The effects of age and IGF-1 availability on the recovery of bone mineral density after lactation

by

Monika Diana Rogowska

M.Sc., Johannes Gutenberg University, Mainz, Germany, 2015

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Spring 2021

Declaration of Committee

Name:	**Monika Diana Rogowska**
Degree:	**Master of Science**
Title:	**The effects of age and IGF-1 availability on the recovery of bone mineral density after lactation**
Committee:	**Chair: Nicholas Dulvy** Professor, Biological Sciences
	Julian Christians Supervisor Professor, Biological Sciences
	Sherryl Bisgrove Committee Member Associate Professor, Biological Sciences
	Robert N. Young Committee Member Professor, Chemistry
	Lisa Julian Examiner Assistant Professor, Biological Sciences

Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

a. human research ethics approval from the Simon Fraser University Office of Research Ethics

or

b. advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University

or has conducted the research

c. as a co-investigator, collaborator, or research assistant in a research project approved in advance.

A copy of the approval letter has been filed with the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Simon Fraser University Library
Burnaby, British Columbia, Canada

Update Spring 2016

Abstract

Lactation causes depletion of the maternal skeleton due to calcium resorption. Once lactation ceases, repair is achieved through bone remodeling, which involves communication between bone cell populations including bone-resorbing osteoclasts and bone-forming osteoblasts. Communication is mediated by factors such as insulin-like growth factor 1 (IGF-1), whose bioavailability is controlled by pregnancy-associated plasma protein A2 (PAPP-A2), which frees IGF-1 by cleaving of insulin-like growth factor binding protein 5 (IGFBP-5). The objectives of this study are to determine whether PAPP-A2 is involved in the recovery of bone after lactation and to test whether recovery is affected by age, using mouse models. PAPP-A2 knockout and control mice were bred at the ages of two, five and seven months and culled at wean or three weeks after to determine what bone traits were affected by breeding. Femurs were measured by microCT and blood was collected for measurement of IGF-1 and IGFBP-5. There was a significant decrease of trabecular BV/TV and trabecular thickness in bred mice compared to nulliparous controls. Trabecular spacing was not affected by breeding. Cortical bone area fraction and thickness was lower in lactating mice. Three weeks after the end of lactation, there was a partial recovery of cortical bone area fraction and cortical thickness, while trabecular bone did not recover. Deletion of *Pappa2* did not affect bone recovery. Age affected the recovery of cortical thickness, which was impaired in mice at the age of five months compared with two or seven months. These results highlight the importance of age at reproduction for evaluation of bone recovery after lactation.

Keywords: bone mineral density, pregnancy-associated plasma protein a2, PAPP-A2, IGF-1, IGFBP-5, bone recovery, bone remodeling, lactation, insulin-like-growth factor 1, insulin-like growth factor binding protein 5

Acknowledgements

I want to thank Prof. Julian Christians for giving me the opportunity to work on this project and his patience with me. I also want to thank the other people who helped out with this thesis, like Alishba Bilal and Uriel Pena. Many thanks also go to Sun Guobin and Nancy Ford from the Centre of High-Throughput Genomics at the University of British Columbia for providing the necessary equipment, as well as the Animal Care Staff at the Simon Fraser University for taking care of our mice.

Table of Contents

List of Tables

List of Figures

List of Acronyms

SFU	Simon Fraser University
AMCs	Age-matched controls
BA/TA	Bone area/Total area
BV/TV	Bone volume/Total volume
BMD	Bone mineral density
BMU	Basic multicellular units
Ct.Ar/Tt.Ar	Cortical area/Total area
Ct.Po	Cortical porosity
Ct.Th	Cortical thickness
ELISA	Enzyme-linked immunosorbent assay
f	Flox
IGF-1	Insulin-like growth factor 1
IGFBP-5	Insulin-like growth factor binding protein 5
MAPK	Mitogen-activated protein kinase
k	Knockout
MSC	Mesenchymal stem cells
OPG	Osteoprotegerin
PAPP-A	Pregnancy-associated plasma protein A
PAPP-A2	Pregnancy-associated plasma protein A2
RANK	Receptor activator of nuclear kappa-B
RANKL	Receptor activator of nuclear kappa-B ligand
Tb.Sp	Trabecular spacing
Tb.Th	Trabecular thickness
TRAF6	TNF receptor-associated factor 6

Chapter 1.

Introduction

1.1. Lactation as a major reproductive investment

Lactation is the secretion of milk from the mammary glands of mammals and is used to sustain neonates after birth. Lactation represents a major reproductive investment by females, as it requires the usage of finite resources (Jasienska, 2009; Ziomkiewicz, Sancilio, Galbarczyk, & Klimek, 2016). Through breastfeeding, the developing neonate is provided with all the necessary nutrients and minerals that it needs to grow and thrive. One such mineral is calcium, which is needed to build the skeleton (Kovacs, 2016). Human neonates up to an age of six months require a calcium intake of 200 mg per day, out of which 120-140 mg are used to build up their skeleton (Kovacs, 2016). This heightened demand for minerals necessitates major changes in the mother's metabolism to accommodate both the needs of her own body as well as those of the neonate. One such adaptation is an increased intestinal absorption of minerals (Boass, Toverud, Pike, & Haussler, 1981). This however isn't enough to supply the demands of the offspring, which leads to a second adaption: the utilization of minerals stored in the skeleton, with the major ones being calcium, magnesium and phosphorus (Confavreux, 2011). Bone loss even occurs in the presence of calcium supplementation. Because the mother's skeleton becomes the main source of calcium for the production of milk, it is depleted during lactation (Kovacs, 2016, 2017). The vast majority of calcium is stored in the bone matrix (Blair et al., 2011) and lactation thus may lead to a weakening of the bone microarchitecture (Salari et. al, 2014).

Throughout life, the skeleton is remodeled through a balance of bone resorption and bone formation. During lactation, bone resorption is increased, leading to a loss in bone mineral density (BMD). Though bone formation is also increased

during lactation, the rate of bone resorption surpasses it, resulting in a net decline of bone mass. After six months of lactation, nursing women lose up to 7% of their bone mass. Similar findings in rodents indicate a loss of up to 30% over a period of three weeks due to a higher number of offspring. BMD decreases by a rate of 1-3% per month, even if calcium supplements are taken (Kovacs, 2017). For comparison, BMD density for menopausal women declines by 1.2% per year for the first 5 years of menopause (Prior, 1998).

In contrast, after offspring are weaned and milk production ceases, BMD recovers rapidly due to a reduction in bone resorption and an elevated rate of bone formation (Liu, Ardeshirpour, VanHouten, Shane, & Wysolmerski, 2012). As reviewed by Kovacs (2016), over 50 studies have shown that bone recovery after weaning is complete, with no permanent reduction in BMD or increased risk of osteoporotic fracture later in life. The loss of 7% of bone mass is repaired during the first 12 months after weaning (Kovacs, 2016; Salari et al., 2014). In cases where osteoporosis is diagnosed during lactation, pharmacological treatments used to increase BMD (e.g., strontium ranelate, bisphosphonates and nasal calcitonin) have little effect on recovery compared to the effects of weaning alone (Kovacs, 2016). Exercise in such women also has no lasting effect on bone mass increase (Kouvelioti et al., 2018). These findings suggest that recovery after lactation is not matched by either drugs or exercise. The knowledge of the mechanisms underlying BMD recovery following lactation could prove useful for the design of more effective treatments to combat bone diseases, such as osteoporosis.

1.2. Bone structure and function

Bones have several functions: they support the body structurally, as muscles attach to our bones via tendons, and they also protect vital organs. While bones are thought of as static structures, this couldn't be further from the truth, as they serve as the site where immune cells and blood cells (i.e., red and white blood cells as well as platelets) are produced in a process called hematopoeisis

(Grabowski, 2009). Bones themselves have different structures - in long bones we find three different regions - the epiphysis at the ends of the bone, with a region called the metaphysis that transitions into the diaphysis, the middle of the bone. Depending on the bone region, we also find different types of bone tissue (Fig.1). Hard bone, or cortical bone, is most prevalent in the diaphysis, and is found as a ring-like structure of hard bone surrounding the inner bone marrow (Fig.1).

The outer layer of hard bone is also known as the periosteum. In cortical bone, about 30% of the bone volume is occupied by vascular channels, like Haversian canals that allows for the presence of nerves and blood channels (Ott, 2018). Trabecular, or spongy bone, is most prevalent in the epiphysis, and is a porous structure with bone tissue connected to each other by thin rods called trabeculae (Fig. 1). Trabecular bone is able to absorb shock much better than cortical bone and is the main load bearing structure in vertebrae (Oftadeh, Perez-Viloria, Villa-Camacho, Vaziri, & Nazarian, 2015). Therefore, if trabecular bone were to lose its structure due to osteoporosis, this could make it a site that becomes more vulnerable to fractures due to a reduction in shock-absorbing trabeculae. Other differences between cortical and trabecular bone include the difference in calcium content, water content and surface area. For example, trabecular bone has more surface area and a higher turnover rate, but a lower calcium content than cortical bone (Ott, 2018). Trabecular bone also has a higher water content than cortical bones (with trabecular bones having as much as 27% of water as cortical bones with 23%) (Oftadeh et al., 2015). In addition to the aforementioned functions, bones also serve as a storage medium for minerals, such as calcium, phosphate and magnesium, which are used for the continued maintenance of our bodies (Florencio-Silva, Sasso, Sasso-Cerri, Simões, & Cerri, 2015a; Sarko, 2005).

1.3. Bone remodeling: Communication between osteoblasts and osteoclasts

There are four different types of bone cells: osteoblasts, osteoclasts, osteocytes and bone lining cells (Downey & Siegel, 2006), all of which are necessary to maintain the balance of bone metabolism, as bone is constantly being worn down and built up again in order to free necessary minerals, or adjust and repair damage. The extracellular matrix in bone becomes mineralized due to the deposition of calcium hydroxyapatite (Caetano-Lopes, Canhão, & Fonseca, 2007).

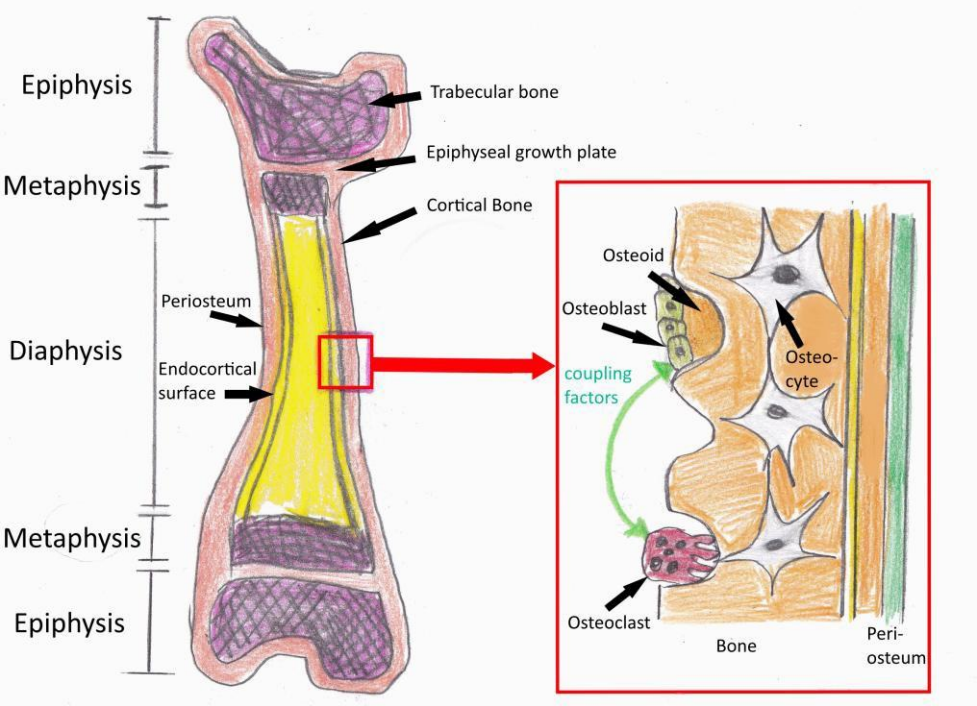

Figure 1:Bone structure, *showing the different bone regions of a typical long bone and the different bone cell populations (picture modified after (Sims & Vrahnas, 2014))*

Bone modeling is the deposition of bone tissue upon a surface that had no prior bone resorption and occurs primarily during growth, while bone remodeling encompasses the two processes of resorption and replacement of bone tissue in the same location without any additional growth occurring (Seeman, 2013). Bone

remodeling is a life-long process that ensures the repair of damage by removing old bone tissue through bone resorption, coupled with the process of bone formation, where new bone tissue is deposited. Osteoblasts are bone cells responsible for the formation of new bone tissue; they are specialized cells that have a large Golgi apparatus and abundance of rough endoplasmatic reticulum when active. Rough endoplasmatic reticulum with its ribosomes ensures the production of proteins necessary for the building of new bone tissue, while the Golgi apparatus sends these products for release outside of the osteoblast cell. Thanks to the Golgi apparatus, osteoblasts are specialized in vesicular transport, with mature osteoblasts being able to secrete bone matrix (Caetano-Lopes et al., 2007). Originally, osteoblasts come from mesenchymal precursor cells which mature in response to two transcription factors called Osterix-1 (OSX1) and the runt-related transcription factor 2 (RUNX2) (Long, 2011). Osteoblasts secreting bone matrix eventually become entrapped in their own secretion, giving rise to osteocytes (Caetano-Lopes et al., 2007) (Fig. 1), which are the most abundant cell type found in bone. Osteocytes act as mechanosensors through their extensions of the plasma membrane and are thought to direct bone resorption as well as bone formation (Knothe Tate, Adamson, Tami, & Bauer, 2004). In contrast, osteoclasts are primarily responsible for the removal of old bone tissue, which is known as bone resorption (Teitelbaum, 2000). They are multinucleated cells that mature from hematopoietic stem cells of the monocyte-macrophage lineage after exposure to macrophage colony stimulating factor (M-CSF). Multinucleation, the formation of cells with more than one nucleus, occurs after induction with Receptor Activator of NF-κB Ligand (RANKL). Osteoclasts are able to remove bone tissue by using enzymes such as carbonic anhydrase (CA) (Asagiri & Takayanagi, 2007), which belongs to a family of metalloenzymes that catalyzes the interconversion between carbon dioxide, water and dissociated ions of carbonic acid, thus maintaining the acid-base balance in fluids (Badger & Price, 1994). In osteoclasts, carbonic anhydrase releases hydrogen ions through the ruffled border into the resorptive cavity, aiding in the dissolution of mineralized bone matrix into their base components.

Osteoclasts and osteoblasts have to communicate with each other for the correct maintenance of bone mineral density (BMD) (Florencio-Silva, Sasso, Sasso-Cerri, Simões, & Cerri, 2015b). It is thought that any miscommunication between these two cell populations can sway the maintenance of BMD into one of two directions: If osteoblasts are more active than osteoclasts, this can eventually lead to osteopetrosis, a bone disease characterized by the deposition of more bone tissue than can be resorbed (Teitelbaum, 2007). If, however, osteoclasts are more active than osteoblasts, this leads to a net loss of BMD, as more bone tissue is resorbed than can be deposited by osteoblasts, which eventually leads to osteoporosis (McClung, 2007). Bone remodeling through communication between osteoblasts and osteoclasts takes place in so-called basic multicellular units (BMUs) (Matsuo & Irie, 2008) and has three phases. In the first phase, osteoclast precursors are recruited to the site that is to be reabsorbed, where they differentiate and mature to begin bone resorption (Nakamura et al., 2007). After bone resorption is finished, the osteoclasts undergo apoptosis, and osteoblasts are activated to deposit new bone tissue. Bone remodeling ends with mineralization of osteoblasts into osteocytes (Matsuo & Irie, 2008).

Bone remodeling is a process that differs depending on the type of bone: trabecular bone is more active in remodeling, which is also the reason why it is less mineralized than cortical bone. The higher surface to volume ratio makes trabecular bone undergo far more remodeling than cortical bone, with up to 26% volume turnover per year, while cortical bone only has a turnover of 3% (Oftadeh et al., 2015). Additionally, differences in bone remodeling result from other structural differences - signals can more readily traverse the matrix volume in trabecular bone, as they are comprised of thin trabecular plates. Cortical bone by contrast has a smaller surface area and large matrix volume, which predisposes it to accumulation of microdamage as signals don't travel as quickly from the bone matrix to the surface where remodeling takes place (Li et al., 2017).

There are many different methods of communication between osteoblasts and osteoclasts. Estrogen, a sex hormone necessary for the maintenance of the

female reproductive system (Archer, Zeleznik, & Rockette, 1988), slows down the rate of bone resorption (Almeida, 2016) (Fig.2). Estrogens promote osteoblastic function, particularly the growth and maturation of osteoblastic precursors. Estrogens also inhibit bone resorption through the control of osteoclasts, particularly by inhibiting cytokines that lead to osteoclast formation and maturation (Khosla, Oursler, & Monroe, 2012).

The function of osteoclasts is promoted by the receptor activator of nuclear factor kappa-B ligand (RANKL), which is a protein of the tumor necrosis factor family (Kong et al., 1999) and thought to be connected to bone loss in cancer (Schmiedel et al., 2013). Osteoclast precursor cells and mature osteoclasts express the receptor activator of nuclear factor kappa-B (RANK) on their surface, which is the receptor for RANKL. When RANKL is produced and released by osteoblasts, it leads to the maturation of osteoclasts, which links bone formation with bone resorption (Ray, 2018). The successful binding of RANKL to RANK leads to the fusion of osteoclastic, mononuclear precursors into multinuclear osteoclasts through the activation of the NF-κB signaling pathway (Fig.2). In this pathway, the TNF Receptor associated factor 6 (TRAF6) is recruited and activates mitogen-activated protein kinase (MAPK) pathways (Abu-Amer, 2013). The importance of RANKL/RANK has been shown in studies where either one of them has been knocked out, which leads to an arrest of osteoclastogenesis and an increase in bone material deposition (Boyce, Xiu, Li, Xing, & Yao, 2015).

Osteoprotegerin (OPG) is a protein that has bone-loss protective functions, which stem from its interference with RANKL binding to its receptor (Fig.2) (Boyce & Xing, 2008). OPG is very similar in structure to RANK (Kong, Boyle, & Penninger, 2000), thus acting as a decoy receptor for RANKL. The binding of RANKL to OPG thus prevents the activation of multinucleation for osteoclastic precursors, leading to a reduction in bone resorption. If OPG is unavailable to control osteoclast maturation, estrogen can suppress the release of RANKL by mature osteoblasts (Streicher et al., 2017). Insulin-like growth factor 1 (IGF-1) is also known to reduce the expression of RANKL, as well as the M-CSF needed for the

production of osteoclasts (Wang et al., 2006). IGF-1 is one of many different factors that can be used by bone cells for communication and acts as a pleiotropic growth factor that mediates the anabolic and mitogenic activity of growth hormone (GH) (Laron, 1999).

