman and Tazelaar, 1999; Hovmark et al., 1986; Kvasnicka et al., 2003; Lawson and Rahn, 1992; Lawson and Steere, 1985; Munson et al., 2012; Oksi et al., 1994; Schlesinger et al., 1985, 1985; Schmitz et al., 2004; Steere, 1995; Steere et al., 1987), and *B. burgdorferi* has been detected in bone marrow and bones of humans, dogs, and the mummy of a Copper Age man (Hoviuis et al., 1999; Keller et al., 2012; Oksi et al., 1994; Schlesinger et al., 1985). However, the effects of *B. burgdorferi* infection on bone microarchitecture and cellular biology have not been widely investigated in humans or in the laboratory mouse.

### 1.6. Mouse Models

#### 1.6.1 Mouse models of Lyme disease

Inbred laboratory mouse strains have been shown to develop some of the pathologies associated with Lyme disease in humans, including arthritis and carditis (Barthold et al., 2010). There are also, however, stark contrasts between disease phenotypes among humans and mice and also across mouse strains. Unlike humans, laboratory mice do not develop EM (due to the fact that mice are small and a whole infected mouse may be considered one large EM) and rarely develop neuroborreliosis (Barthold et al., 2010). Among mouse strains, degree of symptom severity can also vary greatly. C57BL/6 mice, for example, do not develop severe arthritis and carditis, whereas C3H/HeN mice are completely susceptible (Barthold et al., 2010). Both strains, however, still display similar spirochete counts in affected tissues (Ma et al., 1998). BALB/cAnN mice display an arthritis phenotype somewhat "in-between", with arthritis severity and bacterial burden both increasing with infectious dose (Ma et al., 1998). Thus far, there is one known type of inflammatory cytokine postulated to contribute to this phenotypic difference across strains: Type I IFN. Upon infection by *B. burgdorferi*, Type I IFN production is upregulated in C3H/ mice but not in
C57BL/6 mice; BALB/c mice produce low levels compared to C3H (Keane-Myers and Nickell, 1995; Miller et al., 2008). Additionally, Type I IFN receptor-blocking antibodies are known to dramatically reduce arthritis in arthritis-susceptible C3H mice (Miller et al., 2008).

Mouse age is another factor that affects severity of pathology. C3H/He mice infected at 12 weeks of age developed less severe arthritis (and possibly carditis) compared to C3H mice infected at 3 weeks of age (Barthold et al., 2010). To observe maximal pathology, mouse studies of Lyme disease typically use younger mice that are approximately 3 weeks old at the time of infection.

Because of the variability in symptom manifestations across strains and ages, laboratory mice, albeit acknowledged as the most suitable models for observing B. burgdorferi pathology in mammalian hosts, are still considered incomplete models of Lyme disease (Radolf et al., 2012).

### 1.6.2. Mouse models of bone diseases

Mouse models are often used to study bone structure and cellular biology. Adult mice are most typically used starting at 12 weeks of age, since it is at this age where murine bone development becomes less rapid and more stabilized (Willingham et al., 2010), thus reducing the potential for developmental growth to mask experimental effects on bone. Peak BMD, trabecular volume, and cortical size measurements can also be attained between ages 12-24 weeks (Buie et al., 2008).

Among inbred strains, C3H mice and C57BL/6 mice are common choices for studies of bone biology. C3H mice are considered high-density mice while C57BL/6 mice are considered low-density mice, meaning that their femoral bone density differs by as much as 50% (Sheng et al., 1999). This is partially attributed to greater periosteal and endosteal bone formation rates during growth of C3H mice (Sheng et al., 1999). Bone microarchitecture also differs between these strains of mice. C3H mice have thicker femoral and vertebral cortices but fewer trabeculae compared to C57BL/6 mice (Turner et al., 2000).
1.7. Methods to Study Murine Bone Biology

Several methods are commonly used to study murine bone structure and cellular biology. Bone mineral density (BMD), a clinically-applicable indicator of osteoporosis and fracture risk, is often measured areally by dual-energy X-ray absorptiometry (DXA) or volumetrically by micro-computed tomography (μCT) (Blake and Fogelman, 2007; Bouxsein et al., 2010). μCT, in particular, is able to generate three-dimensional reconstructions of a bone's chosen volume of interest from slices of X-ray scans (Bouxsein et al., 2010). This in turn allow for separate microarchitectural analyses of trabecular bone and cortical bone (Bouxsein et al., 2010). Some cortical bone architectural parameters commonly measured include thickness, cross-sectional area, perimeter, and closed porosity (Bouxsein et al., 2010). Some trabecular bone architectural parameters commonly measured include: percent bone volume per total tissue volume measured (BV/TV); bone surface per bone volume ratio (BS/BV); trabecular number (Tb.N); trabecular separation (Tb.Sp); and trabecular pattern factor (Tb.Pf), a parameter that measures intertrabecular connectivity by assessing the relationship between convex and concave trabecular surfaces (Hahn et al., 1992). Except for Tb.Pf, all mentioned trabecular bone parameters can also be measured two-dimensionally using static histomorphometry. Using histological sections stained with specific viewing markers (e.g. TRAP for osteoclasts, Goldner's trichrome for osteoblasts), static histomorphometry allows quantitative evaluation of structural parameters and abundance of specific cell types (e.g. osteoblasts) or other components (e.g. osteoid) at a single time point (Dempster et al., 2013). Histomorphometry can also measure dynamic properties—such as mineral apposition rate (MAR) and bone formation rate (BFR)—in the trabecular region, with the aid of fluorescent cell dyes (e.g calcein) (Dempster et al., 2013). Double-labelling the bone with the dye at two separate time points allows for measurement of bone turnover parameters by measuring the distance between the double labels (Dempster et al., 2013). These parameters can be normalized to a number of referents (i.e. osteoid surface, bone surface, bone volume, tissue volume) (Dempster et al., 2013).
1.8. Objective and Hypothesis of M. Sc. Studies

Objective:
Determine whether *B. burgdorferi* infection affects bone health in mice.

Hypothesis:
*B. burgdorferi* infection lowers bone mineral density and induces bone loss by disrupting the balance between bone formation and bone resorption.
The Lyme disease pathogen *Borrelia burgdorferi* infects murine bone and induces trabecular bone loss

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**Short Title: Lyme disease pathogen induces murine bone loss**

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**Summary of author contributions to the study**

Study conception and design: TTT, MG, TJM
Provision of experimental equipment and materials: LZ, MG, TJM
Data acquisition: TTT, LZ, AB
Data analysis and interpretation: TTT, TJM
Drafting and revision of figures: TTT, TJM
Drafting and revision of manuscript: TTT, TJM
2.1. Abstract

**Objective.** Lyme disease is caused by members of the *Borrelia burgdorferi* sensu lato species complex. Arthritis is a well-known late-stage pathology of Lyme disease, but the effects of *B. burgdorferi* infection on bone at sites other than articular surfaces are largely unknown. In this study, we investigated whether *B. burgdorferi* infection affects bone health in a mouse model of Lyme disease.

**Methods.** In mice inoculated with *B. burgdorferi* or vehicle (mock infection), we measured the presence of *B. burgdorferi* DNA in bones, bone mineral density (BMD), bone formation rates, biomechanical properties, cellular composition, and two- and three-dimensional features of bone microarchitecture.

**Results.** *B. burgdorferi* DNA was detected in bone. Infection was associated with reduced areal and trabecular volumetric BMD in femora but not in vertebrae. Trabecular regions of femora exhibited significant microarchitectural disruption, but infection did not affect BMD, microarchitectural, or biomechanical properties of cortical bone. Bone loss in tibiae was not due to increased osteoclast numbers or bone-resorbing surface area, but was associated with reduced osteoblast numbers, implying that bone loss in long bones was due to impaired bone building, and not excessive bone resorption. Osteoid-producing and mineralization activities of existing osteoblasts were unaffected by infection. Therefore, deterioration of trabecular bone was not dependent on inhibition of osteoblast function, but more likely caused by blockade of osteoblastogenesis, reduced osteoblast survival, and/or induction of osteoblast death.