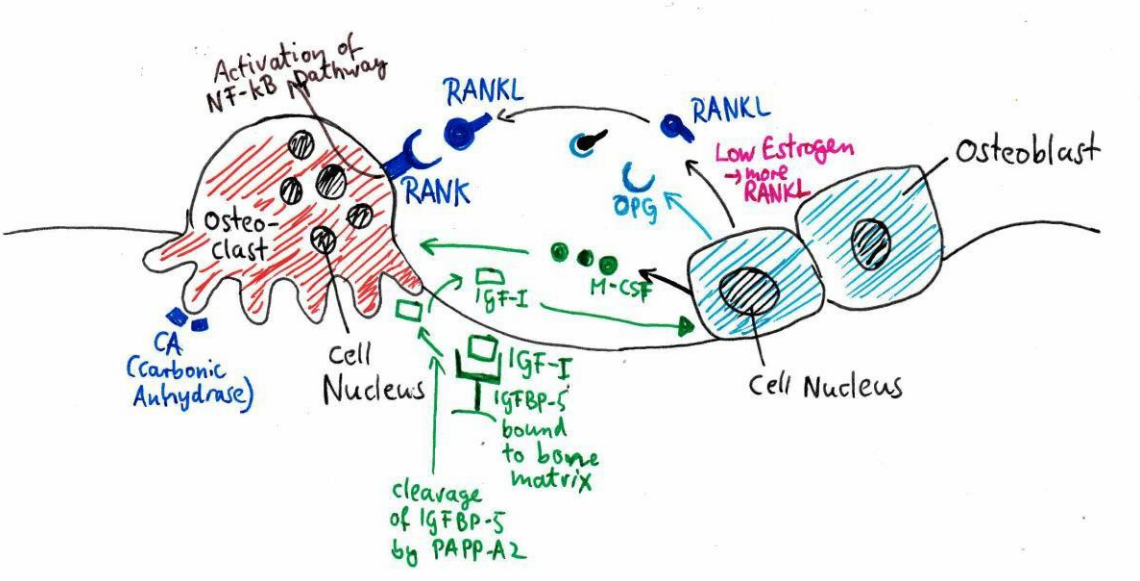

Figure 2: Factors participating in the communcation of osteoblasts and osteoclasts. *RANKL is released by Osteoblasts, with the release increased if estrogen is low. If RANKL isn't stopped by OPG, RANKL binds to RANK on osteoclasts, activating the NF-kB pathway that leads to multinucleation of osteoclast precursors. Carbonic anhydrase (CA) is used by osteoclasts to break down bone, which releases IGF-1 that was trapped inside the bone matrix* (Xian et al., 2013)*.The now freed IGF-1 can then activate more osteoblasts. Picture modified after* (X. Cao, 2011; Han, You, Xing, Zhang, & Zou, 2018)

1.4. The role of the IGF-1/IGFBP axis in bone metabolism

IGF-1 is a polypeptide that is 70 amino acids long and weighs 7649 kDa, and, as the name suggests, belongs to the protein family of insulin-like peptides (Rinderknecht & Humbel, 1978). Structurally, it is very similar to insulin and thus has the ability to bind to the insulin receptor, though with low affinity. It can also bind to its own insulin-like growth factor 1 receptor (IGF1R). Many cell types in different tissues have IGF1R, and through binding to this receptor, intracellular signaling is initiated, which leads to the activation of pathways that stimulate cell growth and cell proliferation and inhibit apoptosis (Peruzzi et al., 1999). IGF-1 is best known as a growth hormone, and stunted growth occurs if there is a lack of

IGF-1 in the system, either through loss of function in IGF-1 or its receptor, leading to dwarfism in some cases (Laron, Pertzelan, Karp, Kowadlo-Silbergeld, & Daughaday, 1971). Recombinant human IGF-1 injections are currently used to help patients with growth deficiencies (Ren, Nie, & Wang, 2016). The IGF-1 that circulates via the blood stream comes in large part from the liver, but it can also be locally released by bone tissue, with both having growth-promoting effects (Ohlsson et al., 2009). Because of the stimulatory effect on different cell types in tissues, IGF-1 and its bioavailability needs to be tightly controlled. This is achieved by insulin-like growth factor binding proteins (IGFBPs), of which there are six (IGFBP-1 to IGFBP-6), though they are structurally very similar and are able to bind both IGF-1 and IGF-2 with high affinity (Forbes, McCarthy, & Norton, 2012). The binding of IGFs to IGFBPs prolongs their half-life, but also inhibits their metabolic and proliferative actions (Bentov & Werner, 2013). For IGFs to be able to act and bind to their target IGFR receptor, their binding proteins must be cleaved.

The loss of IGF-1 action leads to growth deficiency, and more specifically to shorter bones. Mouse studies have shown that IGF-1 deletion mice have significantly lower femur lengths, lowered cortical bone density and thickness, as well as lesser trabecular bone volumes (Baylink, 2003; Yakar et al., 2002). Similarly, human patients with osteoporosis have a significantly lower concentration of serum IGF-1 (Courtland et al., 2013). In bones, the IGF system consists of several components, which includes IGF-1, their respective type 1 receptor as well as all six IGFBPs and IGFBP proteases (Yakar 2005). The two most abundant IGFPBs in bone are IGFBP-4 and IGFBP-5*,* with both of them being stored inside the bone matrix through their ability to bind to hydroxyapatite, the main mineral component of bones (Salih et al., 2005). During bone formation, IGF-1 binds to its type 1 receptor increasing the rate of proliferation, differentiation of osteoblasts by activating the MAPK (mitogen-activated protein kinase) and PI3K (Phosphoinositide 3-kinase) signaling pathways (Govoni, Baylink, & Mohan, 2005; Guntur & Rosen, 2013). This produces an environment in which recruited MSCs (mescenchymal stem cells) can differentiate into

osteoblasts, thus linking bone resorption with bone formation. The administration of GH and IGF-1 significantly increased both bone formation and bone resorption; patients with hip fractures that received treatment with IGF-1 experienced increased bone healing. GH and IGF-1 therapies had a significant anabolic effect (Locatelli & Bianchi, 2014).

Aside from their role in IGF-1 availability, IGFBPs also act independently of IGFs to increase certain parameters of bone formation. IGF-1, IGFBP-3, and IGFBP-5 have a stimulatory effect on bones. Out of the stimulatory IGFBPs, IGFBP-5 is reported to have the most consistent stimulatory effect on bone formation *in vitro* (Govoni et al., 2005), as well as *in vivo*, and patients suffering from osteoporosis have lowered IGFBP-5 concentration in their serum (Jehle et al., 2003).

In order to make IGF-1 available, its binding protein has to be cleaved. This is achieved with the use of proteases, including pregnancy-associated plasma proteins (PAPPAs), of which there are two family members, PAPP-A and PAPP-A2, sharing 45% similarity in amino acid structure. *Pappa* deletion mice have been shown to have shorter femurs and lower body weights than their wild-type counterparts, which is a result of the missing cleavage of IGFBP-4 by the PAPP-A protease (Laursen et al., 2001). IGFBP-4 is a negative regulator of IGF-1, which necessitates cleavage for successful bone growth (Conover, Kiefer, & Zapf, 1993; Mohan et al., 1995). Failure to cleave IGFBP-4 resulted in a loss of 10-13% of volumetric BMD in femurs of *Pappa* knockout mice (Tanner, Hefferan, Rosen, & Conover, 2008). Bone turnover in trabecular bone mass was impaired, and the absence of PAPP-A had little impact on cortical bone modeling (Tanner et al., 2008). PAPP-A2 cleaves IGFBP-5, thus liberating IGF-1 for metabolic actions (Overgaard et al., 2001). The role of the IGFBP-5 protease PAPP-A2 in bone remodeling is not fully known.

Studies focusing on quantitative trait loci (QTL) that affect adult body size have indicated that *Pappa2* is a candidate gene affecting post-natal growth (Christians et al.,2006). It was later confirmed that natural variation in *Pappa2* contributes to

variation in growth, which might explain the association between Pappa2 SNPs and developmental hip dysplasia in humans and birthing in cattle (Christians et al., 2013).

In humans, two homozygous mutations of *PAPPA2* have been identified, p.D643fs25* and p.Ala1033Val. Both of these mutations result in the loss of proteolytic activity of PAPP-A2 due to creating a premature stop codon (Dauber, Muñoz-Calvo, et al., 2016), resulting in growth deficiencies. The role of PAPP-A2 in the general human population has been largely unknown until one study measured the concentration of PAPP-A2 in plasma in the adult general human population. This study aimed to assess whether lifestyle or other biochemical parameters have an effect on this concentration. PAPP-A2 plasma concentrations tended to be higher in women in comparison to men and were positively correlated with age. There was no association between PAPP-A2 plasma concentration and height, suggesting that the relationship between PAPP-A2 and growth does not persist into adulthood when growth ceases (Steinbrecher et al., 2017).

Deletion of *Pappa2* in mice leads to a reduction in IGF-1 bioavailability and an increase in IGFBP-5 concentration, which leads to reduced postnatal growth (Christians et al., 2013; Conover et al., 2011). *Pappa2* deletion in osteoblasts led to a significant decrease in body mass and skeletal size in mice, indicating that postnatal growth of bones is affected by osteoblast-produced PAPP-A2 (Amiri & Christians, 2015; Fujimoto et al., 2019). A constitutive deletion of *Pappa2* in mice of both sexes led to an increase in femoral cortical area fraction. Trabecular bone was reduced by *Pappa2* deletion in young mice. *Pappa2* knockout mice had reduced bone formation markers and reduced bone resorption, which indicated subtle changes in bone morphology due to the resulting increase in IGFBP-5 concentrations (Christians, Amiri, Schipilow, Zhang, & May-Rashke, 2019). An overexpression of IGFBP-5 may reduce BMD via the reduction of bioavailable IGF-1 (Fig.2) (Salih et al., 2005). PAPP-A2 is also a factor in skeletal growth in humans, and decreases from childhood to adolescence. Total IGF-1 increased

from childhood into adolescence, and free IGF-1 levels increased with age (Fujimoto et al., 2020). Many growth disorders are monogenic in nature, and can result in a visible phenotype (Jee, Baron, & Nilsson, 2018). Growth retardation through mutations in the *PAPPA2* gene have also been reported for humans. A homozygous mutation in PAPP-A2 p.D643fs25* lead to a complete lack of circulating PAPP-A2 in two prepubescent children born to nonconsanguineous parents. While their bone length and weight at birth were normal, they showed post-natal and continous growth velocity retardation, in a small chin, moderate microcephaly and long thin digits (Dauber, Muñoz-calvo, et al., 2016). Their growth deficiencies were treated for two years by injection with recombinant human IGF-1, which led to an increase in height and whole-body BMD (Hawkins-Carranza et al., 2018; Mastrangelo et al., 2018). This shows that short stature can be treated with exogenous IGF-1 to elevate the level of free IGF-1 in blood (Le et al., 2017). Alternatively, recombinant human PAPP-A2 can be injected, which results in a dose-dependent increase of IGF-1 (Andrew et al., 2018). While many studies have measured a reduction of free IGF-1 concentrations in *Pappa2*-deficient patients, there aren't many studies that have focused on measuring the concentration of serum PAPP-A2 in humans. Female children experienced a marked pubertal peak in IGF-1 concentrations that may have further effects on their bone structure (Fujimoto et al., 2020). It is not known if PAPP-A2 plays a role in the recovery of BMD after lactation, which warrants further investigation.

1.5. Recovery after lactation and changes with age

It is unknown whether the recovery of BMD after lactation is affected by the age of the nursing mother, but BMD accretion rates are known to vary over the lifespan. 90% of peak BMD in human women is reached by the age of 18 years (National Institutes of Health, 2015). Studies on mothers around and above this age have found a complete recovery of the maternal skeleton after weaning and

that there are no long-term effects of lactation and parity on the risk of developing low BMD in menopausal age (Kovacs, 2016). However, it has been hypothesized that pregnancy prior to reaching peak BMD may have a negative effect on the maximum mineralization achieved. Some studies have found evidence for this hypothesis (Cho et al., 2012; Schnatz, Barker, Marakovits, & O'Sullivan, 2010), while others show the opposite pattern (Chantry, Auinger, & Byrd, 2004). Only a few studies have looked at the effects of lactation on adolescent mothers under the age of 18, and so more research is necessary whether early lactation may have adverse effects on the recovery of BMD. On the opposite end of the spectrum, there have been almost no studies of the effects of lactation on mothers with an advanced age. Given that the age at first pregnancy is increasing (Lampinen, Vehviläinen-Julkunen, & Kankkunen, 2009), further research into this is needed. One short-term study found that bone recovery after lactation at an older maternal age could be impaired (Hopkinson, Butte, Ellis, & Smith, 2000). With increased age, BMD naturally decreases, with women over the age of 40 experiencing BMD loss (Berger et al., 2010, 2008). However, more studies need to be undertaken to confirm whether age has a negative effect on the recovery after lactation in first-time mothers of advanced age. The exact mechanisms that are impacted by increased age also need to be further elucidated. Given known changes in bone with age, it is expected that the recovery following lactation might be impaired at older maternal ages. The properties of bone change with increased age as well.

While the different components of bone are maintained to minimize fractures and increase strength, the properties of the bone geometry and the tissue level geometry of the bones are what determines fracture risk. A shift towards higher mineral content results in stiffer, but more brittle bones, due to changes in the collagen structure of the bone (Boskey & Coleman, 2010). BMD is known to decrease with age and is often used as a marker for possible future fractures in bone health screenings. Cortical bones become weaker and more brittle with age (Tommasini, Nasser, & Jepsen, 2007), as does trabecular bone (Nagaraja, Lin, & Guldberg, 2007). This is caused by the reduced availability of sex hormones with

older age, particularly in females with the onset of menopause. Estrogen is necessary to maintain balance between osteoblasts and osteoclasts and to decrease bone resorption by inhibiting osteoclast formation (Almeida, 2016) and inducing osteoclast apoptosis (Khosla et al., 2012) while also stimulating osteoblastic precursors towards growth and maturation. With these functions of estrogen decreased in older women, the balance in bone maintenance shifts towards more bone resorption than formation, which leads to an increase in fractures in the elderly population (Yates, Karasik, Beck, Cupples, & Kiel, 2007). A decrease in bone density is also observed in mice, where BMD and bone elasticity decreases with age, with the effect being most pronounced older than 50 weeks (Kavukcuoglu, Denhardt, Guzelsu, & Mann, 2007). Age also affects the expression levels of RANKL as well as OPG in both mice and humans. RANKL mRNA levels increase by 4.4-fold in old mice which shifts the balance to increased bone resorption (J. Cao, Venton, Sakata, & Halloran, 2003). OPG levels also decrease, which leads to the activation of more osteoclasts and thus further bone loss (Chung et al., 2014). The decreasing concentrations of IGF-1 with age also affect the balance between osteoclast and osteoblast function.

Genotype also may have a different effect on skeletal growth depending on the age of the subject. In one study, *Pappa2* knockout mice were compared to wildtype mice at three different ages. It was found that knockout mice at the age of ten weeks had increased femoral cortical area fraction, but reduced trabecular thickness. At the age of nineteen weeks, female mice experienced a further increase in cortical area fraction and thickness, as well as a non-significant decrease in trabecular thickness. At thirty weeks of age, these *Pappa2* knockout mice showed further increase in cortical bone parameters, but no further effect on trabecular parameters was observed (Christians et al., 2019). Changes that were observed with increased age may be connected with subtle changes in bone morphology as a result of changed IGFBP-5 levels in knockout mice.

1.6. Pros and cons of the mouse model

In this study, mice were used as a model. Mouse models have been a popular choice for the study of bone fracture healing and bone remodeling research. The benefits of using mice include the easy availability of test subjects, the possibility of genetic modification, easy handling, shorter breeding cycles and fast regeneration. The sequencing of of the mouse genome also revealed a high number of orthologs and homologs to human genes, which makes the mouse a good study model (Haffner-Luntzer et al., 2016). Mice have been used extensively in studies of bone fracture healing, as both mice and humans show an initial inflammatory reaction to bone damage as well as a course of fracture healing with intramembraneous or endochondral ossification (Haffner-Luntzer et al., 2016). The bone regeneration process itself has been found to differ among the available inbred mouse strains. C57BL/6 mice have demonstrated a more rapid bone healing than the DBA/2 or C3H mouse strains (Manigrasso & O'Connor, 2008).

Mice do not show menopause as humans do, and so a special mouse model was created for such research. To study the induction of postmenopausal osteoporosis, ovariectomy is performed in female mice (also known as OVX mice), thus inducing a lack of ovary-derived estrogen. OVX mice show a rapid loss of trabecular bone, shifting the mechanism of bone remodeling towards increased bone resorption, which mimics the human situation of high bone turnover in early menopause (Bain et al., 1993). The one drawback of this method is that the total BMD loss is less than in human patients (Thompson, Simmons, Pirie, & Ke, 1995). The mouse is also used as a model for age-related research on changes in bone since, like aging humans, mice also display a progressive decrease in total bone mass (Silbermann et al., 1987; Weiss et al., 1991).

Though mammals all show the same mechanism of mineral resorption from the maternal skeleton to provide calcium in milk (Kovacs, 2016), one key difference

will be the demand. Mice have a higher demand for calcium delivery due to their larger litter sizes (with around eight to twelve pups per litter), whereas humans typically only give birth to one or two offspring (Kovacs, 2017). Mice also nurse their pups for a shorter amount of time (up to three weeks of lactation), which puts a more intense strain on the maternal skeleton, such that the effects of lactation on bone microarchitecture is more prominent in mice. This has been demonstrated in rodents that were fed a low-calcium diet or had to nurse more pups than normal, which resulted in lowered bone strength (Peng, Garner, Kusy, & Hirsch, 1988). Humans instead have a longer nursing time of between six to twelve months, with the baby requiring around 200 mg of calcium daily in the first six months, and then 120 mg daily in the second six months after (Kovacs, 2016). Human women also experience a negative calcium balance while breastfeeding (Hunscher, 1930). Both humans and rodents also share the mechanism of BMD recovery once lactation ceases. Mice have shown to recover BMD within a timespan of two to four weeks (Ardeshirpour et al., 2007). DXA-measurements during and after lactation in human women showed that BMD-loss caused by lactation is reversed after twelve months post-weaning in most women (Kovacs, 2016)

The focus on mice in my study also enables comparisons with similar studies of bone recovery. One study conducted by Bornstein et al. focused on the skeletal remodeling during and after lactation in mice (Bornstein et al., 2014). The mice used by Bornstein were of the C57BL/6 mouse strain, which is why I used this strain in my own studies. My experiments on recovery after lactation were also modeled after a study conducted by Liu et al.. Though this study used CD1 mice as the experimental animals, the goal of their study was to research bone recovery after lactation at three different bone sites, which included the tibia, third lumbar vertebra and femur. Mice in lactation groups were sacrificed on either the 12th day of lactation or 28 days after weaning (Liu et al., 2012)

1.7. Objectives of this study

The objectives of this study are to determine whether PAPP-A2 is involved in the recovery of bone after lactation and to test whether recovery is affected by age, using mouse models. As mentioned in the previous section above, PAPP-A2 loss-of-function leads to a reduction in skeletal size in mice (Amiri & Christians, 2015). In humans, short stature and reduced bone density have been found to be caused by loss-of-function PAPP-A2, which were improved by IGF-1 treatment.

This suggests that PAPP-A2 may also play a role in the recovery of BMD after lactation. Since experiments with humans are not readily possible, mice are instead used as the model organism, for reasons described above. I used *Pappa2* knockout mice to determine if the loss of PAPP-A2 and the subsequent lower availability of IGF-1 leads to an impairment of BMD recovery after lactation. Furthermore, I assess whether age impairs bone recovery in very young and very old first-time mothers.

Chapter 2. **Methods**

2.1. Mouse work

Two groups of mice with a C57BL/6 background were used: one is homozygous for a deletion of the *Pappa2* gene, while the second group is homozygous for the intact, floxed allele of the gene, as described in (Christians et al., 2013). The Cre/lox recombining system was used for generating knockout mice, with this method being based on Cre, a recombinase protein originating from bacteriophage P1, and loxP, a 34 BP long genetic recognition sequence for Cre (Akagi et al., 1997). Transgenic mice with mouse exon 2 flanked by loxP sites (referred to as flox mice) were developed as previously described (Christians et al., 2013). Flox mice were subsequently mated with mice expressing Cre recombinase, resulting in offspring that have their exon 2 deleted by splicing exon 1 and 3 together to create an early stop codon, resulting in constitutive *Pappa2* knockout mice, meaning mice that show whole-body knockout of this gene (Fig. 3). The Cre transgene was subsequently removed by breeding to produce mice with stable, constitutive *Pappa2* deletion. In this study, flox mice were used as controls to maintain the lines. Previous tests have found no difference between flox and wildtype mice in postnatal growth (Christians, Bath, & Amiri, 2015). At the age of three weeks, mice were weaned and earclipping was performed as a form of identification of individuals. Earclips were stored in Eppendorf tubes at -4°C until DNA extraction. DNA was extracted by adding 60 µl of proteinase K lysis buffer (1 M Tris (pH 8.5, 100 mL), 0.5 M EDTA (10 mL), 5M NaCl (40ml), 20% SDS, 10 mL, 990 mL distilled water) to the earclip samples and incubating those for three to four hours at a temperature of 55°C. The samples were then centrifuged at 13000 rpm for 2 minutes. For inactivation of proteinase K, the samples were incubated at 95°C for ten minutes, flicked and then centrifuged for ten minutes to obtain the supernatants. These were collected with a pipette and mixed with 1 mL distilled water.

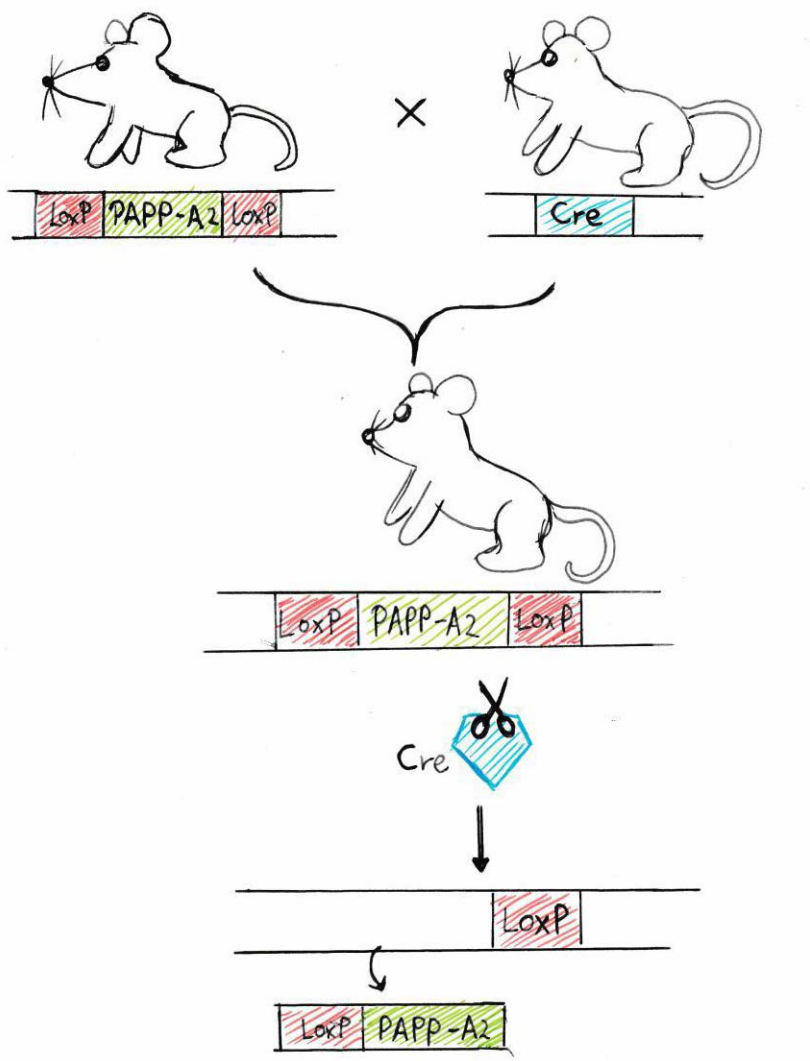

Figure 3: Creation of a Pappa2 knockout mouse *by pairing a mouse with LoxP sites flanking exon 2 of the Pappa2 gene with a mouse containing the gene for Cre recombinase, resulting in offspring with their Pappa2 knocked out.*

2.2. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to determine *Pappa2* genotype (flox vs knockout). The primers used for the PCR were Prox (5' CAG CAA AGG AAA TTT GTG CT 3'), Exon2 (5' GGT CAA ATG AAA CTT CCC TCC 3') and Dist2 (5' CTC TTG CAT GCC TCC ACT AC 3'). For the PCR reaction, each sample has to be set up as Table 1 describes. The specific PCR program that was used is

described in Table 2. The thermal cycler used for the PCRs was the MyCycler Thermal Cycler by BioRad.

Table 1: Composition for the PCR-setup. The recipe given here is for one DNA sample of 3 µl

Components	Amount for one DNA sample of 3µl
Primer	1 µl
dNTP	2µl
Buffer	2µl
MgCl2	1.8µl
H2O	9.1µl
Taq-Polymerase	0.1µl

Table 2: PCR-program used for genotyping. Steps 2 to 4 are repeated for 40 cycles.

Stages	Temperature	Time (in seconds)
1. Initial denaturation	95 °C	240
2. Denaturation	95 °C	30
3. Annealing	55 °C	30
4. Elongation	72 °C	30
5. Final Elongation	72 °C	420

2.3. Agarose gel electrophoresis

A 1% agarose gel is prepared using 1x TBE-Buffer (108g Tris, 55g Boric Acid, 9.5g EDTA (0.5M)) in order to separate DNA according to size. As a dye, 20 µl of GelRed is added to 200 ml of the liquid gel solution before it polymerizes. Before loading 20 µl of the samples onto the gel, they receive 4 µl of 6x Loading Buffer (Thermo Scientific #R0611). 4 µl of the 100 BP ladder (FermentasGeneRuler, #SM0323) is loaded on the gel along with the samples. The run time for the gel electrophoresis is 40 minutes at 120 V. After the run time, the gel can be photographed with the use of UV light (Fig. 4).

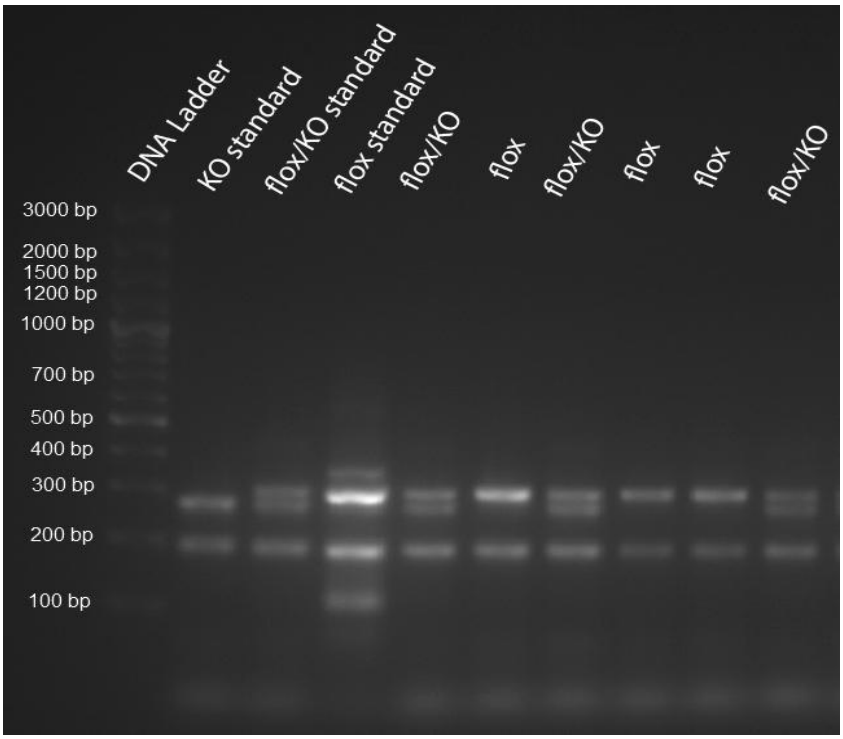

Figure 4: Example of agarose gel used to genotype Pappa2 alleles.

2.4. Bone Work

Mice were bred at the ages of 2 months, 5 months or 7 months to study the effect of age on bone recovery after lactation. Female mice at the age of two months have yet to reach peak BMD, as this occurs at an age of 4 months (Beamer, Donahue, Rosen, & Baylink, 1996), while maximum trabecular bone is achieved

at an age of around 6 to 8 weeks (Glatt, Canalis, Stadmeyer, & Bouxsein, 2007). Therefore mice at 2 months old are comparable to a human of around the age of 18 or less. Mice at the age of 5 months are comparable to a human in their twenties to early thirties, as they have achieved peak BMD and show trabecular bone loss. 7-month-old mice show a further decline in BMD and are reaching the end of their reproductive capabilities, equivalent to a human in their mid-forties. Female littermates that weren't bred served as age-matched controls (AMCs) to assess the effects of breeding and control for age. Litter sizes of bred mice were adjusted so that lactating mice nursed 7 pups. Females were either culled when the pups were three weeks of age, at weaning, or three weeks after weaning to allow for recovery after lactation (Bornstein et al., 2014). At cull, blood was collected by heart puncture and the serum extracted. Culled mice were stored at -20°C until organ removal was carried out. The carcasses were dried at 60°C for two weeks, then given to dermestid beetles to remove soft tissue and obtain the skeleton.

The left femur was obtained after exposure to dermestid beetles and its length was measured using calipers. Bone bundles were created by wrapping three mouse femurs in masking tape, with tiny copper strips used to mark each individual bone. Micro-CT scanning was performed at the Centre for High-Throughput Phenogenomics at the University of British Columbia (UBC), using an an isotropic voxel size of 7.4 μm (Scanco Medical μCT100, Switzerland; 70 kVp, 114 μA, 100 ms integration time). Around 5% of the total bone length was measured to analyse the trabecular (starting at the distal metaphysis) and cortical (starting at the bone mid-shaft) bone (see Fig.5). The region of the trabecular bone scanned was proximal to the distal growth plate which on the micro-CT was 30 layers (222 μm) below from where the four sections of the bone began to fuse, creating a bridge. In the cortical bone, the region scanned was immediately distal to the third trochanter which on the micro-CT was where cross section transitioned from a sharp teardrop shape and began to become rounder. Images were contoured and standard measurements taken, while blinded to the genotype and breeding status of the samples. Cortical measurements included

cortical area fraction (Ct.Ar/Tt.Ar, %), average cortical thickness (Ct.Th, mm), and cortical porosity (Ct.Po, %). Cortical porosity is measured with the formula 1-Bone Volume (BV)/Total Volume (TV), where BV/TV represents the percentage occupied by bone, which would be 100% if the cortical bone was solid and with no pores. Trabecular measurements included bone volume fraction (BV/ TV, %). For trabecular bone, BV represented the volume occupied by bone, whereas TV represented the total volume of the analysed region (including non-bone space). Further trabecular measurements included trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), and trabecular separation (Tb.S, mm) (Bouxsein et al., 2010).

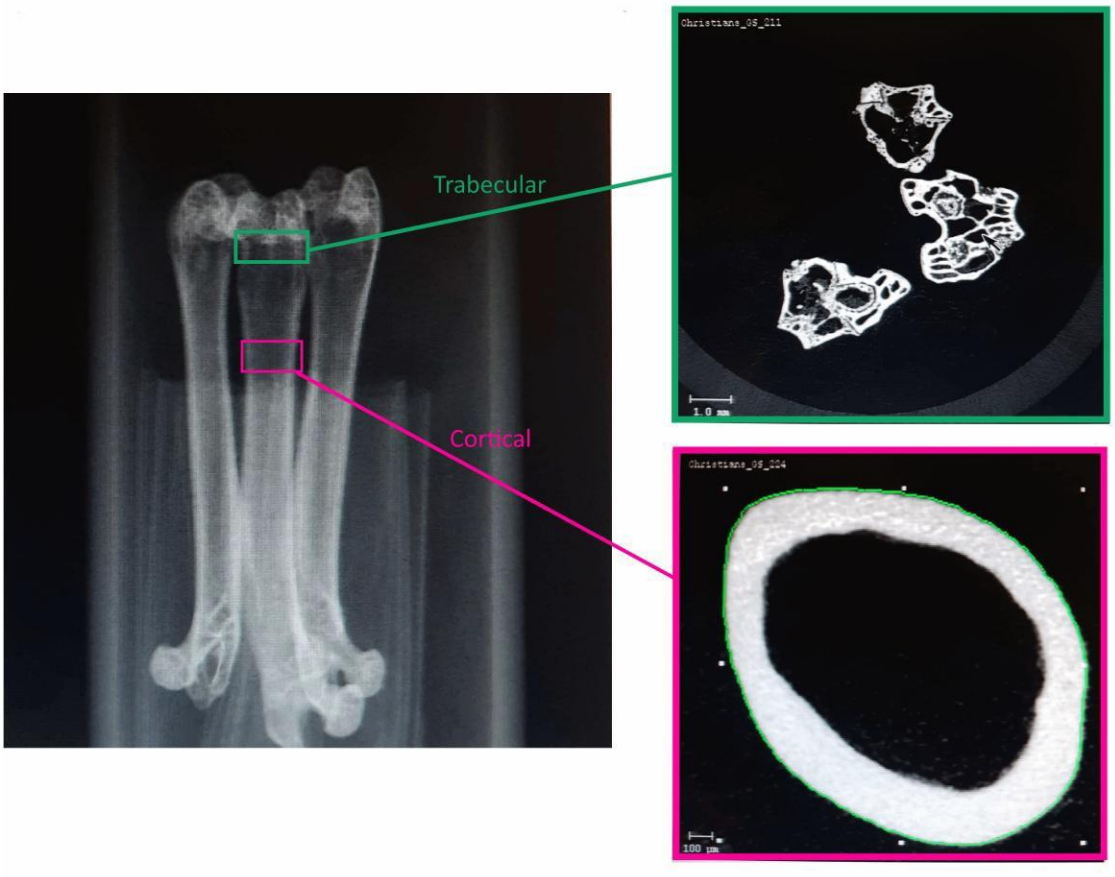

***Figure 5: Investigated bone sites of the mouse femur bundles**. Highlighted in green is the trabecular site with spongy bone tissue, with the corresponding cross-sections shown to the right. The cortical bone site is marked with pink, showing the cross-section to the right of a hollow cylinder of compact bone around empty space.*

2.5. Enzyme-linked Immunosorbent Assay (ELISA)

Serum was kept at -80°C until used for ELISAs. Serum concentrations of total IGF-1 and IGFBP-5 were measured using the IGF-1 DuoSet ELISA kit (#DY791, R&D Systems) and the IGFBP-5 DuoSet ELISA kit (#DY578, R&D Systems), respectively, following the kit procedures. For the IGF-1 ELISA, the first antibody used was the hamster-anti-mouse IGF-1 capture antibody that was provided with the kit and reconstituted with 1 mL PBS. For the IGFBP-5 ELISA, the first antibody used was goat anti-mouse IGFBP-5 antibody, provided with the kit and reconstituted with 1 mL PBS.

The steps for both ELISAs were the same, with only slight differences in the sample preparation between the IGF-1 and IGFBP-5 kits. The 96-well microplate was prepared for the ELISA a day before the experiment by coating it with 100 µL of the working concentration of the capture antibody and incubating it overnight at room temperature. After the end of the incubation period, each well was washed with wash buffer (0.05% Tween in PBS, pH= 7.2-7.4) three times. Any remaining liquid was removed after the last wash by aspiration. In the next step, the plate was blocked with 300 µL of Reagent Diluent in each well and incubated at room temperature for 1 hour. Before the assay procedure, the washing step from before was repeated. For the assay, 100 µL of each sample and IGF-1 standard was added to each well, then the plate was sealed and incubated at room temperature for two hours. The wash step with aspiration was repeated after this incubation step. Next, 100 µL of the detection antibody (biotinylated goat anti-mouse IGF-1 detection antibody for IGF-1 ELISA or biotinylated goat anti-mouse IGFBP-5 detection antibody for IGFBP-5 ELISA) was added to each well and incubated for two hours at room temperature, then the washing step was repeated. Afterwards, 100 µL of the working dilution of streptavidin-HRP was added to each well and incubated for 20 minutes at room

temperature in darkness. The wash/aspiration step was repeated, then 100 µL of the substrate solution provided in the kit was added to each well and incubated for 20 minutes at room temperature. 50 µL of stop solution (2 N H_2SO_4) was added to each well, which turned the blue solution in the well yellow. Gentle tapping of the plate ensured proper mixing. A microplate reader set to 450 nm wavelength was used to measure the optical density of each well. With the help of the IGF-1/IGFBP-5 standard curve run in every microplate, a standard curve was constructed in order to determine the concentration of the protein in the serum samples.

All samples were measured in triplicate, and the concentration was calculated using simple regression, as recommended by the manual. In order to maintain quality between different plates, a control sample was run on each plate. This control sample consisted of a composite of several samples of 30 week old female mice.

2.6. Statistical Analysis

All data were analyzed using general linear models in JMP15 (JMP, version 15.0.0, 390308, SAS Institute), as described in further detail below. Bred mice that lost their pups before weaning, or had less than 5 pups weaned were excluded from the analysis. An alpha level of 0.05 was used to establish significance. Tables containing all relevant information such as reproductive state (bred versus non-bred AMCs), age, weight, pairings, collection date (at wean versus 3 weeks post) and bone data from microCT scans were used in the analysis and loaded in JMP15. General linear models were constructed using fit model, picking the bone data needed (for example trabecular porosity) as the Y value and the traits (state, collection date, genotype) as the model effects. Crosses between traits were also utilized to determine any interactions between different factors. For example, a state*collected interaction tested whether the

effect of breeding state (bred or AMC) depended on the time of collection (i.e., at wean vs. three weeks later), i.e., whether bone recovery had taken place after three weeks of recovery time. Whether deletion of *Pappa2* affected recovery was tested by crossing the model effects of state, collection date and genotype with each other (state*collected*genotype interaction). The model effect of genotype*age was used to determine whether the effect of *Pappa2* deletion at different ages was consistent with previous work. When analyzing only mice collected three weeks after wean, the model effect of state*age answers the question of whether age affects recovery of bone after lactation. Additionally, a comparison of three different groups was made with mice that lost their litter during pregnancy, with mice that successfully bred and lactated as well as with AMCs.

Chapter 3. **Results**

3.1. Traits affected by breeding

To identify traits that were affected by lactation and that showed recovery 3 weeks after lactation, we focused on controls (*flox* mice) at two months of age to facilitate comparison with previous work. Trabecular bone fraction (BV/TV) decreased between wean and 3 weeks later ($p<0.0001$), and was lower in bred mice than in AMCs ($p=0.03$; Table 3; Fig. 6A). Thus, trabecular bone fraction decreased with age and with breeding. However, there was no interaction between reproductive state and collection date, thus no evidence of recovery. Trabecular thickness showed a similar pattern as trabecular bone fraction, showing an effect of breeding and an effect of age, but no interaction between the two (Table 3). Trabecular number and spacing were not affected by breeding (Table 3). Cortical area fraction was significantly reduced by breeding and increased following weaning, but the increase was significantly greater in bred mice (interaction between reproductive state and collection date, showing recovery $p=0.046$; Table 3; Fig. 6B). Cortical thickness showed a similar pattern of recovery as cortical area fraction (interaction $p=0.006$; Table 3; Fig. 6C). Breeding also increased cortical porosity ($p=0.04$) and decreased serum IGF-1 concentration ($p=0.03$; Table 3). logIGFBP-5 concentrations showed a marginally non-significant effect of breeding ($p=0.08$; Table 3). Overall, cortical but not trabecular bone showed recovery from lactation after 3 weeks. In subsequent analyses, I focus on traits affected by breeding in control, two month old mice.

Table 3:Effects of breeding and 3 weeks of recovery on trabecular and cortical bone traits. *Analyses include flox mice at 2 months of age (n=61, 30 mice collected at three weeks post (14 are AMC, 16 are bred), and 31 mice collected at wean (16 mice are AMC, 15 are bred)). P values are from a general linear model including effects of state, collected and state*collected interactions. Significant effects are shown in bold.*

	Bred at wean (mean +/- s.e.)	Bred after 3 weeks (mean +/- s.e)	AMC at wean (mean +/- s.e.)	AMC after 3 weeks (mean +/- s.e.)	State (Bred vs. AMC)	Collection date (at wean vs. after 3 weeks of recovery)	State*Collection date
					P	P	P
Trabecular							
Trabecular Bone Volume Fraction (BV/TV (%))	6.5 ± 0.4	4.1 ± 0.4	7.2 ± 0.4	5.1 ± 0.4	**0.0267**	**<0.0001**	0.75
Trabecular number (Tb.N (mm^{-1}))	2.23 ± 0.11	1.56 ± 0.11	2.26 ± 0.11	1.68 ± 0.11	0.49	**<0.0001**	0.66

Trabecular Thickness (Tb.Th (mm))	0.027 ± 0.001	0.025 ± 0.001	0.03 ± 0.001	0.029 ± 0.001	**<0.0001**	**0.0049**	0.34
Trabecular Spacing (Tb.Sp (mm))	0.45 ± 0.03	0.63 ± 0.03	0.44 ± 0.03	0.59 ± 0.03	0.45	**<0.0001**	0.62
Cortical							
Cortical Area Fraction (Ct.Ar/Tt.Ar (%))	38.2 ± 0.8	43.3 ± 0.7	45.2 ± 0.7	47.2 ± 0.8	**<0.0001**	**<0.0001**	**0.0457**
Cortical Thickness (Ct.Th (mm))	0.153 ± 0.003	0.180 ± 0.003	0.183 ± 0.003	0.194 ± 0.003	**<0.0001**	**<0.0001**	**0.0057**
Cortical Porosity (Ct.Po (%))	5.7 ± 0.4	6.0 ± 0.4	5.0 ± 0.4	5.2 ± 0.4	**0.0426**	0.48	0.91
logIGF-1 Conc.	4.03 ± 0.04	3.87 ± 0.05	4.04 ± 0.05	4.07 ± 0.06	**0.03**	0.18	0.07
logIGFBP5	4.83 ± 0.04	4.83 ± 0.04	4.90 ± 0.04	4.91 ± 0.04	0.08	0.93	0.95

Conc.							