**Conclusion.** Together, these data represent the first evidence that *B. burgdorferi* infection can induce bone loss, and suggest that this phenotype results from inhibition of bone building rather than increased bone resorption.
2.2. Introduction

Lyme disease is the most common vector-borne disease in the northern hemisphere (Mead, 2015). Its causative agents are spirochetes from the *Borrelia burgdorferi* sensu lato species complex (Stanek et al., 2012). These bacteria are not known to secrete conventional toxins, and are therefore postulated to induce pathology primarily by triggering host inflammatory response (Weis and Bockenstedt, 2010). *B. burgdorferi* is transmitted to vertebrates during the blood meal of *Ixodes* ticks, and disseminates from the tick bite site to colonize a variety of host tissues. This can result in diverse clinical manifestations at various infection stages (Shapiro, 2014). In humans, erythema migrans (EM), a bull's-eye-shaped skin lesion, typically appears at the bite site 1-2 weeks post-feeding. If antibiotic treatment does not begin at this early infection stage, *B. burgdorferi* disseminates to more distant tissues, and late-stage manifestations such as Lyme arthritis, carditis, and neuroborreliosis can develop. Of these late-stage symptoms, Lyme arthritis is the most common in North America, affecting as many as 60% of untreated patients (Steere et al., 1987). The pathological effects of *B. burgdorferi* infection on bone tissue outside of articular surfaces, however, have not been widely investigated.

Bone pathologies are observed in diseases associated with other spirochete bacteria, including syphilis and periodontitis (Park et al., 2014; Silva et al., 2015). Bone is central to physiological homeostasis of the whole body and to systemic responses to infection, injury, and a variety of stressors (Redlich and Smolen, 2012). Infections within bone, as well as induction of local and systemic inflammatory responses to infection at other sites, can be accompanied by significant bone loss (Marriott, 2013; Redlich and Smolen, 2012). *B. burgdorferi* has been detected by culture and PCR in bone and marrow of humans, dogs, and the mummy of a Copper Age man (Hovius et al., 1999; Keller et al., 2012; Oksi et al., 1994; Schlesinger et al., 1985). Furthermore, migratory bone pain, bone erosion at articular surfaces, multifocal osteomyelitis, and other bone involvement have been reported in *B. burgdorferi*-infected rodents and humans (Houtman and Tazelaar, 1999; Hovmark et al., 1986; Kvasnicka et al., 2003; Lawson and Rahn, 1992; Lawson and Steere, 1985; Munson et al., 2012; Oksi et al., 1994; Schlesinger et al., 1985, 1985; Schmitz et al., 2004; Steere, 1995;
Steere et al., 1987). In the course of other studies requiring collection of bone marrow, we have noticed that long bones from B. burgdorferi-infected mice occasionally appear to be more brittle than bones of mock-infected controls. This prompted us to examine whether B. burgdorferi induces structural and cellular pathology in murine bones.

2.3. Results

B. burgdorferi infection reduces serum alkaline phosphatase activity, a biomarker of bone formation

Alkaline phosphatase (ALP) catalyzes monoester hydrolysis and is involved in many physiological processes, including bone formation (Millán, 2006; Millán and Whyte, 2016). Unlike in humans, where serum ALP is derived primarily from liver, in mice serum ALP consists mainly of tissue-non-specific bone isotype (bone TNAP), and can therefore serve as a biomarker of bone formation (Ferreira et al., 2015; Linder et al., 2013).

In 16 week-old adult male C3H/HeN mice infected with 10-10^6 B31-derived B. burgdorferi for three weeks, serum ALP activity declined with increasing infectious dose, especially for mice inoculated with ≥10^3 bacteria (Fig. 1A). In mice infected with 10^4 B. burgdorferi, serum ALP activity declined with increasing infection duration, with the most prominent reduction in activity observed at three weeks post-inoculation (Fig. 1B), when pathological outcomes of B. burgdorferi infection are typically most pronounced in C3H mice (Barthold et al., 2010). Juvenile mice, which are typically more commonly used for Lyme disease studies (Barthold et al., 1990), also displayed reduced serum ALP activity after 4 weeks of infection with 10^4 B. burgdorferi (Fig. 1C). The effect of B. burgdorferi infection on serum ALP activity was less prominent than in older mice, likely because bones in juvenile animals are still undergoing rapid bone formation and skeletal development (Willingham et al., 2010). These data prompted us to investigate whether B. burgdorferi colonizes bone and/or affects bone health.
Figure 2-1: Effect of *B. burgdorferi* infection on ALP, a serum marker of bone formation

Mean ± SEM serum alkaline phosphatase (ALP) activity, normalized to mean baseline ALP activity at time of infection (0 weeks: A, B) or in mock-infected mice (C). (A-B) Serum ALP activity in adult (16-17 week old) male C3H/HeN mice infected for 3 weeks with $10^1$-$10^6$ B31-derived GCB726 *B. burgdorferi* (A) or for 1, 3 and 8 weeks with $10^4$ bacteria (B). N=5 mice/group (A), 10 mice/group (B). Statistics: Pearson correlation analysis ($r$). * indicates p<0.05 vs dose (A) and time (B). (C) Serum ALP activity in juvenile (3-4 week old) male C3H/HeN mice infected for 4 weeks with $10^4$ *B. burgdorferi*. Mock: age-matched mice inoculated with vehicle alone. N=10 mice/group. Statistics: two-tailed unpaired $t$ test. * indicates p<0.05 vs mock.
Bone colonization by *B. burgdorferi*

We examined *B. burgdorferi* colonization and infection effects on bone in 12-week-old adult male C3H/HeN mice. At this age murine bone development stabilizes, and peak bone mineral density (BMD), trabecular volume and cortical thickness are attained (Buie et al., 2008; Willingham et al., 2010). Mice were infected for four weeks with 1x10^4 B31-derived *B. burgdorferi*. Bone morphology, cellular composition, mineralization, growth rate, and biomechanical properties were investigated in long bones (tibiae, femora) and vertebrae, since long bones and vertebrae can exhibit different degrees of response to external stimuli. For example, rosiglitazone, a common antidiabetic drug, increased fracture risks in women in the limbs but not in vertebrae (Viberti et al., 2002).

We first investigated whether *B. burgdorferi* could colonize murine bone. DNA from clean, crushed L6 vertebrae and distal halves of tibiae was extracted, and total copies of a portion of the *B. burgdorferi* flaB gene in each sample were measured by quantitative real-time PCR (qPCR). To compare *B. burgdorferi* DNA load in different bones, flaB copy number values were adjusted for total μg of DNA from each bone sample (Fig. 2). flaB DNA copy number was significantly greater compared to background in mock-infected animals in both the tibiae and vertebrae of infected mice, but no significant differences in copy number were detected between bone types (Fig. 2). These data showed that *B. burgdorferi* DNA could be detected in bones outside of articular surfaces.
Figure 2-2: Detection of *B. burgdorferi* DNA in bones

Quantitative real-time PCR measurement of copies of *B. burgdorferi flaB* DNA sequence in distal tibial halves and L6 vertebrae from 12 week-old male C3H/HeN mice four weeks after inoculation with $10^4$ *B. burgdorferi* or vehicle alone (mock). *flaB* number was adjusted for total µg of DNA in each sample. Bars correspond to medians for individual values within each group (shown). N=14-15 per group. Statistics: two-tailed unpaired Mann-Whitney test. * indicates p<0.05 vs mock.
*B. burgdorferi* infection induces osteopenia in the trabecular region of long bones

To determine if *B. burgdorferi* bone colonization induced pathology, we measured areal and volumetric bone mineral density (aBMD and vBMD) in the left femora and L5 vertebrae from mock-infected and infected animals, using dual-energy X-ray absorptiometry (DXA) and micro-computed tomography (μCT). The effects of *B. burgdorferi* infection on trabecular and cortical bone were also compared using three-dimensional microarchitectural reconstructions from μCT. To distinguish between potential effects on trabecular and cortical bone, μCT analysis of cortical bone was performed in femora at the mid-shaft (composed mainly of cortical bone), whereas analysis of trabecular bone in femora was conducted at the distal metaphyses, where trabecular bone is more abundant (36). BMD values were expressed as a percentage of the values for mock-infected animals (Fig. 3A).