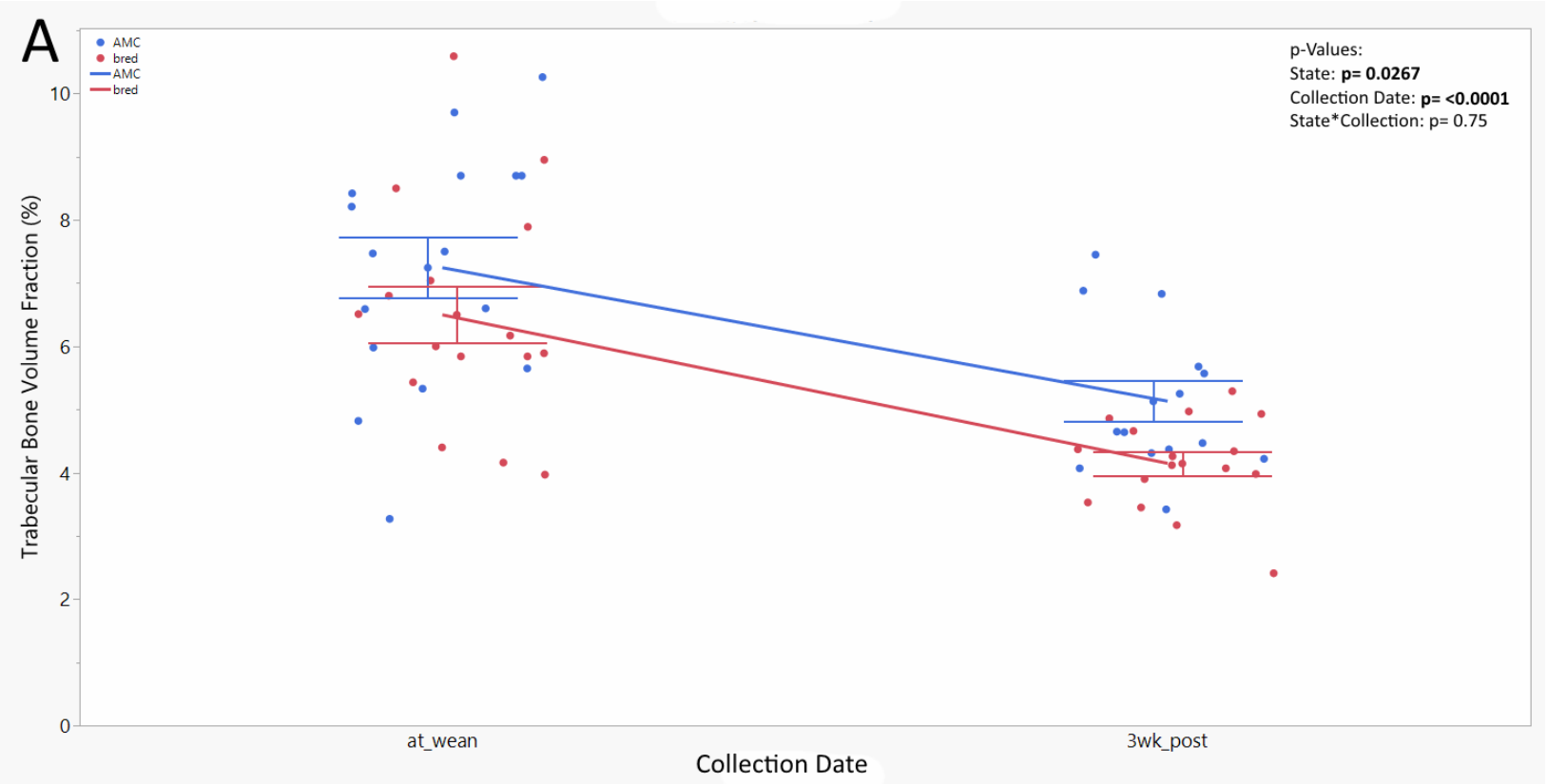

Figure 6A:Effects of breeding, lactation and recovery time on trabecular bone fraction in 2 month old flox mice, *AMC are shown in blue while their bred counterparts are shown in red. The error bars show the standard error from the mean. P-values for state, collection date and state*collection date test whether there is an effect of state, collection date, and whether the effect of state differs between collection date, respectively.*

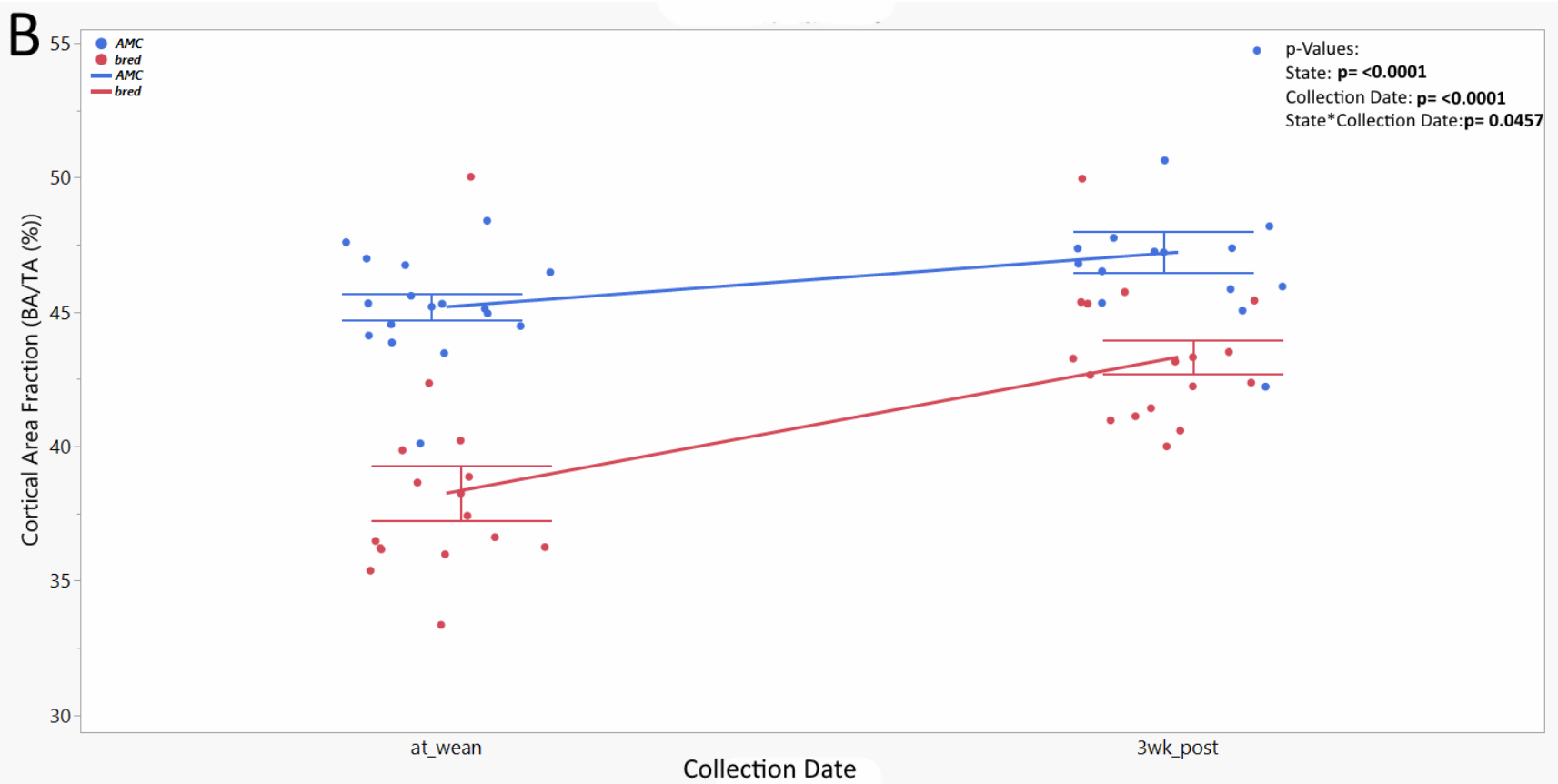

Figure 6B:Effects of breeding, lactation and recovery time on area fraction in 2-month old flox mice. *AMC are shown in blue while their bred counterparts are shown in red. P-values for state, collection date and state*collection date test whether there is an effect of state, collection date, and whether the effect of state differs between collection date, respectively.*

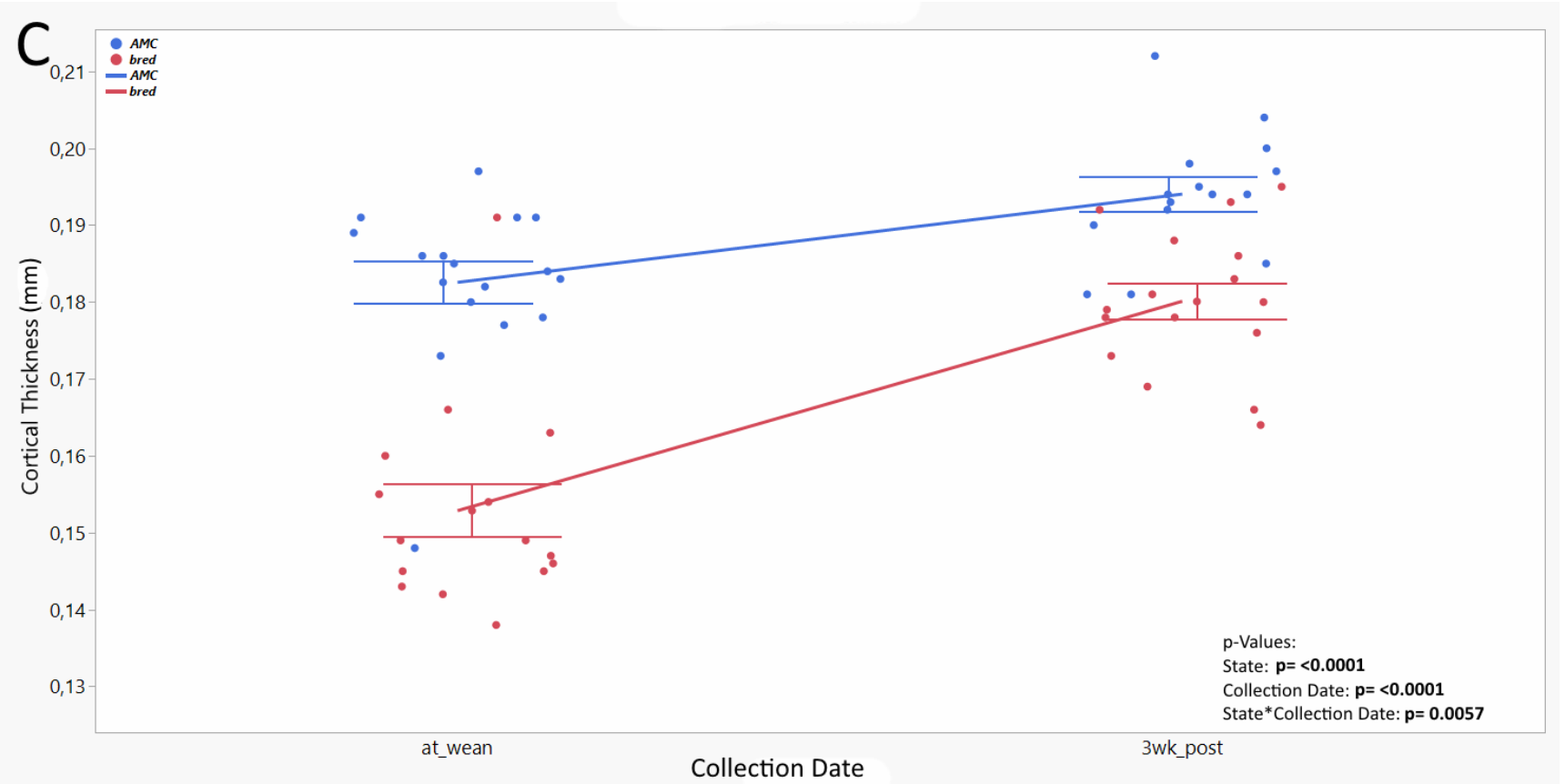

Figure 6C: Effects of breeding, lactation and recovery time on trabecular cortical thickness in 2-month old flox mice. *AMC are shown in blue while their bred counterparts are shown in red. P-values for state, collection date and state*collection date test whether there is an effect of state, collection date, and whether the effect of state differs between collection date, respectively.*

3.2. The effects of *Pappa2* deletion on bone recovery

To test the effect of *Pappa2* deletion on bone recovery, I examined mice at the age of two months and at the age of five months (Fig. 7A-7G) and tested effects of breeding state (bred or AMC), collection date (at wean or 3 weeks after), genotype (flox or knockout), the two-way interaction between state and collection date (to test for recovery) and the three-way interaction between state, collection date and genotype (to test whether recovery differed between genotypes). Trabecular bone volume fraction (BV/TV) is shown in Fig. 7A. As in controls, there was no recovery for mice at the age of two months (state*collection date p=0.50), and there was no difference in recovery between genotypes (three-way interaction p=0.83). Both flox and ko mice show a decline in trabecular BV/TV in the three weeks after wean ($p < 0.0001$), with age-matched controls showing higher BV/TV values than their bred counterparts ($p = 0.0006$ Fig. 7A, top). At the age of five months, we see a similar decline in trabecular BV/TV in the three weeks after wean ($p = 0.1$), with no difference between bred mice and age-matched controls ($p = 0.37$). Bred mice show a decline in trabecular BV/TV, though the interaction between state and collection date ($p = 0.388$) and the interaction between state, collection date and genotype ($p = 0.819$) was not significant (Fig.7A, bottom).

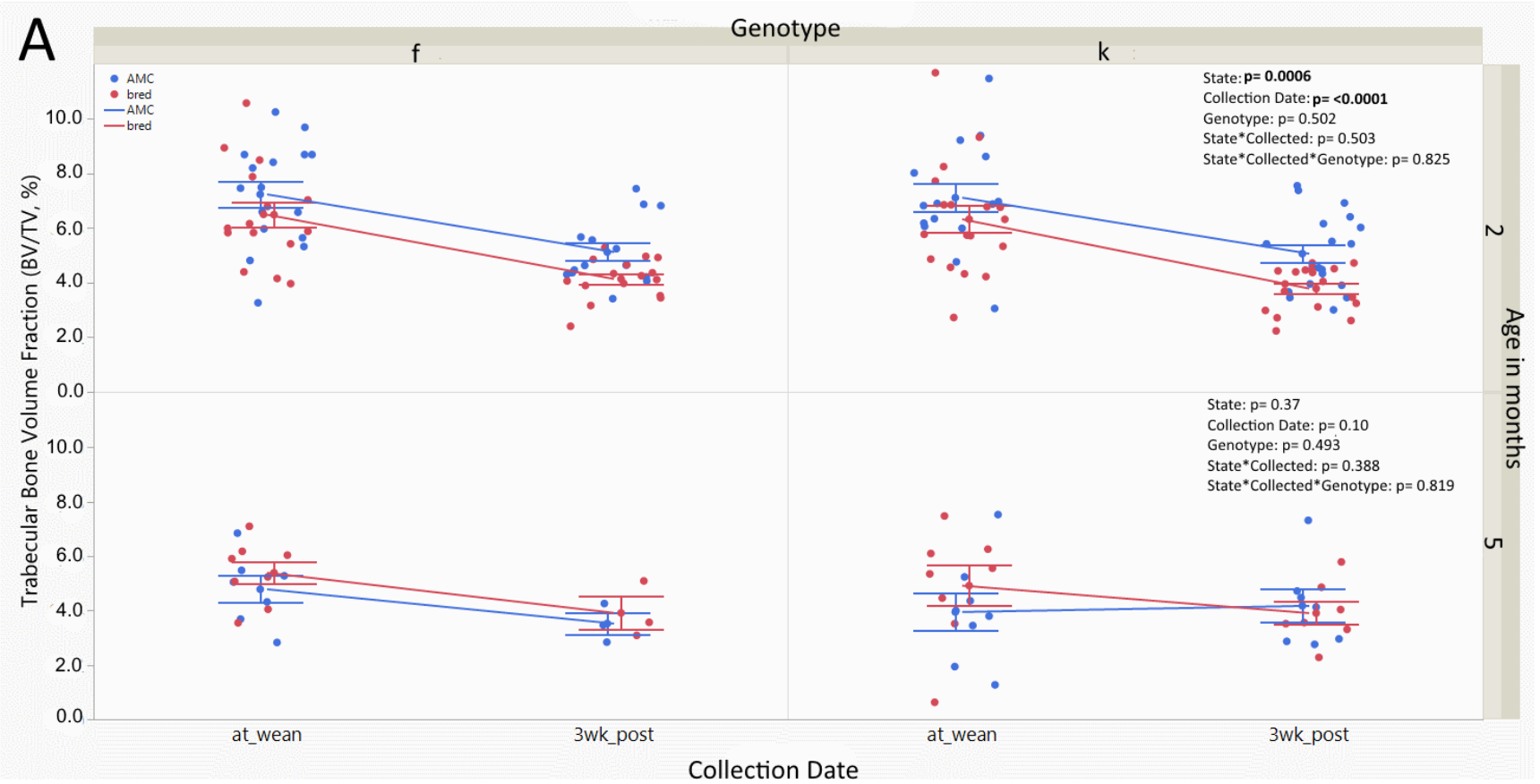

Figure 7A:Effect of Pappa2 deletion and lactation on trabecular bone volume fraction. *AMCs are shown in blue, bred mice are shown in red. Bone parameters were measured at 2 months of age (n=128, 64 mice collected at 3 weeks post (30 flox (16 bred, 14 AMCs), 34 knockouts (17 bred mice, 17 AMCs); 64 mice were collected at wean (31 flox (16 being AMC, 15 bred) and 33 knockouts (18 bred mice, 15 AMCs)) and at 5 months of age (n=51, 21 flox mice with 6 collected at 3 weeks post (3 bred and 3 AMCs) and 15 collected at wean (7 AMCs and 8 bred), 30 knockout mice, with 14 collected at 3 weeks (7 bred and 7 AMCs) post and 16 collected at wean (8 bred and 8 AMCs). P-values for state, collection date, genotype and state*collection date and state*collection date*genotype test whether there is an effect of state, collection date, genotype, whether the effect of state differs between collection date, and whether the latter difference depends on genotype, respectively.*