To determine if changes in BMD in infected mice were clinically significant, we also calculated T-scores from the BMD values for vertebrae and femora. A T-score represents the number of standard deviations in the individual’s BMD below the mean BMD of healthy young adults. T-scores 1-2.5 standard deviations lower than normal BMD are indicative of osteopenia, and T-scores larger than 2.5 correspond to osteoporosis (Kanis and Glüer, 2000). Means of individual T-scores were compared between *B. burgdorferi*-infected and mock-infected mice (Fig. 3B).

These experiments revealed that BMD of femoral trabecular bone was significantly lower in infected than in mock-infected mice (Fig. 3A). Trabecular BMD in vertebrae of infected animals was reduced but not significantly, and cortical BMD was not affected by *B. burgdorferi* infection (Fig. 3A). Examination of mean T-scores for bones from infected mice indicated that significant osteopenia was present in femora but not vertebrae (Fig. 3B). Representative reconstructed 3D models also showed visibly reduced bone volume and compactness by *B. burgdorferi* infection in the trabecular bone of femora, but the difference appears less prominent in vertebral trabecular bone (Fig. 3C-D). We concluded that *B. burgdorferi* infection was associated with clinically significant osteopenia in long bones but not vertebrae, and that bone loss was confined to trabecular bone.
Figure 2-3: Effect of *B. burgdorferi* infection on bone mineral density

Areal and volumetric bone mineral density (BMD) measured by DXA and µCT, respectively, in distal femoral metaphyses and L5 vertebrae from 12 week-old C3H/HeN male mice infected for four weeks with $10^4$ *B. burgdorferi* or mock-infected with vehicle alone. Values are normalized to mean values for mock-infected mice within each bone type and measurement method group. Primary data are provided in Table S1. (A-B) Mean ±SEM areal and volumetric BMDs (A) and BMD T-scores (B). (C-D) Representative 3D µCT models of trabecular bone from femoral metaphyses (C) and vertebrae (D). Scale bar and ticks: 200 μm. Additional representative 3D µCT models of cortical bone are presented in Figure S1A. N=14-15 mice per group. Statistics: two-way ANOVA with Holm-Sidak post-test. * indicates p<0.05 vs mock within bone and measurement type. # indicates p<0.05 long bones vs vertebrae.
Effects of *B. burgdorferi* infection on trabecular and cortical microarchitecture in long bones and vertebrae

To assess if trabecular bone microarchitecture was deteriorated by infection, we measured the following parameters: BV/TV (the fraction of bone volume (BV) within total measured tissue volume (TV) of interest); BS/BV (the ratio of the bone surface (BS) to total bone volume (BV)); trabecular number (Tb.N, the average number of trabeculae per mm); trabecular separation (Tb.Sp, mean distance between trabeculae); trabecular pattern factor (Tb.Pf, represents intertrabecular connectivity by assessing the relationship between convex and concave trabecular surfaces) (Hahn et al., 1992). BV/TV, BS/BV, Tb.N., Tb.Sp. and Tb.Pf. were measured in three dimensions in left femora and L5 vertebrae using μCT. The same parameters, except Tb.Pf., were also measured in two dimensions in the proximal region of left tibiae and L3 vertebrae by histomorphometry performed in trichrome-stained bone sections. This permitted evaluation of bone structure by multiple methods, as well as comparison of effects of infection in different long bones.

μCT analyses of trabecular bone from distal metaphyses of femora showed that bone comprised a significantly smaller proportion of total measured tissue volume in infected mice compared to mock-infected controls (Fig. 4A: BV/TV). Infection was also associated with significantly increased bone surface per bone volume ratios in the femora (Fig. 4B: BS/BV). Trabecular number (Tb.N) was significantly lower in femora of infected animals (Fig. 4C). Moreover, the trabecular pattern factor (Tb.Pf) parameter was greater in infected femora (Fig. 4D), indicating more poorly-connected spongy lattices (Hahn et al., 1992). A corresponding increase in trabecular separation (Tb.Sp) was observed in infected femora, although this difference was not statistically significant (Fig. 4E). Histomorphometry analyses of trabecular bone from trichrome-stained sections of proximal tibiae also revealed significant reductions in BV/TV in infected mice (Fig. 4A). BS/BV (Fig. 4B) and Tb.Sp (Fig. 4E) values were increased by a similar percentage in femora (μCT) and tibiae (histomorphometry) of infected animals, but the changes observed in tibiae were not significant, possibly due to inherently greater variation in histomorphometry sectioning and measurements. Tb.N did not differ in tibiae and femora of mock-infected and infected mice (Fig. 4C). Collectively, these analyses indicated that trabecular bone in the long bones of *B. burgdorferi* infected mice was
more porous and was composed of smaller, more poorly connected trabeculae, consistent with the observed osteopenia.

In contrast to long bones, neither μCT nor histomorphometry analyses of vertebrae revealed significant infection-dependent changes in BV/TV, BS/BV, Tb.Sp, and Tb.Pf (Fig. 4A-B, D). Representative sections also show that bone volume was reduced more dramatically by infection in tibiae than in vertebrae (Fig. 4F-G). Additionally, Tb.N was similar in μCT-analyzed vertebrae from both experimental groups, but was significantly greater in the vertebrae of infected than mock-infected mice when analysis was performed by histomorphometry (Fig. 4C). The discrepancy in these results suggests that the effect of infection on trabecular number was likely mild, and was possibly less frequently observed in some vertebrae than others. Together these data showed that *B. burgdorferi* infection did not affect vertebral trabecular bone microarchitecture as strongly as it affected long bone microarchitecture, consistent with the results of BMD assays.

To assess the effects of *B. burgdorferi* infection on cortical bone microarchitecture, we measured cortical bone perimeter (B.Pm), thickness, cross-sectional area, and anteroposterior (AP) and mediolateral (ML) diameters in femoral mid-shafts by μCT. None of these properties differed significantly in infected and mock-infected groups (Table S1). Furthermore, three-point bending at the mid-shafts of the same femora revealed that infection did not affect cortical bone biomechanical properties (Table 1). Together with the results of BMD assays, these results implied that *B. burgdorferi* infection did not affect the structure or function of cortical bone.

Collectively, these data indicated that *B. burgdorferi* infection significantly disrupted the trabecular microarchitecture of long bones, without affecting the vertebrae or cortical bone.
Figure 2-4: Effect of *B. burgdorferi* infection on trabecular bone microarchitecture

Microarchitectural parameters of trabecular bone (A-E) measured by µCT (distal femoral metaphyses and L5 vertebrae) and histomorphometric analysis of trichrome-stained sections (proximal tibial metaphyses and L3 vertebrae). Shown are mean ± SEM of values normalized to means for mock-infected mice. Primary data are provided in Table S1-S2. N=14-15 mice per group. Statistics: two-way ANOVA with Holm-Sidak post-test. * indicates p<0.05 vs mock within bone and measurement type. # indicates p<0.05 long bones vs vertebrae. (F-G) Representative sections from trichrome-stained left tibiae (F) and L3 vertebrae (G). Scale bar: 100 μm. Additional representative TRAP-stained sections are presented in Figure S1B.
Table 2-1: Biomechanical properties of femoral mid-shafts assessed by three-point bending