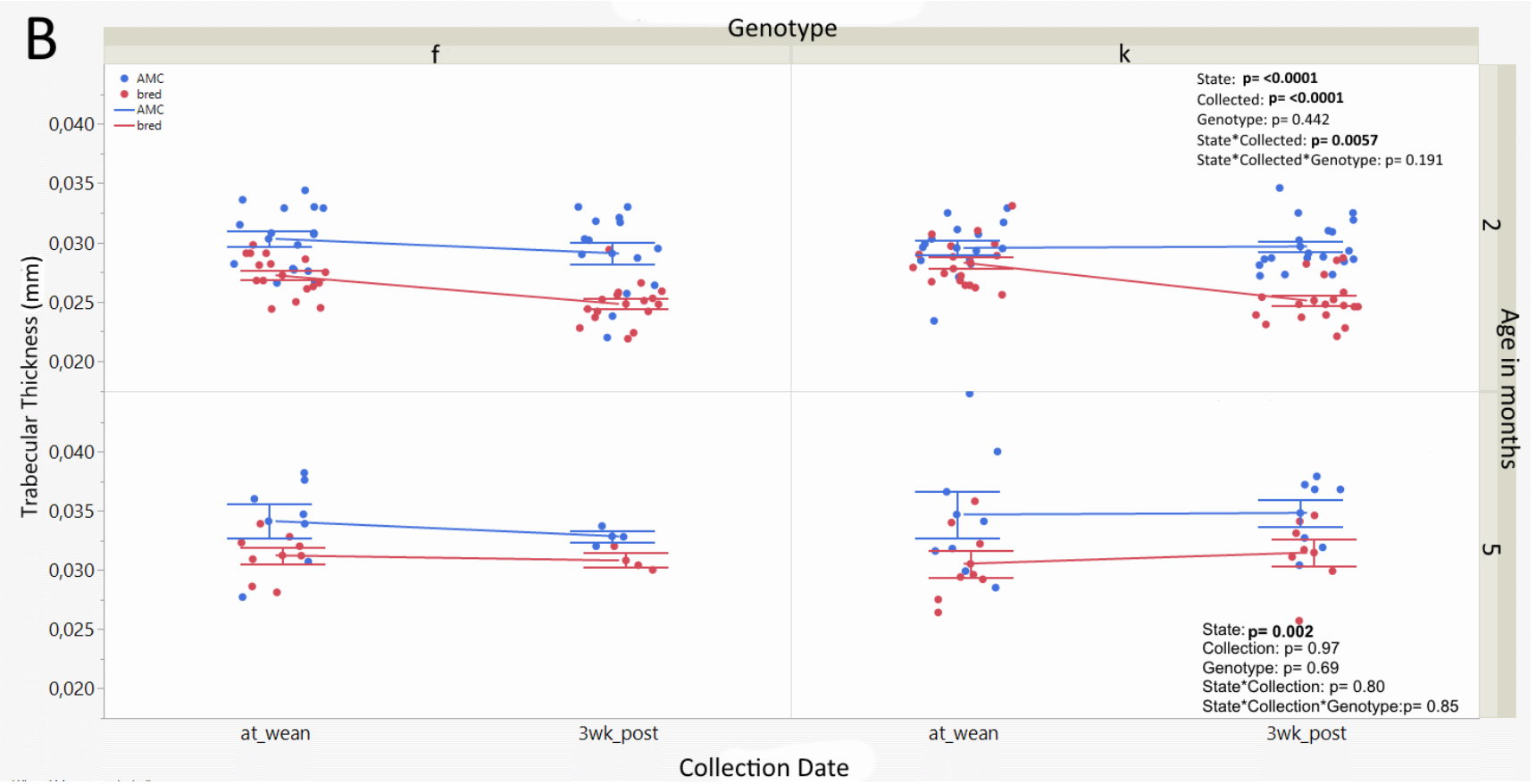

Figure 7B: Effect of Pappa2 deletion and lactation on trabecular bone thickness (in µm). *AMCs are shown in blue, bred mice are shown in red. Bone parameters were measured at 2 months of age (n=128, 64 mice collected at 3 weeks post (30 flox (16 bred, 14 AMCs), 34 knockouts (17 bred mice, 17 AMCs); 64 mice were collected at wean (31 flox (16 being AMC, 15 bred) and 33 knockouts (18 bred mice, 15 AMCs)) and at 5 months of age (n=51, 21 flox mice with 6 collected at 3 weeks post (3 bred and 3 AMCs) and 15 collected at wean (7 AMCs and 8 bred), 30 knockout mice, with 14 collected at 3 weeks (7 bred and 7 AMCs) post and 16 collected at wean (8 bred and 8 AMCs). P-values for state, collection date, genotype and state*collection date and state*collection date*genotype test whether there is an effect of state, collection date, genotype and whether the effect of state differs between collection date and genotype, respectively.*

Trabecular thickness for two month old mice was higher for AMC mice than bred counterparts ($p < 0.0001$; Fig. 7B, top). There was a significant interaction between breeding state and collection date ($p = 0.006$), where the decrease in trabecular thickness after wean was greater in bred mice than AMC. However, this interaction did not differ between genotypes (three-way interaction $p = 0.19$). In five month old mice, trabecular thickness was higher in AMC mice than in bred mice ($p = 0.002$), but there was no decline after wean ($p = 0.97$), in contrast to two month old mice (Fig.7B, bottom). There was no state by collection date interaction for trabecular thickness in 5 month old mice ($p = 0.80$) and the interaction between state, collection date and genotype was not significant ($p = 0.85$).

In two month old mice, cortical bone area fraction (BA/TA in %) was significantly affected by breeding ($p < 0.0001$) and genotype ($p < 0.0001$). Bred mice show lower cortical bone volume fraction than their AMCs. Knockout mice have a higher values than flox mice (Fig. 7C, top). Mice that had been collected three weeks after weaning showed higher cortical BA/TA than mice collected at wean ($p < 0.0001$), with a greater increase in bred mice, indicating recovery (interaction $p = 0.0022$), but this recovery did not differ between genotypes ($p = 0.68$).
In five month old mice, there was a significant effect of reproductive state, with AMC mice having a higher cortical BA/TA than their bred counterparts ($p < 0.0001$). BA/TA did not change with collection date ($p = 0.15$), though there was a significant difference between genotypes ($p = 0.044$), with higher values in knockout mice (Fig.7C, bottom). Overall however, there was no significant recovery observed ($p = 0.21$), and there was no significant difference in recovery between genotypes ($p=0.76$).

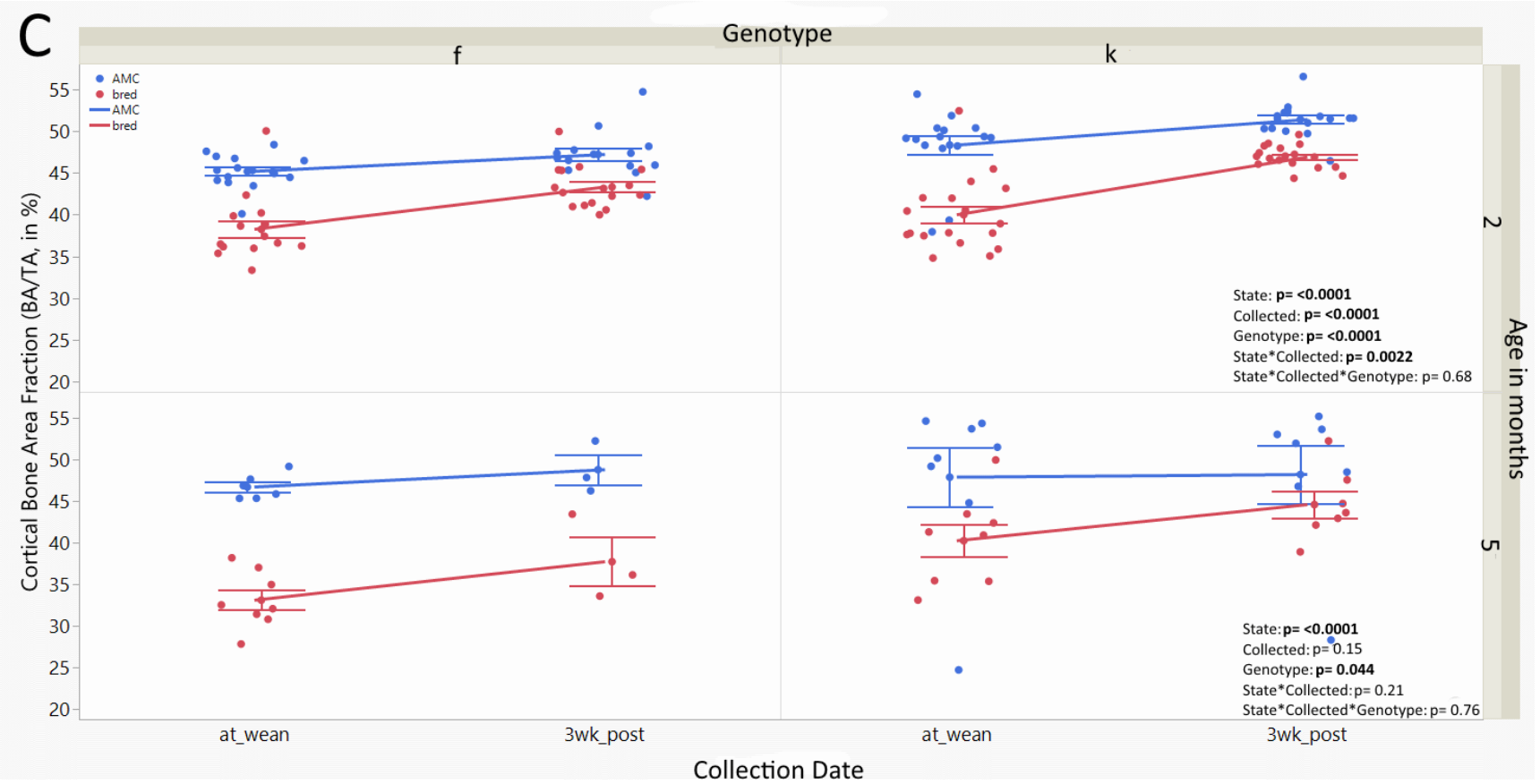

Figure 7C: Effect of* Pappa2 *deletion and lactation on cortical bone area fraction. *AMCs are shown in blue, bred mice are shown in red. Bone parameters were measured at 2 months of age (n=128, 64 mice collected at 3 weeks post (30 flox (16 bred, 14 AMCs), 34 knockouts (17 bred mice, 17 AMCs); 64 mice were collected at wean (31 flox (16 being AMC, 15 bred) and 33 knockouts (18 bred mice, 15 AMCs)) and at 5 months of age (n=51, 21 flox mice with 6 collected at 3 weeks post (3 bred and 3 AMCs) and 15 collected at wean (7 AMCs and 8 bred), 30 knockout mice, with 14 collected at 3 weeks (7 bred and 7 AMCs) post and 16 collected at wean (8 bred and 8 AMCs).A general linear model including effects of State, collected, genotype and State*Collected, Genotype*state*collected interactions was used. Significant effects are shown in bold. P-values for state, collection date, genotype and state*collection date and state*collection date*genotype test whether there is an effect of state, collection date, genotype and whether the effect of state differs between collection date and genotype, respectively.*

Cortical thickness in two month old mice was significantly lower in bred mice than in AMCs ($p < 0.0001$; Fig. 7D). Mice collected three weeks after wean showed higher cortical thickness than mice collected at wean ($p < 0.0001$), and this increase was greater for bred mice, indicating recovery (interaction $p = 0.0006$). There was no significant difference between the two genotypes ($p = 0.28$), and no effect of genotype on recovery ($p = 0.90$) (Fig. 7D, top). In five month old mice, cortical thickness was significantly higher in AMCs than in bred mice ($p < 0.0001$). Mice collected three weeks after weaning showed an increase in cortical thickness ($p= 0.0161$). While this increase tended to be greater for bred mice, the interaction was marginally non-significant ($p = 0.08$) and the interaction between state, collection date and genotype was not significant ($p= 0.72$). Knockout mice showed higher cortical thickness than flox mice ($p= 0.0066$; Fig.7D, bottom).

Bred mice showed significantly higher cortical porosity at two months than AMC mice ($p < 0.0001$), and porosity declined three weeks after wean ($p < 0.0001$; Fig. 7E, top), with the decline being greater in bred mice (interaction $p = 0.0106$), such that bred mice and AMC had similar porosity after three weeks of recovery. There was no significant difference between genotypes, and no interaction between state, collection date and genotype ($p = 0.74$). In five month old mice, bred mice had higher cortical porosity than AMCs (Fig. 7E, bottom) ($p < 0.0001$). There was no change in cortical porosity over three weeks ($p = 0.10$), and no interaction between breeding and collection date ($p = 0.12$). There was a significant effect of genotype ($p < 0.0001$), with flox mice displaying higher cortical porosity than ko mice.

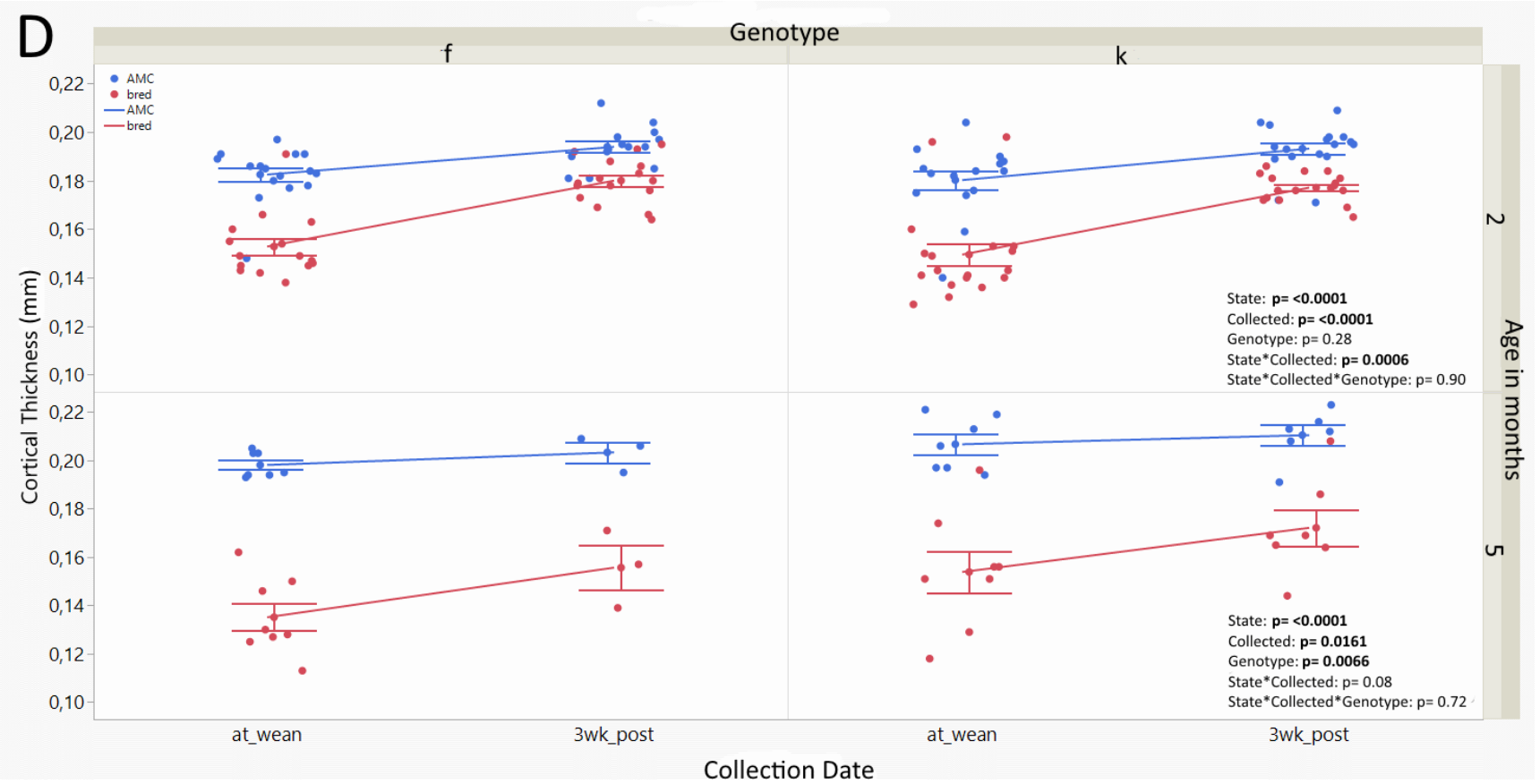

Figure 7D: Effect of* Pappa2 *deletion and lactation on cortical thickness (in mm). *AMCs are shown in blue, bred mice are shown in red. Bone parameters were measured at 2 months of age (n=128, 64 mice collected at 3 weeks post (30 flox (16 bred, 14 AMCs), 34 knockouts (17 bred mice, 17 AMCs); 64 mice were collected at wean (31 flox (16 being AMC, 15 bred) and 33 knockouts (18 bred mice, 15 AMCs)) and at 5 months of age (n=51, 21 flox mice with 6 collected at 3 weeks post (3 bred and 3 AMCs) and 15 collected at wean (7 AMCs and 8 bred), 30 knockout mice, with 14 collected at 3 weeks (7 bred and 7 AMCs) post and 16 collected at wean (8 bred and 8 AMCs). P-values for state, collection date, genotype and state*collection date and state*collection date*genotype test whether there is an effect of state, collection date, genotype and whether the effect of state differs between collection date and genotype, respectively.*

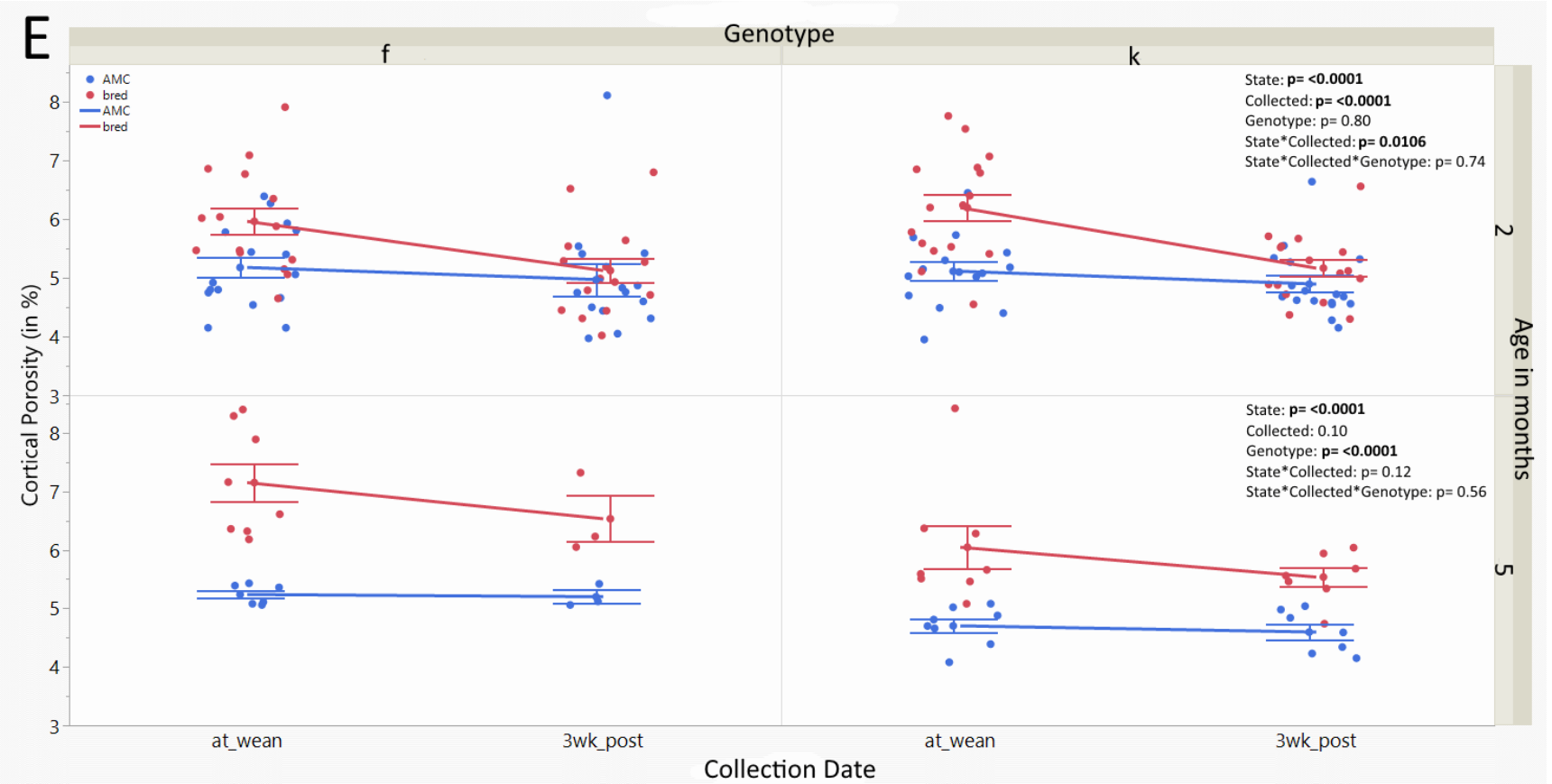

Figure 7E: Effect of Pappa2 deletion and lactation on cortical porosity (%). *AMCs are shown in blue, bred mice are shown in red. Bone parameters were measured at 2 months of age (n=128, 64 mice collected at 3 weeks post (30 flox (16 bred, 14 AMCs), 34 knockouts (17 bred mice, 17 AMCs); 64 mice were collected at wean (31 flox (16 being AMC, 15 bred) and 33 knockouts (18 bred mice, 15 AMCs)) and at 5 months of age (n=51, 21 flox mice with 6 collected at 3 weeks post (3 bred and 3 AMCs) and 15 collected at wean (7 AMCs and 8 bred), 30 knockout mice, with 14 collected at 3 weeks (7 bred and 7 AMCs) post and 16 collected at wean (8 bred and 8 AMCs). P-values for state, collection date, genotype and state*collection date and state*collection date*genotype test whether there is an effect of state, collection date, genotype and whether the effect of state differs between collection date and genotype, respectively.*

In total IGF-1 serum concentrations measured in two month old mice (Fig.7F, top), there was no significant effect of breeding ($p = 0.77$). However, mice collected at the later time point showed lowered concentrations ($p= 0.0494$). There was also a significant effect of genotype on IGF-1 concentrations - knockout mice had a lower total IGF-1 serum concentration ($p = 0.0006$). There was no interaction between state and collected, and no interaction between state, collected and genotype (Fig. 7F, top row).

At the age of five months, there was no significant effect of breeding (Fig. 7F, bottom) ($p = 0.45$). Mice collected three weeks later showed increased IGF-1 concentrations ($p = 0.0066$). Knockout mice have a lower IGF-1 concentration than flox mice do ($p <0.0001$). There was no interaction between state and collected, and no interaction between state, collected and genotype (Fig. 7F, bottom row).

The only factor with a significant effect on IGFBP-5 concentration was genotype at five months of age (Fig. 7G). *Pappa2* knockout mice have a higher IGFBP-5 concentration than flox mice ($p = 0.0122$).

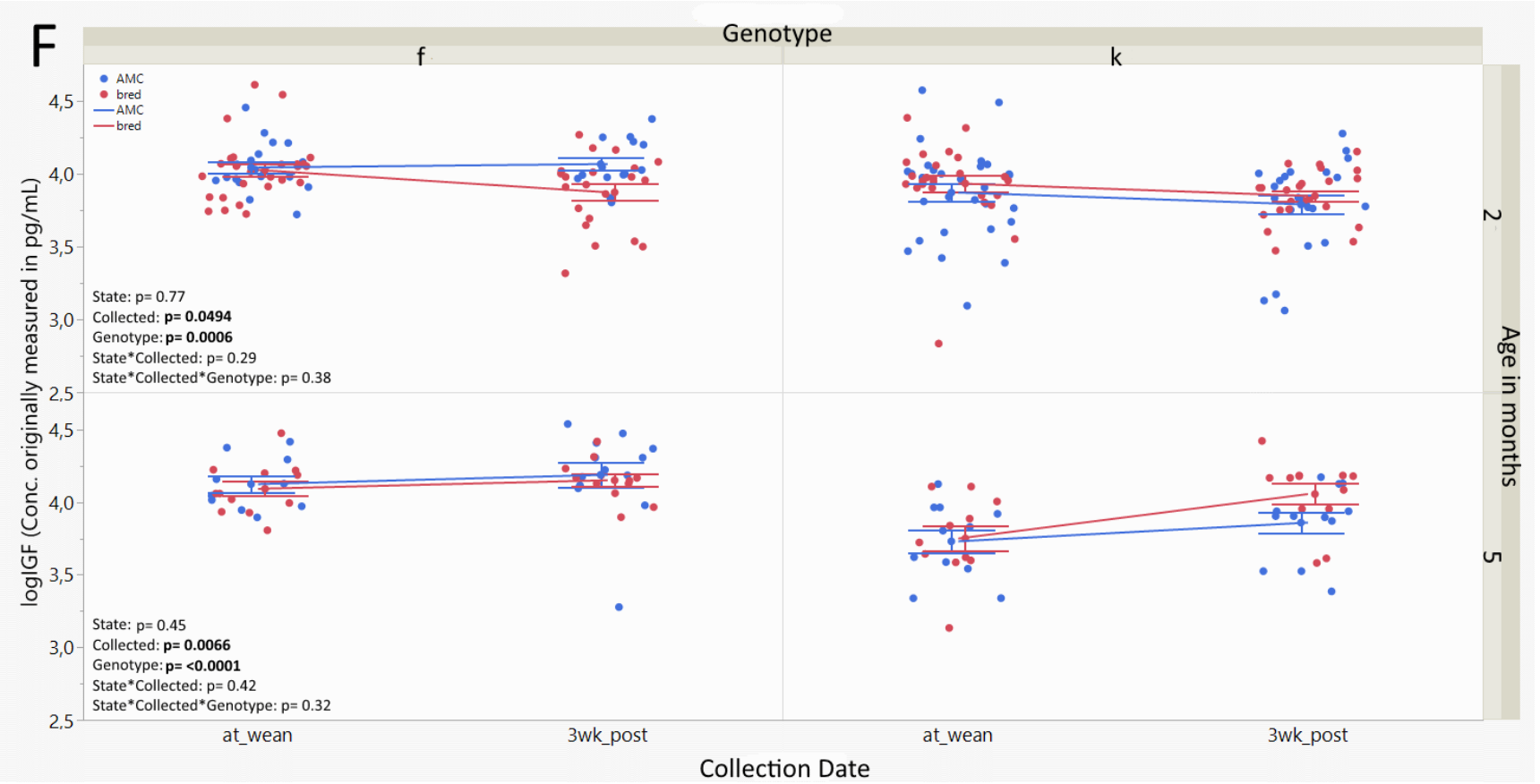

Figure 7F: Effect of Pappa2 deletion and lactation on logIGF-1 serum concentrations. *AMCs are shown in blue, bred mice are shown in red. Bone parameters were measured at 2 months of age (n=128, 64 mice collected at 3 weeks post (30 flox (16 bred, 14 AMCs), 34 knockouts (17 bred mice, 17 AMCs); 64 mice were collected at wean (31 flox (16 being AMC, 15 bred) and 33 knockouts (18 bred mice, 15 AMCs)) and at 5 months of age (n=51, 21 flox mice with 6 collected at 3 weeks post (3 bred and 3 AMCs) and 15 collected at wean (7 AMCs and 8 bred), 30 knockout mice, with 14 collected at 3 weeks (7 bred and 7 AMCs) post and 16 collected at wean (8 bred and 8 AMCs). P-values for state, collection date, genotype and state*collection date and state*collection date*genotype test whether there is an effect of state, collection date, genotype and whether the effect of state differs between collection date and genotype, respectively.*

Figure 7G:Effect of* Pappa2 *deletion and lactation on logIGFBP-5 serum concentrations. *AMCs are shown in blue, bred mice are shown in red. Bone parameters were measured at 2 months of age (n=128, 64 mice collected at 3 weeks post (30 flox (16 bred, 14 AMCs), 34 knockouts (17 bred mice, 17 AMCs); 64 mice were collected at wean (31 flox (16 being AMC, 15 bred) and 33 knockouts (18 bred mice, 15 AMCs)) and at 5 months of age (n=51, 21 flox mice with 6 collected at 3 weeks post (3 bred and 3 AMCs) and 15 collected at wean (7 AMCs and 8 bred), 30 knockout mice, with 14 collected at 3 weeks (7 bred and 7 AMCs) post and 16 collected at wean (8 bred and 8 AMCs). P-values for state, collection date, genotype and state*collection date and state*collection date*genotype test whether there is an effect of state, collection date, genotype and whether the effect of state differs between collection date and genotype, respectively.*

3.3. Effects of *Pappa2* deletion

Next, to compare with results of a previous study (Christians et al., 2019), I assessed the effects of *Pappa2* deletion in AMC mice at all three ages (two months, five months and seven months old), pooling mice collected at wean or 3 weeks later (Table 4). Trabecular bone volume fraction was not significantly affected by genotype ($p = 0.53$) (see Table 4, Fig 8A). Mice of both genotypes showed a higher BV/TV at two months than at the ages of five and seven months ($p < 0.0001$), but there was no significant interaction between genotype and age ($p = 0.20$). Similar results were seen with trabecular thickness, with no significant difference between flox and knockout mice ($p = 0.88$, Table 4). Trabecular thickness was lower at two months than at the ages of five and seven months ($p < 0.0001$), though the interaction between age and genotype was non-significant ($p = 0.45$).

Trabecular spacing was significantly affected by genotype ($p < 0.0001$) and a genotype by age interaction ($p < 0.0001$), with knockout mice showing higher trabecular spacing at seven months of age (Fig. 8B). Trabecular spacing also increased with age ($p < 0.0001$) (Fig. 8B).

Table 4: Effects of* Pappa2 *deletion. *Shown are bone parameters of mice at 2 months (62 mice total, with 31 being collected at wean (16 flox and 15 ko mice) and 31 collected 3 wks post (14 were flox and 17 were ko)), 5 months (25 mice total, with 15 collected at wean (7 mice were flox, 8 mice were ko), and 10 collected 3wks post (3 flox mice and 7 ko mice)) and 7 months of age (14 mice total, with 1 collected at wean (which was flox), and 13 mice collected at 3wks post (6 mice were flox, 7 were ko)), of mice that were not bred. A general linear model including effects of genotype, sex, and the genotype*sex interaction was used. Traits where the effect of genotype is significant are shown in bold. P-values for age reflect the test of variation among all 3 ages, and not pair-wise comparisons between ages.*

	2 months		5 months		7 months		Genotype (flox vs. knockout)	Age (2 months vs. 5 months vs. 7 months)	Genotype* Age
	Flox (mean +/- stderr)	KO (mean +/- stderr)	Flox (mean +/- stderr)	KO (mean +/- stderr)	Flox (mean +/- stderr)	KO (mean +/- stderr)	P	P	P
Trabecular									
BV/TV (%)	6.26 ± 0.32	5.9 ± 0.3	4.41 ± 0.56	4.06 ± 0.46	4.34 ± 0.63	2.24 ± 0.67	0.53	**<0.0001**	0.20

Tb.Th (mm)	0.0297 ± 0.0006	0.0296 ± 0.0005	0.0337 ± 0.001	0.0347 ± 0.0008	0.0353 ± 0.0011	0.0337± 0.0012	0.88	**<0.0001**	0.45
Tb.Sp (mm)	0.51 ± 0.06	0.54 ± 0.06	0.81 ± 0.11	1.06 ± 0.09	0.87 ± 0.12	1.87 ± 0.13	**<0.0001**	**<0.0001**	**<0.0001**
Cortical									
Ct.Ar/Tt.Ar (%)	46.14 ± 0.82	49.97 ± 0.79	47.38 ± 1.50	48.02 ± 1.16	43.60 ± 1.59	52.22 ± 1.83	0.95	**0.0001**	**0.0381**
Ct.Th (mm)	0.188 ± 0.002	0.187± 0.002	0.200± 0.004	0.209± 0.003	0.194± 0.004	0.214 ± 0.005	**<0.0001**	**0.002**	**0.0099**
Ct.Po (%)	5.08± 0.11	4.99 ± 0.11	5.22 ± 0.21	4.65 ± 0.16	5.15 ± 0.24	4.50 ± 0.24	**0.0049**	0.48	0.14

logIGF-1 Conc.	4.05 ± 0.05	3.84 ± 0.04	4.16 ± 0.05	3.80 ± 0.05	4.33 ± 0.09	3.95 ± 0.07	**<0.0001**	**0.0081**	0.20
logIGFBP5 Conc.	4.91 ± 0.03	4.92 ± 0.03	4.75 ± 0.04	4.86 ± 0.05	4.84 ± 0.07	4.77 ± 0.09	0.72	**0.0086**	0.32

Cortical bone area fraction was not significantly affected by genotype ($p = 0.95$), although there was a significant interaction between age and genotype ($p = 0.0381$), with knockout mice having higher cortical BA/TA than flox mice at two and seven months (Fig. 8C, red lines). Age has a significant effect on cortical BA/TA ($p= 0.0001$). (Fig. 8C).

Cortical thickness differed significantly between genotypes ($p < 0.0001$), and there was a significant interaction between genotype and age ($p = 0.0099$), with with knockout mice having a higher cortical thickness at older ages. Age was also a significant factor ($p= 0.002$), as cortical thickness increased with increasing age (Fig. 8D). The logarithmic values of the IGF-1 concentrations show that flox mice in general have a significantly higher IGF-1 concentration than their knockout counterparts ($p <0.0001$, Table 2). IGF-1 concentrations also increased with age ($p = 0.0081$), though the interaction between age and genotype was non-significant ($p = 0.199$). IGFBP-5 concentrations did not differ significantly between genotypes ($p = 0.72$), though they were higher at two months that at older ages ($p = 0.0086$). There was no significant interaction between genotype and age ($p = 0.32$).

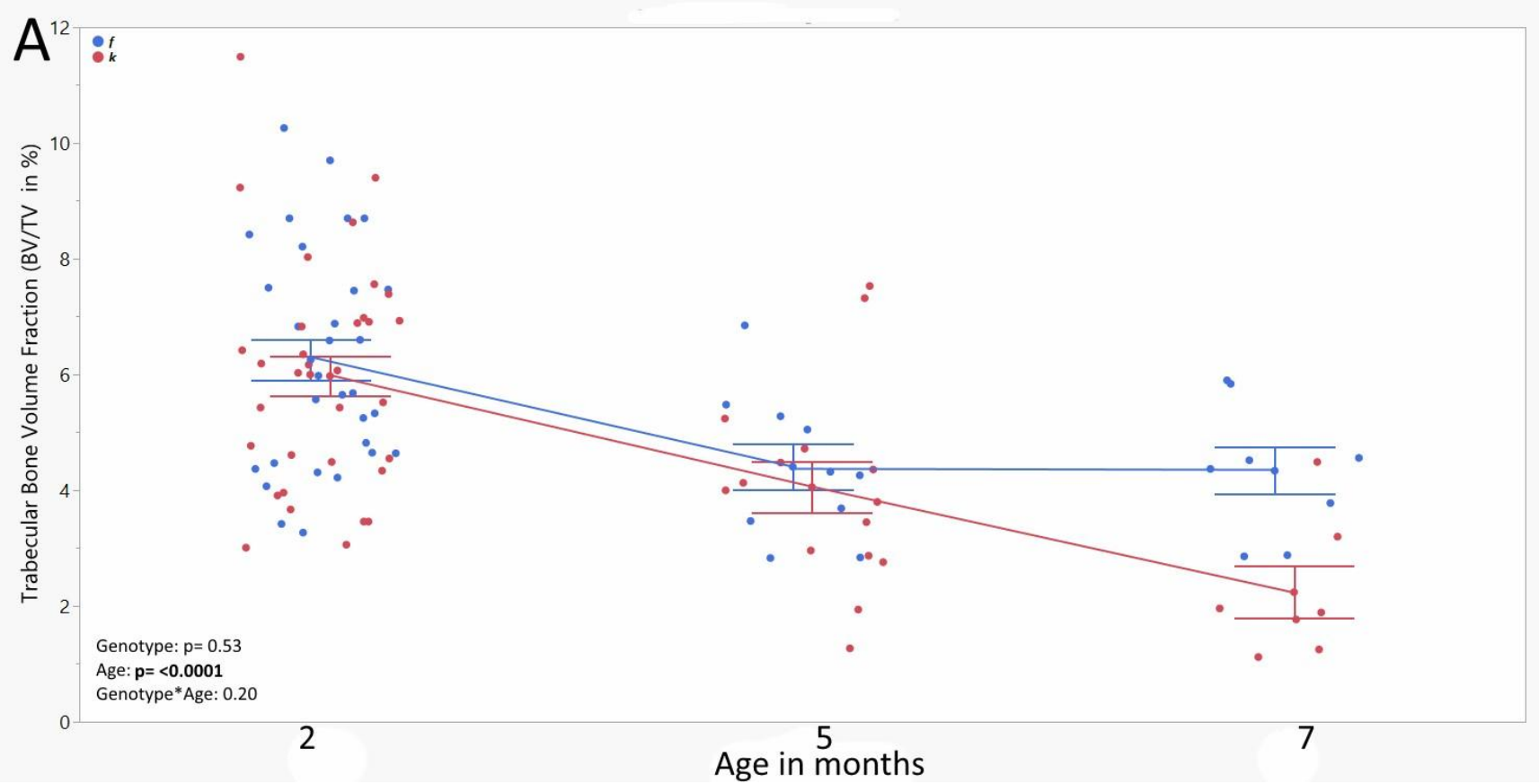

Figure 8A Effects of* Pappa2 *deletion on trabecular bone volume fraction (BV/TV) in %. *Flox mice are marked with a blue color, while knockout mice are marked with red. P-values for age, genotype and age*genotype test whether there is an effect of genotype and age and whether the effect of genotype differs between ages, respectively*

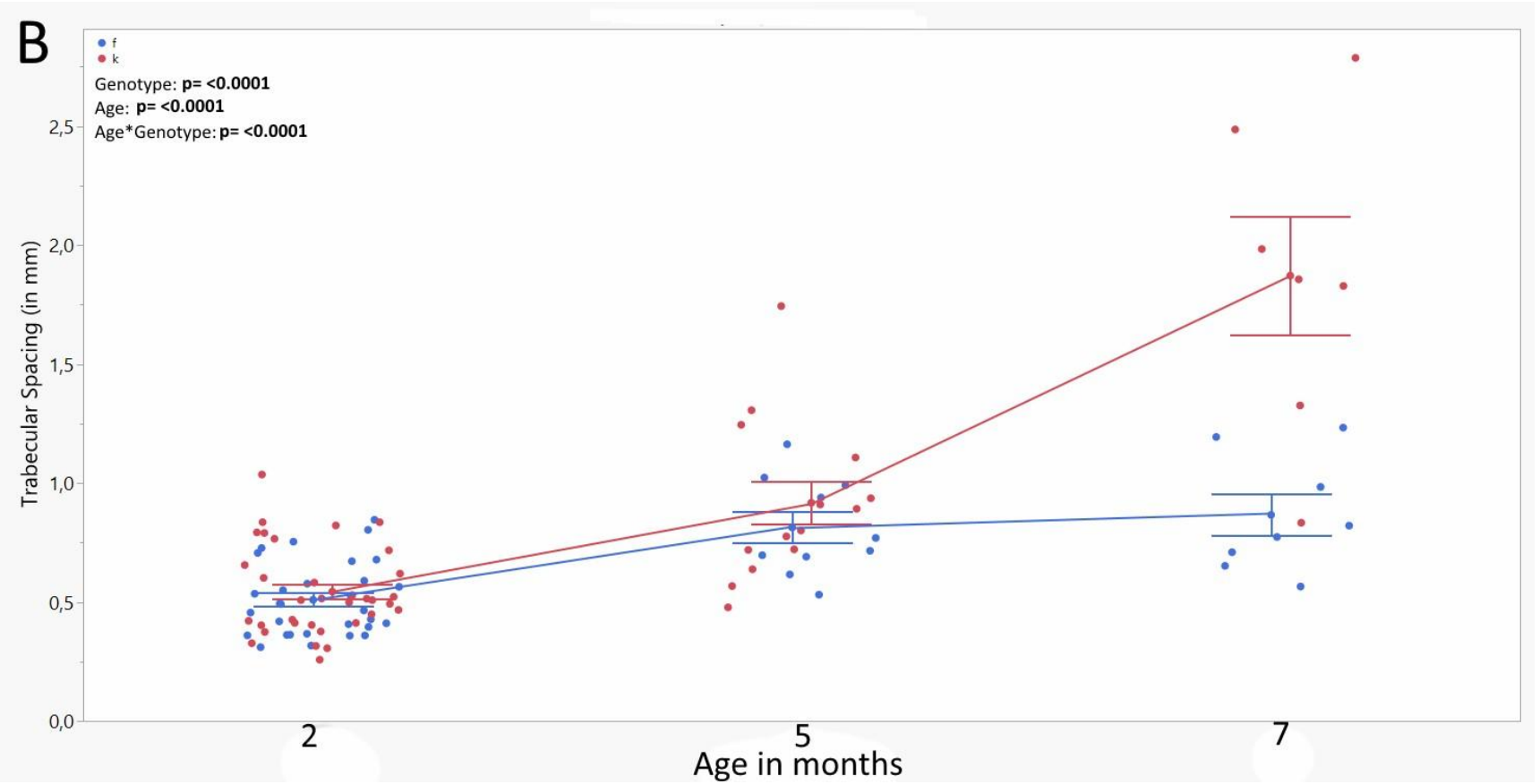

Figure 8B: Effects of Pappa2 deletion on trabecular spacing (in mm). *Flox mice are shown in blue while their knockout counterparts are shown in red, all mice seen here were unbred. P-values for age, genotype and age*genotype test whether there is an effect of genotype and age and whether the effect of state differs between age and genotype, respectively*