<table>
<thead>
<tr>
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<th>Mock-infected</th>
<th>Infected</th>
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<tr>
<td><strong>Structural properties</strong></td>
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<tr>
<td>Peak load (N)</td>
<td>21.78 ± 0.68</td>
<td>22.15 ± 0.34</td>
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<tr>
<td>Failure displacement (mm)</td>
<td>0.13 ± 0.0036</td>
<td>0.14 ± 0.0031</td>
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<tr>
<td>Work to failure (mJ)</td>
<td>1.48 ± 0.077</td>
<td>1.58 ± 0.053</td>
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<tr>
<td>Stiffness (N/mm)</td>
<td>198.50 ± 4.72</td>
<td>193.10 ± 3.40</td>
</tr>
<tr>
<td><strong>Mechanical properties</strong></td>
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<tr>
<td>Ultimate stress (MPa)</td>
<td>183.40 ± 5.23</td>
<td>191.20 ± 7.38</td>
</tr>
<tr>
<td>Failure strain (%)</td>
<td>1.94 ± 0.064</td>
<td>1.99 ± 0.056</td>
</tr>
<tr>
<td>Toughness (mJ/mm³)</td>
<td>168.70 ± 10.56</td>
<td>166.50 ± 8.07</td>
</tr>
<tr>
<td>Young’s modulus (GPa)</td>
<td>11.16 ± 0.17</td>
<td>11.41 ± 0.23</td>
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Values represent mean ± SEM. No significant differences were found between mock and infected groups for any measured parameter (two-tailed unpaired t tests: p>0.05). N: newtons; mJ: millijoules; MPa: megapascals; GPa: gigapascals.
Bone loss in long bones of B. burgdorferi-infected mice is associated with reduced numbers of osteoblasts, but does not affect osteoblast activity or osteoclast numbers

Healthy bone remodelling in response to stressors results from balance between bone-degrading activities of osteoclasts and the bone-building activities of osteoblasts (Clarke, 2008). Increased osteoclastogenesis and osteoclast activity can result in increased number and size of osteoclasts at the bone surface, which lead to net bone resorption. This is frequently observed in bone loss due to local or systemic inflammation (Redlich and Smolen, 2012). By contrast, osteoblast apoptosis (i.e. decreased osteoblast numbers) and inhibition of osteoblast function (i.e. lower osteoid production, slower mineralization rate) are more prominent in bone loss associated with direct infection of bone (i.e. osteomyelitis) (Marriott, 2013).

To determine whether osteopenia in trabecular regions of long bones in B. burgdorferi-infected mice was associated with changes in bone cell numbers, we measured osteoclast number per bone surface (Oc.N/B.Pm) in tartrate-resistant acid phosphatase (TRAP)-stained sections of right proximal tibial metaphyses and L4 vertebrae, and osteoblast number per bone surface (Ob.N/B.Pm) in trichrome-stained sections of the left proximal tibial metaphyses and L3 vertebrae (Fig. 5A-B). We also measured the ratio of total osteoclast surface to total bone surface (Oc.S/BS) and osteoid surface to bone surface (OS/BS) from the same sections (Fig. 5C-D).

Since B. burgdorferi-induced pathology in most tissues is primarily due to host inflammatory responses to these bacteria (Weis and Bockenstedt, 2010), we expected that bone loss in infected mice would be accompanied by significant osteoclast-driven bone resorption. However, we found that osteoclast numbers (Fig. 5A) and coverage of bone surfaces (Fig. 5C) did not significantly increase in either the tibiae or vertebrae of infected mice. Instead, we observed that bone loss was accompanied by reduced osteoblast numbers at bone surfaces in tibiae (Fig. 5B). Osteoblast numbers in vertebrae were significantly greater than in tibiae (Fig. 5B), suggesting that differences in osteoblast number possibly contributed to differences in the bone loss phenotypes in these bones.
Figure 2-5: Effect of *B. burgdorferi* infection on cellular composition and activity in bone

(A-D) Static histomorphometry analysis of numbers of osteoclasts and osteoblasts per bone surface (A-B), and osteoclast and osteoid surface per bone surface (C-D). Osteoclast parameters were measured in TRAP-stained sections of right proximal tibial metaphyses and L4 vertebrae. Osteoblast and osteoid parameters were measured in trichrome-stained sections of left proximal tibial metaphyses and L3 vertebrae. (E-G) Dynamic histomorphometry analysis of mineral apposition rate/day (E), mineralization surface/osteoid surface (F) and bone formation rate per osteoid surface (G) in left proximal tibial metaphyses, measured by fluorescent calcein green imaging. For all groups, mean ± SEM values for parameters normalized to the mean value for mock-infected mice are shown. Primary data are provided in Table S2. Statistics: two-way ANOVA with Holm-Sidak post-test (A-B); two-tailed unpaired *t* tests (C-E). * indicates *p*<0.05 vs mock within bone and measurement type. # indicates *p*<0.05 long bones vs vertebrae.
Despite reduced osteoblast numbers in the tibiae (Fig. 5B), osteoid production in bones of infected mice was not impaired (Fig. 5D). This implied that infection did not inhibit the activity of the osteoblasts present in bone. To further test this conclusion, rates of mineralized bone formation over time were examined via dynamic histomorphometry in calcein-labelled bone (Fig. 5E-G). Mineralization apposition rates (MAR) in infected and mock-infected animals were similar (Fig 5E), as was the ratio of mineralization surface area to osteoid surface area (MS/OS: Fig 5F). Furthermore, infection did not reduce bone formation rates/osteoid surface (BFR/OS: Fig. 5G), implying that growth rates for mineralized bone were not affected when adjusted for osteoid volume. Thus, we concluded that although osteoblast numbers were reduced, osteoid production and mineralization were not affected by infection. Collectively, these data indicated that B. burgdorferi infection-dependent bone loss was associated with reductions in osteoblast numbers in long bones, but not with increased osteoclastogenesis, increased osteoclast activity, or impaired osteoblast activity.

2.4. Discussion

In this study, we found that B. burgdorferi infection in mice is associated with infectious dose- and time-dependent reductions in ALP, a serum biomarker of bone formation in mice, as well as osteopenia and disrupted trabecular microarchitecture in long bones but not in vertebrae. These effects were not associated with increased osteoclast-dependent bone resorption, inhibition of osteoblast synthesis of osteoid, or bone mineralization. Instead, trabecular bone loss was associated with osteoblast number reduction in long bones but not in vertebrae. It is unclear whether the differences in effect in long bones and vertebrae were directly related to B. burgdorferi infection or due to other variables, since long bones and vertebrae have been observed to be affected differently by systemic factors (e.g. rosiglitazone treatment) for reasons that remain unknown (Viberti et al., 2002). We also found that B. burgdorferi DNA is present in multiple bones from individual mice, suggesting that these bacteria spread to bone by a hematogenous route. However, the presence of similar amounts of B. burgdorferi DNA in both long bones and vertebrae implies that the presence of B. burgdorferi DNA alone is not sufficient to induce bone loss in all bone types. This, in turn, suggests that differences in host responses to infection and/or bone remodelling
dynamics in different bones are likely a major determinant of bone pathology severity associated with *B. burgdorferi* infection.

Although bone pain and bone involvement have been described in patients with Lyme disease (Houtman and Tazelaar, 1999; Hovmark et al., 1986; Kvasnicka et al., 2003; Lawson and Rahn, 1992; Lawson and Steere, 1985; Oksi et al., 1994; Schlesinger et al., 1985; Schmitz et al., 2004; Steere, 1995; Steere et al., 1987), and *B. burgdorferi* has been detected in human bone and bone marrow (18,19,23), the possibility that *B. burgdorferi* may infect and cause pathology in bone at sites other than articular surfaces has not been widely investigated. We found that *B. burgdorferi* infection in mice causes a level of osteopenia in the trabecular regions of long bones that would be considered a clinically significant finding in humans. However, structural and biomechanical properties of cortical bone were not affected by infection. It will be important to determine if conditions such as prolonged *B. burgdorferi* infection or infection in the context of pre-existing osteopenia or osteoporosis would eventually result in a clinically symptomatic phenotype in cortical bone. It may also be useful to determine if *B. burgdorferi* infection in humans is associated with increased rates and severity of osteopenia and osteoporosis, and/or increased risk of fracture outcomes.