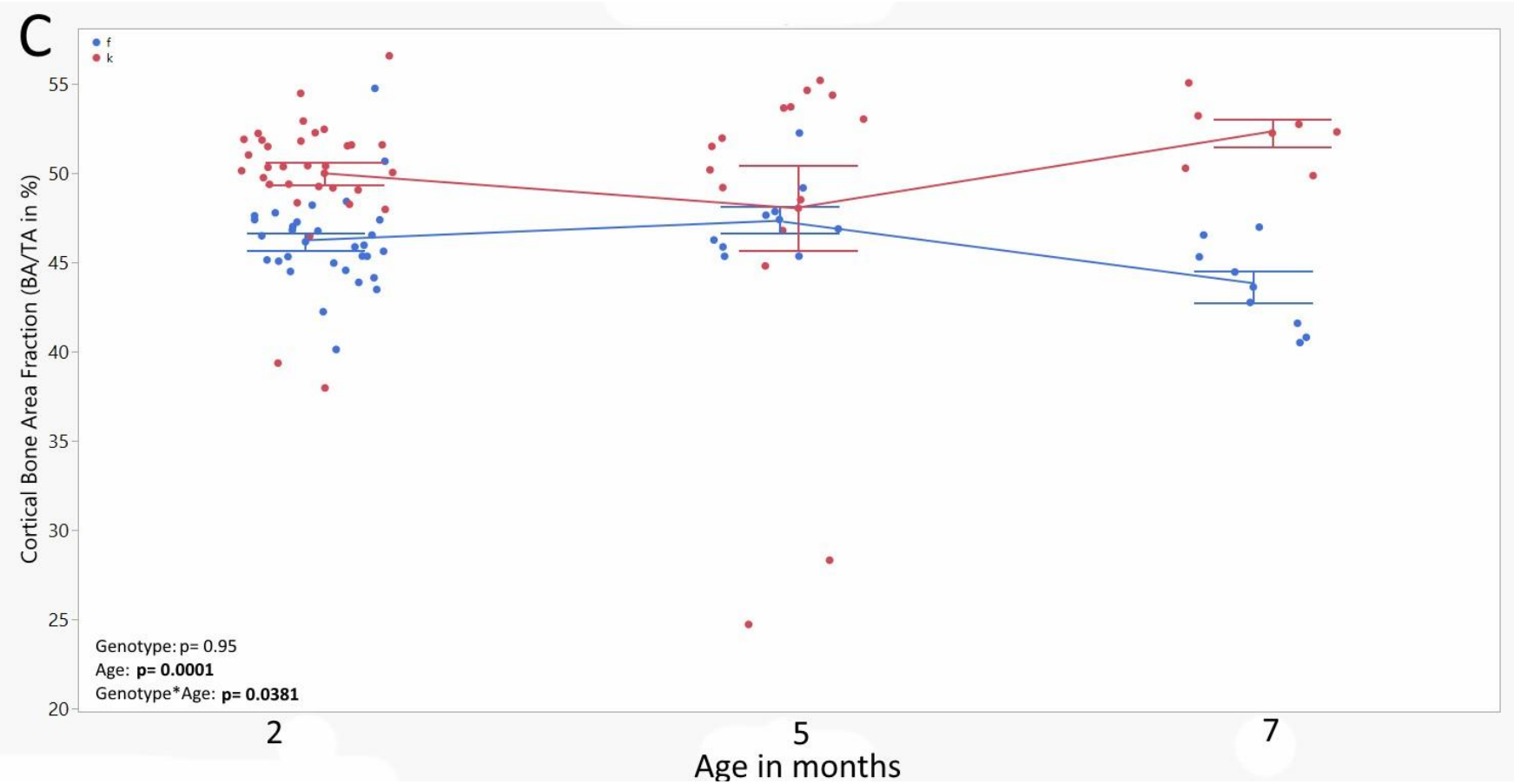

Figure 8C: Effects of* Pappa2 *deletion on cortical bone area fraction (BA/TA) in %. *Flox mice are marked with a blue color, while knockout mice are marked with red, all mice seen here were unbred. P-values for age, genotype and age*genotype test whether there is an effect of genotype and age and whether the effect of state differs between age and genotype, respectively*

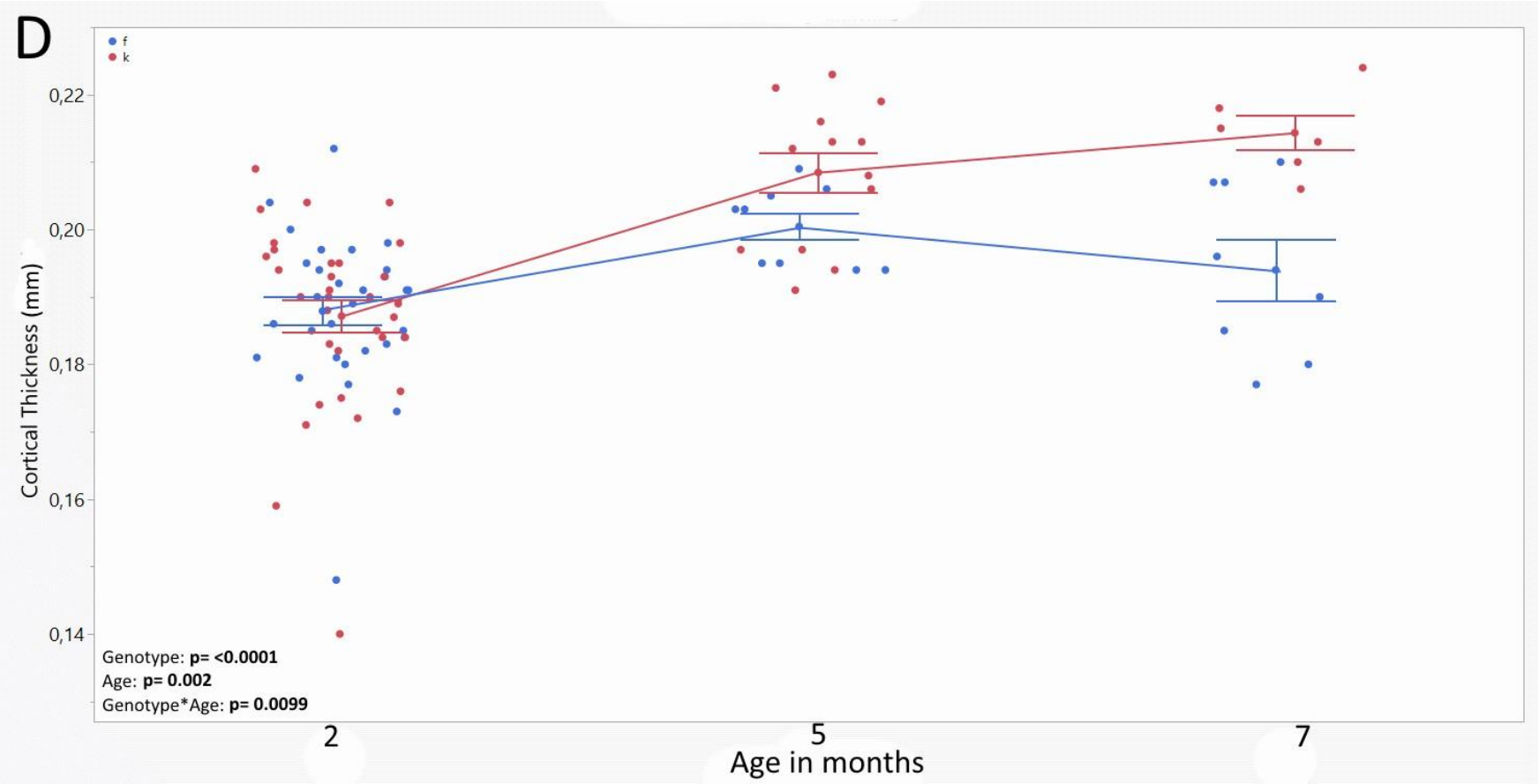

Figure 8D: Effects of* Pappa2 *deletion on cortical thickness (in mm). *Flox mice are marked with a blue color, while knockout mice are marked with red, all mice seen here were unbred. P-values for age, genotype and age*genotype test whether there is an effect of genotype and age and whether the effect of state differs between age and genotype, respectively*

3.4. The effect of age on recovery

To investigate the effect of age on recovery in mice, I examined mice at two, five and seven months of age collected three weeks after weaning (7 month females were not collected at wean) (Fig. 9A-G). Since this analysis focused on females at one time point after weaning, recovery was assessed by the effect of reproductive state (i.e., a difference between bred mice and AMCs, both three weeks after weaning), and effects of age on recovery would be reflected by the reproductive state by age interaction.

At two months, trabecular BV/TV is significantly lower in bred mice than in AMC, suggesting incomplete recovery, whereas there is little difference between bred mice and AMC at five and seven months, although trabecular BV/TV is lower at these ages compared with AMC at two months ($p = 0.0008$) (Fig. 9A).Trabecular BV/TV showed a significant interaction between state and age ($p=0.02$; Fig. 9A), suggesting that the extent of recovery differs between ages. The interaction between reproductive state, age and genotype was non-significant ($p= 0.73$).
Trabecular thickness also showed a significant interaction between state and age ($p= 0.0057$; Fig. 9B), suggesting that the extent of recovery differs between ages. Trabecular thickness is significantly lower in bred mice than in AMC at two months, with a smaller difference at five months and almost no difference at seven months (Fig. 9B). Age showed a significant effect ($p <0.0001$), with trabecular thickness increasing with age.

Cortical bone area fraction did not show a significant interaction between state and age ($p = 0.29$; Fig. 9C), suggesting no effect of age on recovery. Cortical bone area fraction was significantly affected by reproductive state ($p <0.0001$), age ($p=0.002$) and genotype ($p <0.0001$) (Fig. 9C).

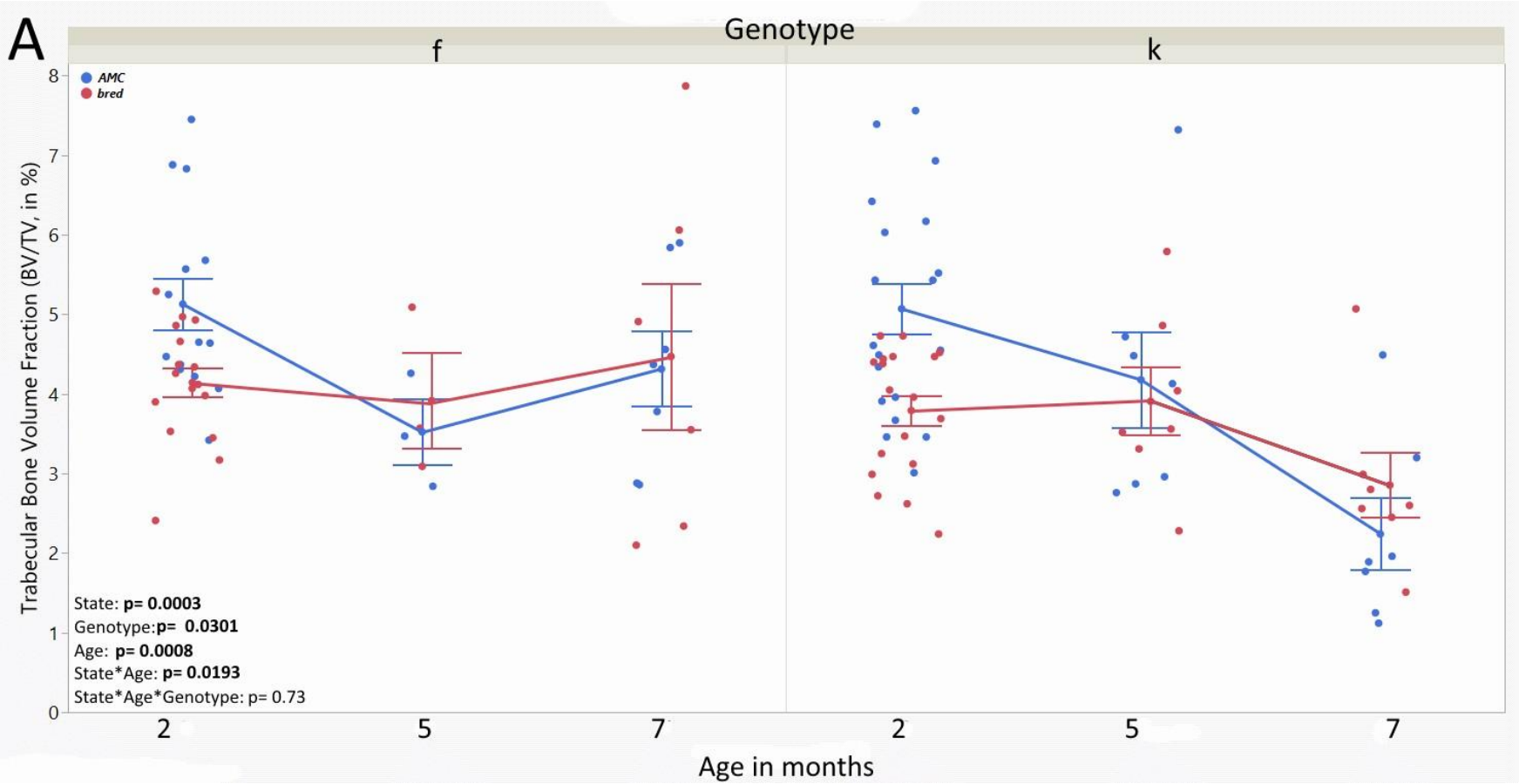

Figure 9A: The effect of age on recovery of trabecular bone volume fraction. *Presented are mice at the ages of 2 months, 5 months and 7 months who have been given a time of three weeks for recovery after lactation (n= 111; 64 mice at the age of 2 months (17 mice were knockouts and AMCs, 17 were knockouts and bred, 14 mice were flox and AMC, 16 mice were flox and bred), 20 mice at the age of 5 months (3 mice were flox and AMCs, 7 mice where knockout and AMCs, 7 mice were knockout and bred, 3 mice were flox and bred), 26 at 7 months old (with 6 mice being flox and AMC, 6 mice being flox and bred, 7 mice being knockout and AMC and 7 mice being knockout and bred). P-values for state, genotype, age, state* age and state*age*genotype test whether there is an effect of state, genotype and age and whether the effect of state differs between ages, and whether the latter effect depends on genotype, respectively.*

Trabecular thickness is significantly affected by the reproductive state of the mice, with flox mice in general showing an increased thickness (blue lines, Fig. 9B). Genotype played no significant role on trabecular thickness, which is visible by the very similar graphs for both genotypes shown ($p= 0.92$, Fig. 9B). Age showed a significant effect ($p <0.0001$), with trabecular thickness increasing with age. There is a form of bone recovery that is dependent on the interaction of state and age ($p= 0.0057$), though the addition of genotype as a factor does not make their interaction significant ($p= 0.90$). AMC mice showed an increase in trabecular thickness, though it plateaus out somewhat at the age of 7 months (Fig.9B, left). Bred mice show a higher recovery than nulliparous mice, and their means even surpass that of their AMCs.

Cortical bone area fraction is significantly affected by reproductive state ($p <0.0001$), age ($p=0.002$) and genotype ($p <0.0001$) (Fig. 9C). However, the effect of breeding didn't significantly differ with age ($p=0.29$) and the interaction between state, age and genotype was non-significant ($p=0.09$). Nulliparous mice show higher cortical BA/TA than bred mice at all measured ages and between different genotypes, though knockout mice have a higher cortical BA/TA than flox mice. Knockout mice at the age of two months have a similar difference between AMC and bred mice as at five months. At seven months, the difference between AMC knockout mice and bred knockout mice is the highest (Fig. 9C, right). In flox mice, at the ages of two and seven months, the difference between bred and AMC mice is similar, but at the age of five months, the difference in cortical BA/TA is the highest (Fig. 9C, left).

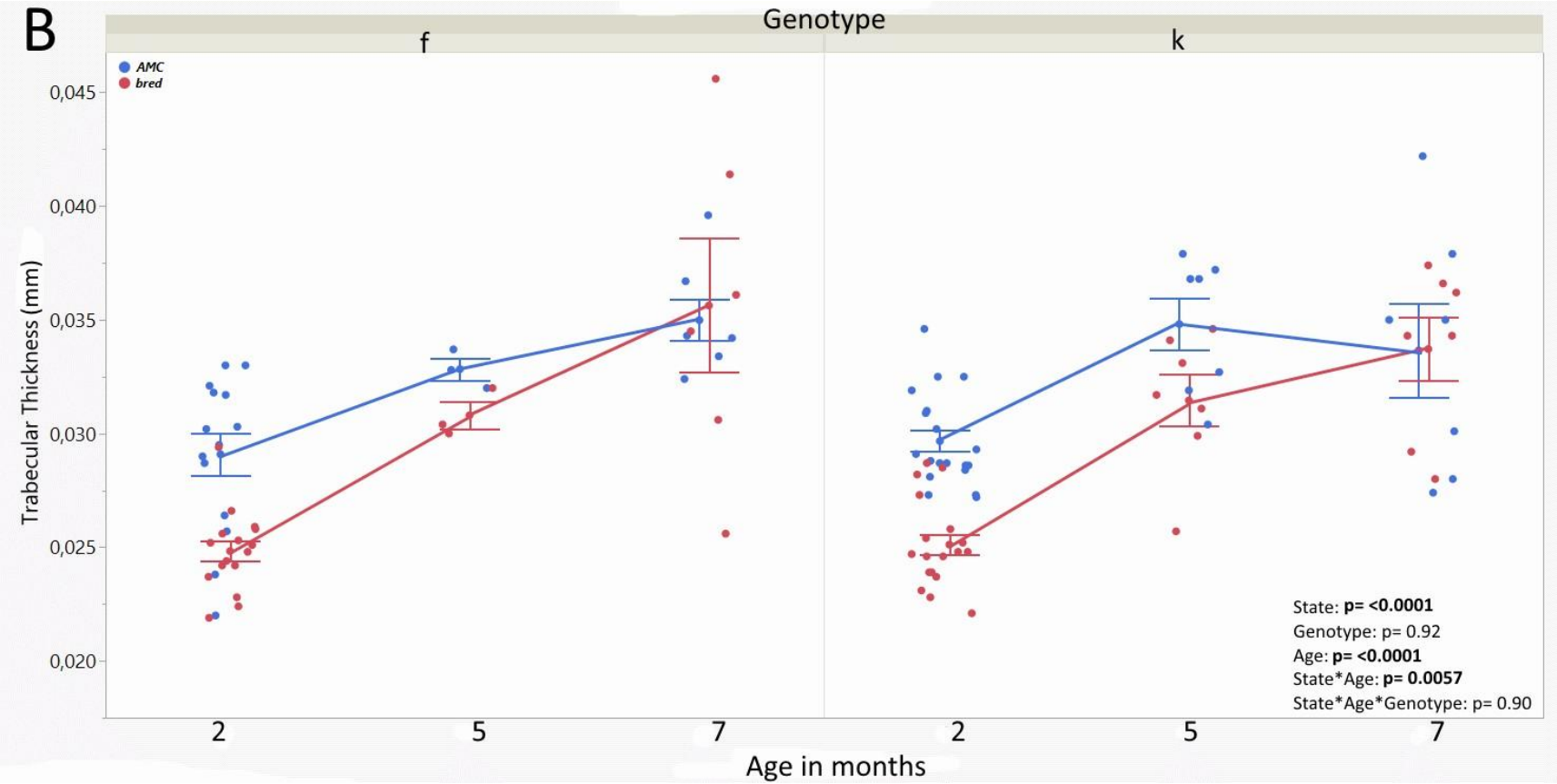

Figure 9B: The effect of age on recovery of trabecular thickness. *Presented are mice at the ages of 2 months, 5 months and 7 months who have been given a time of three weeks for recovery after lactation (n= 111; 64 mice at the age of 2 months (17 mice were knockouts and AMCs, 17 were knockouts and bred, 14 mice were flox and AMC, 16 mice were flox and bred), 20 mice at the age of 5 months (3 mice were flox and AMCs, 7 mice where knockout and AMCs, 7 mice were knockout and bred, 3 mice were flox and bred), 26 at 7 months old (with 6 mice being flox and AMC, 6 mice being flox and bred, 7 mice being knockout and AMC and 7 mice being knockout and bred). P-values for state, genotype, age and state* age and state*age*genotype test whether there is an effect of stategenotype and age and whether the effect of state differs between age and genotype, respectively.*