Bone loss results from imbalance between the bone-resorbing activities of osteoclasts and bone-building functions of osteoblasts. Our data suggested that bone loss at 4 weeks post-infection was not primarily due to increased osteoclastogenesis or osteoclast activation, although it is possible that osteoclast-driven bone loss occurred earlier in infection. This is plausible because TNF-\(\alpha\), IL-1 and IL-6, which are systemically and rapidly upregulated in response to *B. burgdorferi* infection (Benhnia et al., 2005; Isogai et al., 1996), are three of the major inflammatory cytokines that stimulate osteoclastogenesis and osteoclast activation (Redlich and Smolen, 2012). These cytokines also suppress differentiation of osteoblasts (Redlich and Smolen, 2012), consistent with our observation that osteoblast numbers were reduced in bones which exhibited osteopenia. However, regardless of whether increased osteoclast-dependent resorption was responsible for early bone loss in infection, data from the present study suggest that osteoblast deficiency made important, long-lasting contributions to bone loss.
The major causes of osteoblast-related abnormalities in bone disease are loss or reduction of signals required for osteoblast differentiation (e.g. hormones, local regulatory proteins such as bone morphogenetic proteins), differentiation of bone marrow mesenchymal stem cells into cells of non-osteoblastic lineage, premature osteoblast cell death, and inhibition of osteoblast activities (i.e. osteoid synthesis and mineralization) (Marie, 2015). We did not find that osteoid production or mineralization by the osteoblasts present in bones of infected mice were impaired, implying that osteoblast abnormalities occurred instead due to suppression of osteoblastogenesis or reduced osteoblast viability. It is unknown if *B. burgdorferi* infection affects mesenchymal stem cell differentiation or other major regulators of osteoblastogenesis such as growth hormones, growth factors, estrogen, and parathyroid hormone (Marie, 2015). However, one of *B. burgdorferi*’s complement-regulating surface proteins, CRASP-1, is known to bind bone morphogenetic protein-2 (BMP-2) (Hallstrom et al., 2010), a potent inducer of osteoblast differentiation (Marie, 2015). Treatment with vitamin D, another key regulator of osteoblastogenesis, has also been shown to reduce the severity of Lyme arthritis in mouse models (Cantorna et al., 1998). Additionally, direct or indirect bacterial induction of cell death in osteoblast lineage cells is a known important mediator of bone loss seen in osteomyelitis caused by other bacterial species (Marriott, 2013). *B. burgdorferi* is known to induce cell death in a number of cell types, including glia, neurons, oligodendrocytes, lymphocytes, monocytes and macrophages (Buffen et al., 2013; Cruz et al., 2008; Glickstein and Coburn, 2006; Perticarari et al., 2003; Ramesh et al., 2008; Thai et al., 2011), but the effect of this bacterium on osteoblast biology and cell death have not yet been examined. These literature observations, together with our findings, warrant future investigations on the effect of *B. burgdorferi* infection on osteoblastogenesis and osteoblast viability.

In summary, this paper presents the first animal study directly demonstrating an effect of *B. burgdorferi* infection on bone tissue deterioration at sites other than articular surfaces involved in Lyme arthritis. Since these observations may have implications for our understanding of Lyme disease in humans, further investigation of the mechanisms underlying bone loss in response to *B. burgdorferi* infection is warranted.
2.5 Materials and Methods

*Ethics statements*

All animal procedures were approved by the University of Toronto Animal Care Committee (Protocol #20011501). Mice were housed in a biosafety level 2 room in groups of 2-4 per cage, with *ad libitum* access to food and water. Mice were anesthetized to a surgical plane of anesthesia by intraperitoneal injection of 200 mg/kg ketamine hydrochloride (Rogar/STB, Montréal, QC, Canada) and 10 mg/kg xylazine (MTC Pharmaceuticals, Cambridge, ON, Canada) before tissue harvesting and euthanasia. The authors declare no financial or other conflicts of interest.

*Animal husbandry and dietary conditions*

Upon arrival, four-week-old male C3H/HeNCrI mice (Charles River, Montréal, QC, Canada) were fed standard chow (Teklad 2018 Rodent Chow, Harlan Laboratories, Mississauga, ON, Canada). At 12 weeks of age, mice were switched to maintenance diet containing 0.6% calcium (Teklad 2014 Rodent Chow, Harlan Laboratories) until sacrifice.

*B. burgdorferi cultivation and mouse infections*

The *B. burgdorferi* strain used in this study was B31 5A4-derived GCB726 (Moriarty et al., 2008). As described (Javid et al., 2016), this strain was cultivated to log phase in Barbour-Stoenner-Kelly-II (BSK-II) medium before infection experiments. At 12 weeks of age, randomly assigned mice were inoculated subcutaneously at the dorsal lumbar midline with *B. burgdorferi* or BSK-II medium alone (mock). With the exception of the experiments presented in Fig. 1, mice were infected with $10^4$ *B. burgdorferi* for a period of 4 weeks. Each of these mice was also intraperitoneally injected with freshly-prepared 30 mg/kg 0.6% calcein green (Sigma-Aldrich Canada, Oakville, ON) 9 and 2 days prior to sacrifice, to label newly formed bone.
Harvesting and preparation of sera and bones

Blood was collected by cardiac puncture from anaesthetized mice before euthanasia by cervical dislocation, as described (Javid et al., 2016). Serum was harvested after overnight incubation at 4°C by centrifugation at 10,000 xg for 10 min.

The following bones were collected for all other analyses in this study: femora, tibiae, and L3-L6 vertebrae. The left femora and L5 vertebral columns were cleaned of adherent soft tissue, wrapped in saline-soaked gauze, and stored at -20°C. These bones were thawed overnight at 4°C before DXA, μCT, and biomechanical analyses. Tibiae were excised and clipped at the mid-shaft into proximal and distal halves. Proximal metaphyses of the right tibiae and L4 vertebral columns were fixed immediately in 10% buffered formalin (Sigma), and decalcified in 14% EDTA pH 7.4 at 4°C for 21 days with solutions replaced every two days. These samples were then processed by the Toronto Centre for Phenogenomics (see Bone histomorphometry section). Proximal metaphyses of the left tibiae and L3 vertebral columns were fixed in 70% ethanol and stored at 4°C until submission to the Toronto Centre for Phenogenomics for processing (see details in Bone histomorphometry section). Distal tibial halves and L6 vertebral bodies cleaned of adherent soft tissue were stored at -80°C until DNA extraction for qPCR analysis.

Serum alkaline phosphatase (ALP) activity assays

Serum ALP activity was measured in technical duplicates using a colorimetric ALP assay kit (Abcam, Cambridge, UK) following manufacturer's instructions.

DNA extraction and quantitative PCR (qPCR) measurement B. burgdorferi DNA copy number

Bones were pulverized with a mortar and pestle on dry ice. DNA was extracted from powdered bone using Qiagen DNeasy tissue extraction kits (Toronto, ON, Canada) as per manufacturer’s instructions. Concentration of extracted DNA were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Measurement of numbers of copies of B. burgdorferi flaB DNA by qPCR was performed in technical sextuplicates as described (Javid et al., 2016), using triplicate logarithmic dilutions (10^1-10^6...
copies) of plasmid pTM222 (contained a copy of flaB DNA sequence) as a standard curve. The mean of technical replicates was used for subsequent analyses.

**Bone mineral density (BMD) and morphometry measurements**

Measurement of areal bone mineral density (aBMD: g/cm$^2$) in left femora and L5 vertebral bodies was performed by dual-energy X-ray absorptiometry (DXA), using a PIXIImus mouse bone densitometer (GE Medical Systems, Madison, WI). Measurement of volumetric bone mineral density (vBMD: g/cm$^3$) and morphometry from left femora and L5 vertebral bodies and cortical bone from left femora was performed after DXA analysis, by microcomputed tomography (μCT), using a SkyScan 1174 μCT scanner (Bruker microCT, Kontich, Belgium). Images were acquired at 50 kV and 800 μA, and a 0.25 mm aluminum filter was applied for noise removal. Two hydroxyapatite (HA) phantoms were scanned and used to calculate vBMD. Structural indices were computed using the SkyScan CT Analyzer software. Following image acquisition, three-dimensional reconstruction was performed to calculate bone microarchitecture parameters. Morphometry measurements were performed with global thresholding to exclude soft tissue (trabecular bone: 0.9 g HA/cm$^3$, gray value 65; cortical bone: 1.1 g HA/cm$^3$, gray value 100).

For trabecular bone analyses, distal femoral metaphyses and L5 were scanned at a voxel size of 6.1 μm$^3$. The volume of interest (VOI) for femora consisted of a 400-slice (2.4 mm) section extending 50 slices (0.3 mm) from the first cartilage bridge of femur metaphyses. The VOI for L5 was between the growth plates, with a 50-slice (0.3 mm) offset from each. For cortical bone analyses, femoral mid-diaphyses were scanned at 11.6 μm$^3$. The VOI for cortical bone was a 100-slice (1.16 mm) section at the mid-diaphysis.

**Biomechanical testing**

After μCT analysis, biomechanical properties of the left femora were assessed by three-point bending at the mid-shaft, using an Instron ElectroPuls E1000 machine (Instron Corp., Canton, MA, USA) with a 100 N load cell. Femora were placed anterior face up on two supports 7 mm apart. A pre-test of 10 cycles (maximum 5 N load, minimum 2 N load) was applied to each femur. A destructive load was then vertically applied to the mid-shaft at 2.0 mm/min.
until failure. Load deformation curves derived from biomechanical testing were generated using accompanying Bluehill 3 software (Instron). Parameters were calculated using geometry measurements for cortical bone using μCT.

**Bone histomorphometry**
Tartrate-resistant acid phosphatase (TRAP)-staining for identification of osteoclasts was performed on 5 μm-thick sections cut from formalin-fixed, decalcified right tibiae and L4 vertebrae embedded in paraffin. Ethanol-fixed, undecalcified left tibiae and L3 vertebrae were embedded in methylmethacrylate, sectioned, and stained with Goldner's trichrome (4 μm-thick sections) or left unstained for measurement of calcein fluorescence (7 μm-thick sections). All histology services were performed by the Toronto Phenogenomics Centre.

Static and dynamic histomorphometry analyses of TRAP-stained, trichrome-stained, and calcein green-labelled sections were performed manually using BioQuantOsteo v11.2.6. All histomorphometric measurements were performed and analyzed following guidelines from the American Society of Bone and Mineral Research (Dempster et al., 2013).

**Statistical analyses**
Statistical analyses were performed in GraphPad Prism v.6.0 (GraphPad Software, La Jolla, CA, USA). Specific tests used for analyses are indicated in figure and table legends. Data were considered statistically significant at a confidence level of 95% (p<0.05).
2.6. Acknowledgements


Conflicts of Interest

The authors declare no competing financial or other conflicts of interest.

Author Contributions

Conceptualization (TTT, MG, TJM), methodology (TTT, LZ, AB), formal analysis (TTT, AB, TJM), investigation (TTT, LZ, AB), resources (MG, TJM), writing: original draft (TTT), review and editing (TTT, LZ, MG, TJM), visualization (TTT, TJM), supervision (MG, TJM), project administration (TJM), funding acquisition (MG, TJM).
2.7. Supplemental Figures and Tables

Figure 2-S1: Representative images: 3D models of cortical bone and TRAP-stained trabecular bone sections

(A) 3D models of cortical bone at femoral mid-diaphyses, obtained by μCT. Scale bar and ticks: 200 μm. (B) TRAP-stained right tibiae and L4 vertebrae. Magnification: 10x. Scale bar: 100 μm.
Table 2-S1: Femoral and vertebral microarchitectural properties measured by DXA and µCT

<table>
<thead>
<tr>
<th></th>
<th>Mock-infected</th>
<th>Infected</th>
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</thead>
<tbody>
<tr>
<td><strong>Trabecular bone</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Left femora</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Areal BMD (g/cm²)</td>
<td>0.06884 ± 0.0006818</td>
<td>0.06489 ± 0.00709350 *</td>
</tr>
<tr>
<td>Volumetric BMD (g/cm³)</td>
<td>0.3333 ± 0.005851</td>
<td>0.3086 ± 0.007279 *</td>
</tr>
<tr>
<td>Bone volume/tissue volume (%)</td>
<td>13.05 ± 0.5153</td>
<td>10.99 ± 0.4666 *</td>
</tr>
<tr>
<td>Bone surface/bone volume (mm⁻¹)</td>
<td>62.93 ± 0.06614</td>
<td>65.75 ± 0.9486 *</td>
</tr>
<tr>
<td>Trabecular number (mm⁻¹)</td>
<td>2.021 ± 0.08938</td>
<td>1.737 ± 0.07168 *</td>
</tr>
<tr>
<td>Trabecular pattern factor (mm⁻¹)</td>
<td>21.35 ± 0.5229</td>
<td>23.44 ± 0.6548 *</td>
</tr>
<tr>
<td>Trabecular separation (mm)</td>
<td>0.2246 ± 0.005959</td>
<td>0.2401 ± 0.005144 (p=0.06)</td>
</tr>
<tr>
<td><strong>L5 vertebrae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Areal BMD (g/cm²)</td>
<td>0.03046 ± 0.0009634</td>
<td>0.02760 ± 0.001129 (p=0.07)</td>
</tr>
<tr>
<td>Volumetric BMD (g/cm³)</td>
<td>0.2864 ± 0.007408</td>
<td>0.2734 ± 0.01068</td>
</tr>
<tr>
<td>Bone volume/tissue volume (%)</td>
<td>14.87 ± 0.4204</td>
<td>15.04 ± 0.7367</td>
</tr>
<tr>
<td>Bone surface/bone volume (mm⁻¹)</td>
<td>2.498 ± 0.06333</td>
<td>2.514 ± 0.1042</td>
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<tr>
<td>Trabecular number (mm⁻¹)</td>
<td>15.83 ± 0.6179</td>
<td>15.62 ± 1.034</td>
</tr>
<tr>
<td>Trabecular pattern factor (mm⁻¹)</td>
<td>64.37 ± 0.8855</td>
<td>63.87 ± 1.437</td>
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<td>Trabecular separation (mm)</td>
<td>0.2898 ± 0.008307</td>
<td>0.2804 ± 0.008937</td>
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<td><strong>Cortical bone: Left femora</strong></td>
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<tr>
<td>Volumetric BMD (g/cm³)</td>
<td>1.340 ± 0.009270</td>
<td>1.341 ± 0.01983</td>
</tr>
<tr>
<td>Periosteal perimeter (mm)</td>
<td>8.208 ± 0.1239</td>
<td>8.109 ± 0.1006</td>
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<tr>
<td>Cortical thickness (mm)</td>
<td>0.3305 ± 0.003702</td>
<td>0.3329 ± 0.005164</td>
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<tr>
<td>Cross-sectional bone area (mm²)</td>
<td>1.089 ± 0.01415</td>
<td>1.084 ± 0.01951</td>
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<tr>
<td>Anteroposterior (AP) diameter (mm)</td>
<td>1.184 ± 0.01222</td>
<td>1.195 ± 0.01219</td>
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<tr>
<td>Mediolateral (ML) diameter (mm)</td>
<td>1.746 ± 0.01864</td>
<td>1.718 ± 0.01597</td>
</tr>
<tr>
<td>Closed porosity (%)</td>
<td>0.5965 ± 0.1996</td>
<td>0.4428 ± 0.09315</td>
</tr>
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</table>

Mean ± SEM values for all parameters. All parameters except areal BMD were measured by µCT. Statistics: two-tailed unpaired t test. *: p<0.05 vs mock.
Table 2-S2: Histomorphometry measurements: tibiae and vertebrae

<table>
<thead>
<tr>
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<th>Mock-infected</th>
<th>Infected</th>
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<tr>
<td><strong>Static histomorphometry: TRAP-stained slides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Right tibiae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone volume/tissue volume (%)</td>
<td>26.47 ± 1.510</td>
<td>29.55 ± 1.507</td>
</tr>
<tr>
<td>Bone surface/bone volume (mm(^{-1}))</td>
<td>29.39 ± 1.938</td>
<td>26.50 ± 1.108</td>
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<tr>
<td>Osteoclast number/bone surface (mm(^{-1}))</td>
<td>5.192 ± 0.5730</td>
<td>4.631 ± 0.3032</td>
</tr>
<tr>
<td>Osteoclast surface/bone surface (%)</td>
<td>8.002 ± 0.5789</td>
<td>9.112 ± 0.7641</td>
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<tr>
<td><strong>L4 vertebrae</strong></td>
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<td></td>
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<tr>
<td>Bone volume/tissue volume (%)</td>
<td>27.93 ± 1.771</td>
<td>27.73 ± 2.362</td>
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<tr>
<td>Bone surface/bone volume (mm(^{-1}))</td>
<td>35.77 ± 1.644</td>
<td>37.85 ± 3.051</td>
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<tr>
<td>Osteoclast number/bone surface (mm(^{-1}))</td>
<td>4.266 ± 0.3157</td>
<td>4.169 ± 0.2208</td>
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<tr>
<td>Osteoclast surface/bone surface (%)</td>
<td>7.142 ± 0.7310</td>
<td>7.922 ± 0.5416</td>
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<td><strong>Static histomorphometry: Trichrome-stained slides</strong></td>
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<td><strong>Left tibiae</strong></td>
<td></td>
<td></td>
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<tr>
<td>Bone volume/tissue volume (%)</td>
<td>20.56 ± 2.708</td>
<td>14.91 ± 1.239 *</td>
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<tr>
<td>Bone surface/bone volume (mm(^{-1}))</td>
<td>39.67 ± 2.656</td>
<td>45.12 ± 1.761 (p=0.09)</td>
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<td>Trabecular number (mm(^{-1}))</td>
<td>4.727 ± 0.08585</td>
<td>4.793 ± 0.1481</td>
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<tr>
<td>Trabecular separation (mm)</td>
<td>0.1065 ± 0.005811</td>
<td>0.1206 ± 0.006056</td>
</tr>
<tr>
<td>Osteoblast number/bone surface (mm(^{-1}))</td>
<td>0.9075 ± 0.1202</td>
<td>0.7235 ± 0.1691 *</td>
</tr>
<tr>
<td>Osteoid volume/bone volume (%)</td>
<td>1.894 ± 0.2970</td>
<td>2.026 ± 0.4509</td>
</tr>
<tr>
<td><strong>L3 vertebrae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone volume/tissue volume (%)</td>
<td>23.95 ± 2.017</td>
<td>22.70 ± 1.870</td>
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<tr>
<td>Bone surface/bone volume (mm(^{-1}))</td>
<td>40.52 ± 1.964</td>
<td>44.56 ± 2.311</td>
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<td>Trabecular number (mm(^{-1}))</td>
<td>5.412 ± 0.08608</td>
<td>5.768 ± 0.1213 *</td>
</tr>
<tr>
<td>Trabecular separation (mm)</td>
<td>0.08375 ± 0.003938</td>
<td>0.08183 ± 0.004512</td>
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<tr>
<td>Osteoblast number/bone surface (mm(^{-1}))</td>
<td>0.6267 ± 0.09381</td>
<td>0.8079 ± 0.1125</td>
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<tr>
<td>Osteoid volume/bone volume (%)</td>
<td>1.348 ± 0.2377</td>
<td>1.766 ± 0.2443</td>
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<tr>
<td><strong>Dynamic histomorphometry: Calcein imaging</strong></td>
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<tr>
<td><strong>Left tibiae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineral apposition rate (mcm/day)</td>
<td>1.329 ± 0.1051</td>
<td>1.405 ± 0.07492</td>
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<tr>
<td>Mineralization surface/osteoid surface (%)</td>
<td>202.7 ± 45.16</td>
<td>351.4 ± 77.75 (p=0.13)</td>
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<tr>
<td>Bone formation rate/osteoid surface (mcm/day)</td>
<td>2.858 ± 0.6685</td>
<td>5.026 ± 1.154 (p=0.14)</td>
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<tr>
<td>Mineralization surface/bone surface (%)</td>
<td>16.32 ± 0.8185</td>
<td>19.54 ± 1.229 (p=0.051)</td>
</tr>
<tr>
<td>Bone formation rate/bone surface (mcm/day)</td>
<td>0.1365 ± 0.01186</td>
<td>0.1723 ± 0.01428 (p=0.076)</td>
</tr>
</tbody>
</table>

Mean ± SEM values for all parameters. Statistics: two-tailed unpaired t test. *: p<0.05 vs mock.
Chapter 3
Thesis Discussion

3.1. Thesis Discussion

Lyme disease is the most prevalent vector-borne disease in the northern hemisphere (Radolf et al., 2012). Due to a rapid spread in tick habitat as a result of climate change (Leighton et al., 2012), more areas are becoming exposed to ticks that carry the etiologic agent, *Borrelia burgdorferi*. In the last few years, Lyme disease has become more of a public concern and a media sensation. Though the disease is growing in prominence, much still remains to be understood about *B. burgdorferi* pathogenesis mechanisms. Additionally, there is still room for further understanding of the pathological symptoms on mammalian hosts, which can range quite widely due to the multi-system involvement of *B. burgdorferi* infection. My M.Sc. research project focused on bone tissue involvement, and overall concluded that *B. burgdorferi* infection can impair bone health in mice.

Before discussing details of the present study, however, it is important to highlight its significance in the field and the rationale for conducting this study. Although Lyme arthritis is a well-known and well-studied late-stage manifestation of Lyme disease (Steere et al., 1987), the effects of Lyme disease and *B. burgdorferi* on bone tissue outside of joints have not been extensively reported. There have been very few documented human Lyme disease cases featuring bone defects as manifestations (Houtman and Tazelaar, 1999; Hovmark et al., 1986; Kvasnicka et al., 2003; Lawson and Rahn, 1992; Lawson and Steere, 1985; Munson et al., 2012; Oksi et al., 1994; Schlesinger et al., 1985, 1985; Schmitz et al., 2004; Steere, 1995; Steere et al., 1987). Of these, few have offered direct evidence of *B. burgdorferi* presence in the bone. No mouse study has ever been performed investigating whether *B. burgdorferi* infects bone and affects the health of bone outside of articular surfaces eroded by Lyme arthritis. This study was an attempt to instigate development in this potentially novel area of research.

Our interest in this underexplored area of the field was sparked by anecdotal observations from previous experiments. First, while collecting bone marrow from the long bones of
C57BL/6 mice—a strain which does not develop severe Lyme arthritis (Ma et al., 1998)—we noticed that the bones were more brittle and snapped more easily. Second, we measured serum alkaline phosphatase (ALP) activity in arthritis-susceptible C3H/HeN mice, initially as an assessment for liver function as clinically performed in humans. Instead of an expected elevation, which is a clinical sign for compromised hepatocyte integrity (Yam, 1974), we found a significant dose- and time-dependent decrease in serum ALP activity of infected mice (Fig. 2-1A-B). We then became aware that serum ALP in mice in fact is more representative of the bone-specific tissue-nonspecific isotype (bone TNAP) (Linder et al., 2013). Connecting these ALP-related observations with earlier anecdotal observations, we hypothesized that B. burgdorferi infection may be compromising bone health across different strains of mice independent of their susceptibility to Lyme arthritis, and decided to perform a number of detailed structural, cellular, and molecular analyses to test our hypotheses.

Microarchitecture analyses of adult C3H/HeN mouse bones by DXA and μCT found that trabecular bone mineral density, volume, surface, number, and compactness were all significantly affected by a 4-week-long B. burgdorferi infection in the femora but less so in the lumbar vertebrae (Fig. 2-3; Fig. 2-4). In contrast, cortical bone of the femora of B. burgdorferi-infected mice showed no significant changes in any of the measured parameters, including BMD, perimeter, thickness, diameters, and porosity (Table 2-1; Fig. 2-S1A). Three-point bending analysis also revealed no differences in bone biomechanics at the mid-diaphyses (composed mainly of cortical bone) of the same femora (Table 2-2). These findings can be generalized into two major conclusions. The first is that a 4-week B. burgdorferi infection appears to impair trabecular bone more than cortical bone. The second is that a 4-week B. burgdorferi infection appears to affect long bones more severely than lumbar vertebral bones. These conclusions will now be discussed separately.

The confinement of infection-related deterioration to solely the trabecular bone appears to be consistent with symptoms of early osteomyelitis. Specifically, the first stage of clinical osteomyelitis—as classified by the Cierny-Mader system—is characterized by deterioration of the medullary (trabecular) bone only (Calhoun et al., 2009; Cierny et al., 2003). Since, during the course of this study, we were also able to obtain direct evidence of B. burgdorferi
presence in the long bones by qPCR (Fig. 2-2), it is highly likely that the trabecular bone defects we observed result from osteomyelitis due to direct bone infection by the pathogen. This marks the first study linking Lyme disease to osteomyelitis in the mouse model, and is quite significant for the Lyme disease field—where so few cases of Lyme disease-induced osteomyelitis have been documented that even clinical literature studying the phenotype were vocal about potential uncertainty regarding the connection (Oksi et al., 1994).

The apparent restriction of infection-related pathology to the femora but not the vertebrae also offers interesting perspectives to the connection between Lyme disease and osteomyelitis. One way by which osteomyelitis can be classified is the primary anatomical location at which it occurs (Kremers et al., 2015). Long bone osteomyelitis and vertebral osteomyelitis are both defined phenotypes that each have dedicated literature. Interestingly, according to an epidemiological review of clinical cases of osteomyelitis in the United States between 1969 and 2009, infection in most cases (94%) involved only one bone type (e.g. long bones only, or axial locations only) (Kremers et al., 2015). There have also been other recorded incidences of systemic influences impacting one bone type more than another. A recent example involves the drug rosiglitazone (RSG), which is an insulin-sensitizine treatment for type 2 diabetes mellitus (hereafter referred to as diabetes) (Sardone et al., 2011). The A Diabetes Outcome Progression Trial (ADOPT) study, a randomized, double-blind, parallel-group study in a sample of 3,600 diabetes patients, showed that women taken RSG experienced more limb fractures but not vertebral fractures (Viberti et al., 2002). The mechanism behind the apparently different susceptibilities of long bones and vertebrae to fracture remains unidentified. It is possible that our observation of deterioration in the murine femora alone is reflective of unknown, potentially intrinsic differences in damage susceptibility between bone types, or due to other factors (discussed later). Further investigations are necessary to confirm this hypothesis.

There is, however, a major limitation of our study in relation to the above findings that must be addressed. This is the duration of infection. In this study, all mice were only infected (or mock-infected) for a period of 4 weeks. It is absolutely necessary that the architectural analyses we conducted be repeated in the future with different infection durations (longer
and shorter). Not only will this help us visualize whether *B. burgdorferi*-induced bone pathology becomes more severe and eventually grow to affect cortical bone, it will also enable us to see whether defects in the vertebrae would emerge over time. The latter is particularly important to address, given some key structural differences between the long bones and the vertebrae. The vertebrae are considered "irregular bones", one of four main categories of bone (which also includes long bones) (Clarke, 2008). Their trabecular bone to cortical bone ratio is much higher than that of long bones (Clarke, 2008), and they are known to have higher turnover rates in rats (Li and Klein, 1990). Given that *B. burgdorferi* infection in the present study only appeared to induce trabecular bone loss, it is possible that any trabecular bone loss in the vertebrae may have been masked by a high turnover rate. Lengthening the duration of infection would allow us to assess this hypothesis, and may even help us distinguish whether the observed lack of pathology in the vertebrae is related to structural differences from long bones or other mechanisms.

Apart from the large-scale analyses of bone structure in *B. burgdorferi*-infected mice, we also investigated microscopic differences on the cellular level. We found that osteoblast number (Ob.N) was significantly reduced by *B. burgdorferi* infection (**Fig. 2-5A**); serum ALP, a bone formation marker, also appeared to be less active in infected mice (**Fig. 2-1C**). There were no significant differences, however, in osteoclast biology parameters measured in the tibiae of infected mice (**Fig. 2-5A-B**). Again, it is yet unclear whether lengthening the duration of infection will impact the severity of the effects on the biology of both cell types, and extending infection duration is a definite future step. Nevertheless, our observations raise some interesting parallels between *B. burgdorferi* and *Staphylococcus aureus*, the causal pathogen most frequently isolated in clinical cases of osteomyelitis (Hatzenbuehler and Pulling, 2011; Olson and Horswill, 2013). *S. aureus* has been shown to have a distinctive ability to adhere to, invade, and drive apoptosis of osteoblasts both *in vitro* and *in vivo* (Jevon et al., 1999; Shi and Zhang, 2012; Tucker et al., 2000; Wright and Nair, 2010), most likely using a fibronectin-related adhesion pathway (Josse et al., 2015; Khalil et al., 2007). Incidentally, a recent study (and the most detailed study on *B. burgdorferi* invasion of cells) was able to demonstrate *B. burgdorferi* invasion of fibroblasts and endothelial cells via a $\beta_1$-dependent mechanism (Wu et al., 2011). This internalization, however, did not appear to be
entirely dependent on the $\alpha_5\beta_1$ integrin (which mediates *S. aureus* internalization) or BBK32, a well-known borrelial FnBP. Nevertheless, performing similar assays in osteoblasts and comparing invasion to that observed in other cells may offer new insight into both *B. burgdorferi*-bone interaction and any potential relationship between *B. burgdorferi* invasion and FnBPs.

Another interesting parallel between *S. aureus* and *B. burgdorferi*, though not directly addressed by this study, is their ability to form biofilm. Formation of biofilm by *S. aureus* in the bone has long been established as the main reason they are extremely difficult to eradicate in clinical cases of chronic osteomyelitis (Anwar et al., 1990; Ellington et al., 2006; Gristina et al., 1985); formation of biofilm by *B. burgdorferi* has only been recently characterized *in vitro* (Sapi et al., 2012), and has not been extensively studied since then. These preliminary parallels between *B. burgdorferi* and *S. aureus* warrant further investigation by osteoblast internalization assays. If similar invasion mechanisms are elucidated in *B. burgdorferi* in the future, the finding will be significant for several reasons. First, it will broaden our mechanistic understanding of *B. burgdorferi*-host cell interaction. Second, new methods of *in vitro* studying of *B. burgdorferi* adhesion and invasion using osteoblasts can potentially be developed. Third, should *B. burgdorferi* be found to form biofilm in the bone, we may even cultivate new perspectives on the poorly-understood, controversial concept of post-Lyme disease syndrome. Patients suffering post-Lyme disease syndrome experience nonspecific symptoms despite displaying little evidence of *Borrelia* infection from bodily fluids (Fallon et al., 2008; Marques, 2008), and these symptoms are only described as post-Lyme disease syndrome when there are no conditions other than previous contraction of Lyme disease to explain their presence (Marques, 2008). Interestingly, musculoskeletal pain is known as one of the most common complaints (Marques, 2008). This further adds to the possibility that *B. burgdorferi* infection may be using the skeleton as a site for chronic persistence, though this hypothesis is, of course, merely a speculation that needs more detailed experimentation to be justified.

In summary, the present study is the first descriptive, controlled animal study ever performed to investigate whether *B. burgdorferi* can cause murine bone deterioration outside of joints.
We observed that *B. burgdorferi* appears to induce early stage osteomyelitis in adult mice after an infection period as short as 4 weeks. Many future experiments still need to be conducted in order to better understand and explore the mechanistic relationship between *B. burgdorferi* infection, bone health, and osteomyelitis over varying time periods and in different mouse models. Nevertheless, this study is a good beginning for a bridging between Lyme disease and bone biology. By establishing the connection, it was able to broaden our understanding of Lyme disease in rodents, open a door to a new area of mechanistic investigation, and offer some translational implications for better understanding Lyme disease in humans.
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mineral apposition rates are greater in C3H/HeJ (high-density) than C57BL/6J (low-density) mice during growth. Bone 25, 421–429.