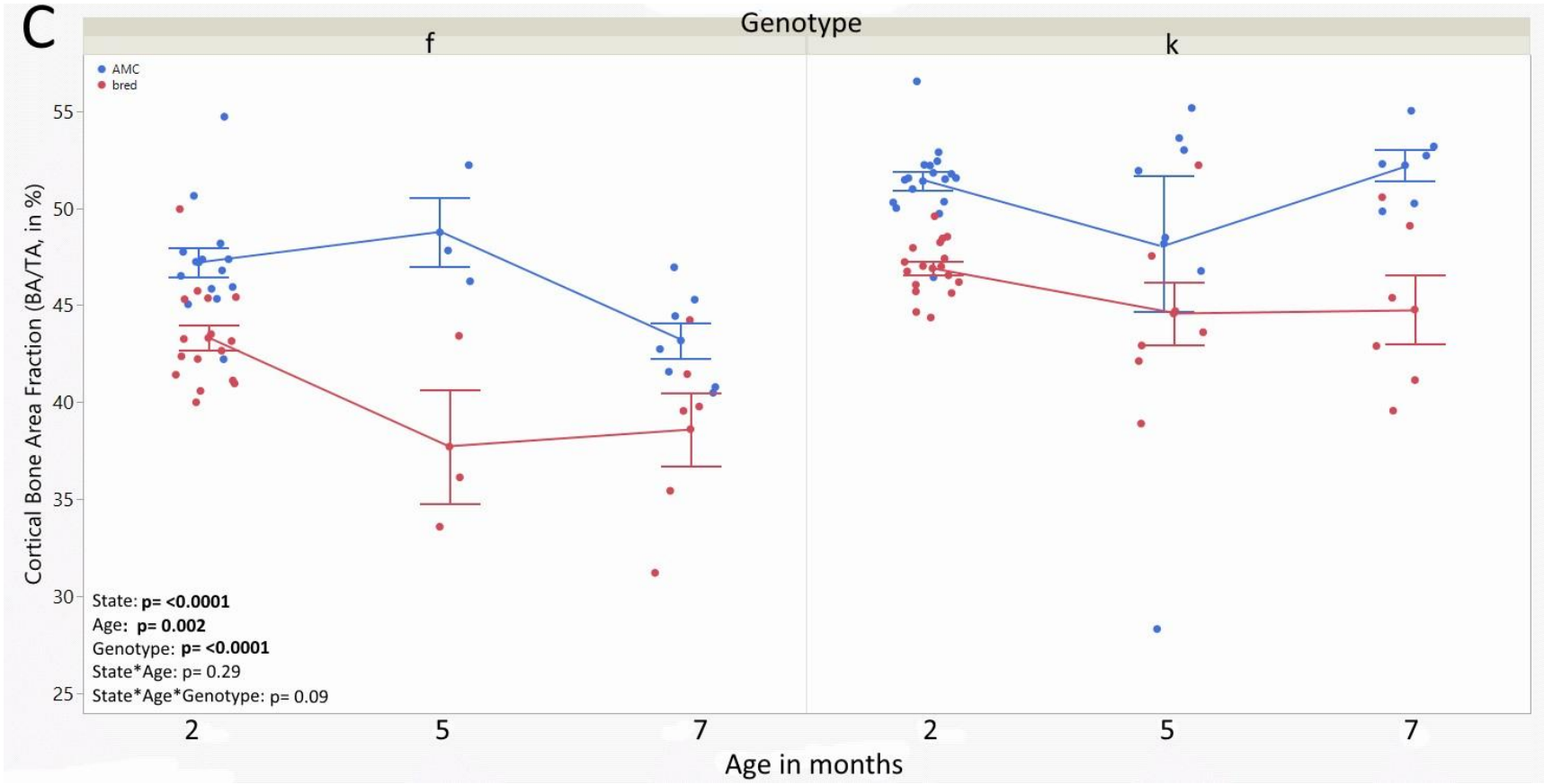

Figure 9C:The effect of age on recovery of cortical bone area fraction. *Presented are mice at the ages of 2 months, 5 months and 7 months who have been given a time of three weeks for recovery after lactation (n= 111; 64 mice at the age of 2 months (17 mice were knockouts and AMCs, 17 were knockouts and bred, 14 mice were flox and AMC, 16 mice were flox and bred), 20 mice at the age of 5 months (3 mice were flox and AMCs, 7 mice where knockout and AMCs, 7 mice were knockout and bred, 3 mice were flox and bred), 26 at 7 months old (with 6 mice being flox and AMC, 6 mice being flox and bred, 7 mice being knockout and AMC and 7 mice being knockout and bred). P-values for state, genotype, age and state* age and state*age*genotype test whether there is an effect of stategenotype and age and whether the effect of state differs between age and genotype, respectively.*

Cortical thickness showed a significant interaction between state and age ($p<0.0001$; Fig. 9D), with a greater difference between bred and AMC mice at 5 months of age. Genotype also had a significant effect ($p=0.0479$), with knockout AMC mice having a higher cortical thickness than flox mice (Fig. 9D).

Cortical porosity showed a significant interaction between state and age ($p = 0.0132$), with no effect of breeding at 2 months, but higher porosity among bred mice at 5 and 7 months (Fig. 9E). Between the two genotypes measured, knockout mice showed lower cortical porosity than flox mice ($p = 0.021$). Age had no significant effect on cortical porosity ($p = 0.09$). IGF-1 concentrations showed no interaction between state and age ($p = 0.22$; Fig. 9F), but increased with age ($p <0.0001$), and were lower in knockout mice ($p <0.0001$). For IGFBP-5 concentrations, the interaction between state and age was non-significant ($p = 0.72$; Fig. 9G), but IGFBP-5 decreases with age ($p = 0.0124$).

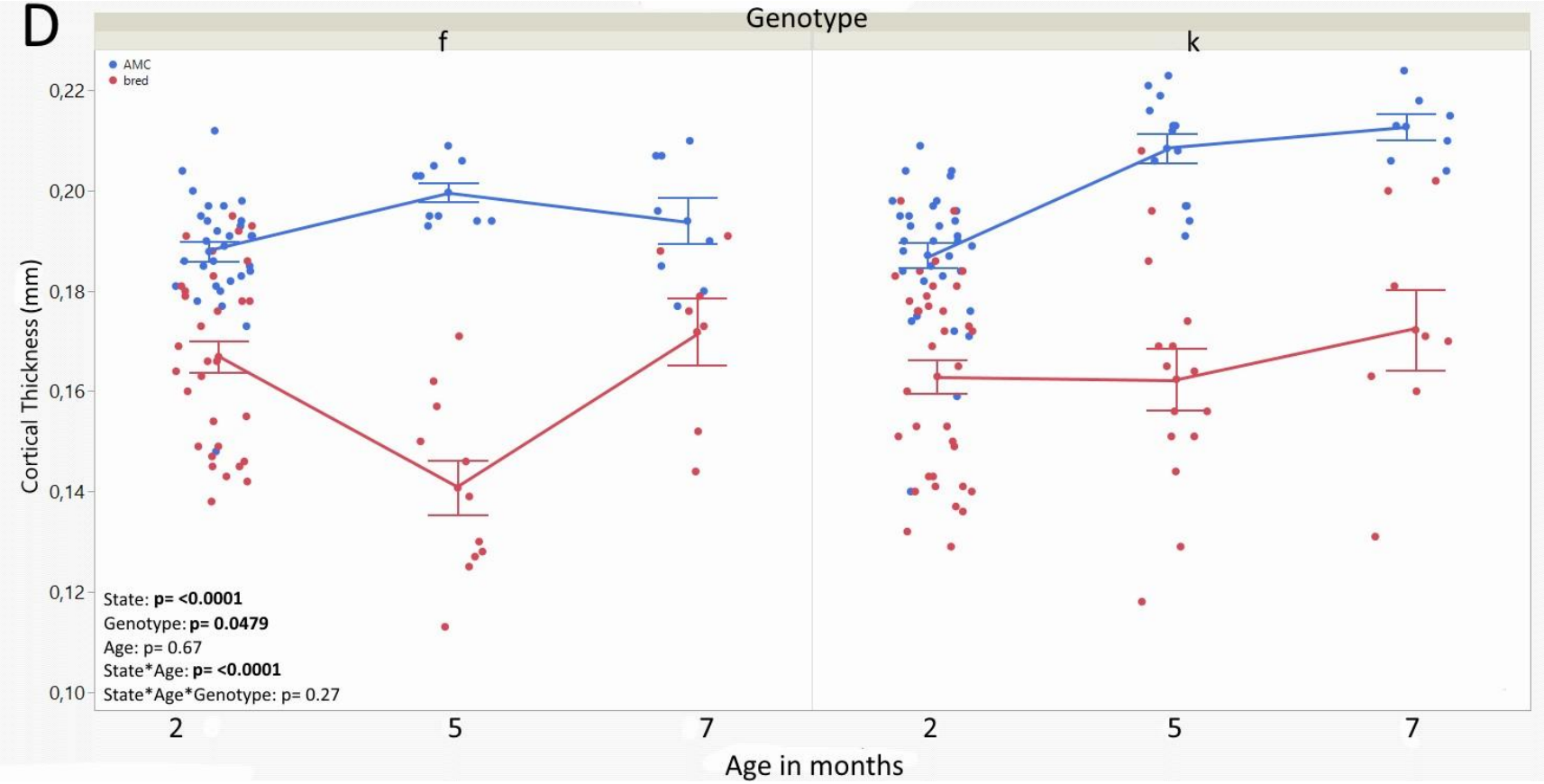

Figure 9D:The effect of age on recovery of cortical thickness. *Presented are mice at the ages of 2 months, 5 months and 7 months who have been given a time of three weeks for recovery after lactation (n= 111; 64 mice at the age of 2 months (17 mice were knockouts and AMCs, 17 were knockouts and bred, 14 mice were flox and AMC, 16 mice were flox and bred), 20 mice at the age of 5 months (3 mice were flox and AMCs, 7 mice where knockout and AMCs, 7 mice were knockout and bred, 3 mice were flox and bred), 26 at 7 months old (with 6 mice being flox and AMC, 6 mice being flox and bred, 7 mice being knockout and AMC and 7 mice being knockout and bred). P-values for state, genotype, age and state* age and state*age*genotype test whether there is an effect of stategenotype and age and whether the effect of state differs between age and genotype, respectively.*

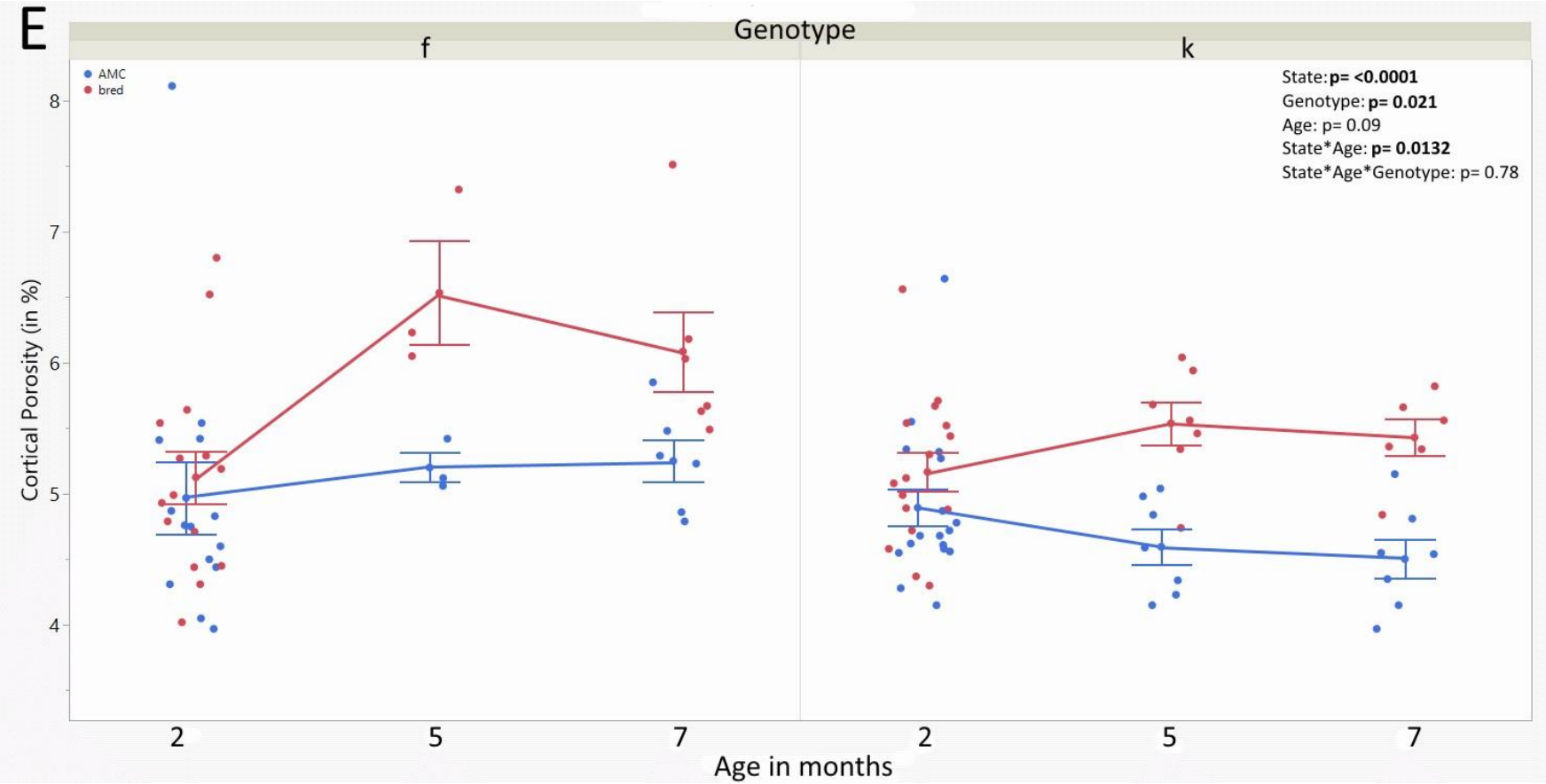

Figure 9E:The effect of age on recovery of cortical porosity. *Presented are mice at the ages of 2 months, 5 months and 7 months who have been given a time of three weeks for recovery after lactation (n= 111; 64 mice at the age of 2 months (17 mice were knockouts and AMCs, 17 were knockouts and bred, 14 mice were flox and AMC, 16 mice were flox and bred), 20 mice at the age of 5 months (3 mice were flox and AMCs, 7 mice where knockout and AMCs, 7 mice were knockout and bred, 3 mice were flox and bred), 26 at 7 months old (with 6 mice being flox and AMC, 6 mice being flox and bred, 7 mice being knockout and AMC and 7 mice being knockout and bred). P-values for state, genotype, age and state* age and state*age*genotype test whether there is an effect of stategenotype and age and whether the effect of state differs between age and genotype, respectively.*

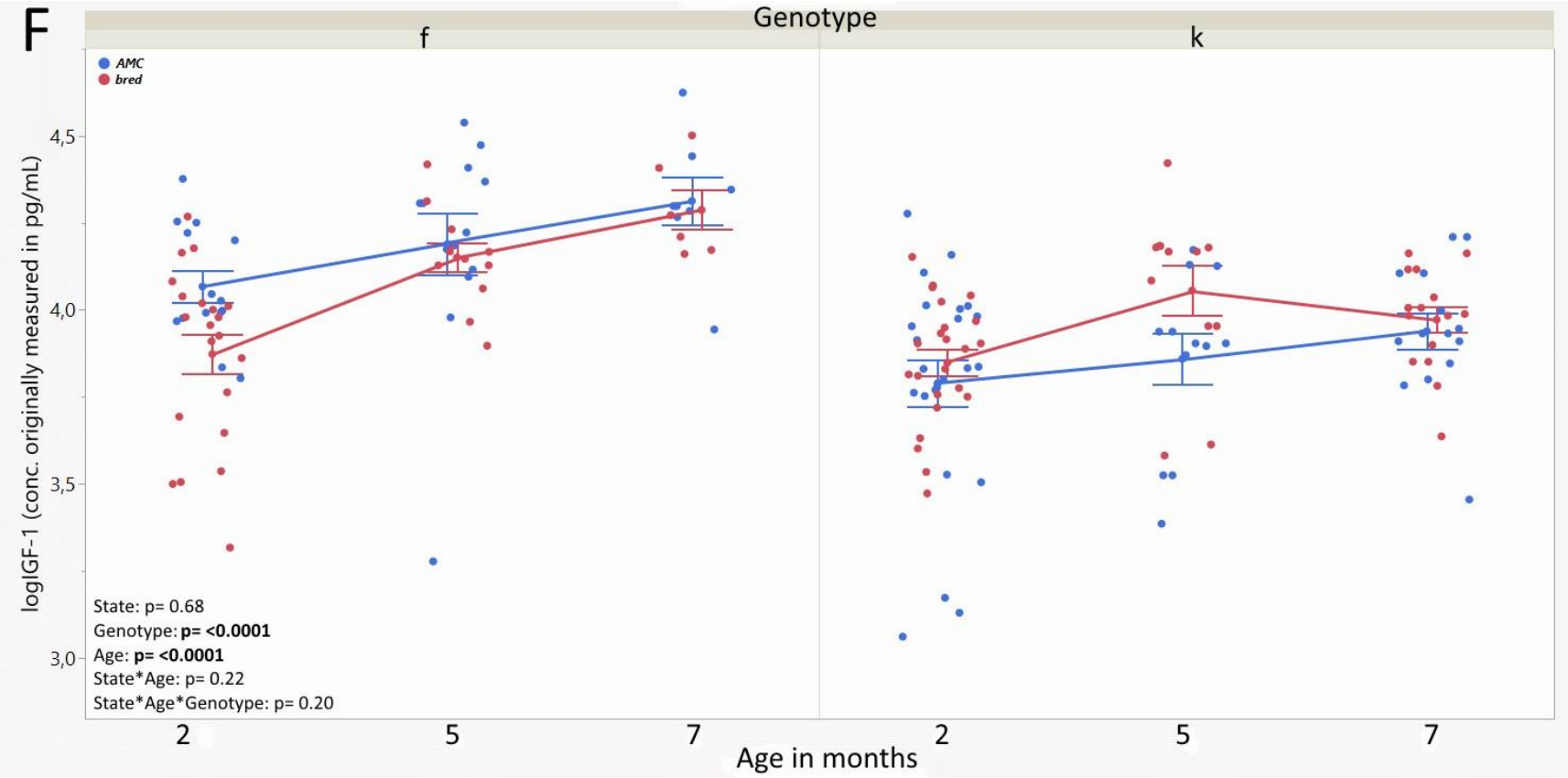

Figure 9F: The effect of age on recovery of serum logIGF-1 concentrations. *Presented are mice at the ages of 2 months, 5 months and 7 months who have been given a time of three weeks for recovery after lactation (n= 111; 64 mice at the age of 2 months (17 mice were knockouts and AMCs, 17 were knockouts and bred, 14 mice were flox and AMC, 16 mice were flox and bred), 20 mice at the age of 5 months (3 mice were flox and AMCs, 7 mice where knockout and AMCs, 7 mice were knockout and bred, 3 mice were flox and bred), 26 at 7 months old (with 6 mice being flox and AMC, 6 mice being flox and bred, 7 mice being knockout and AMC and 7 mice being knockout and bred).). P-values for state, genotype, age and state* age and state*age*genotype test whether there is an effect of stategenotype and age and whether the effect of state differs between age and genotype, respectively*

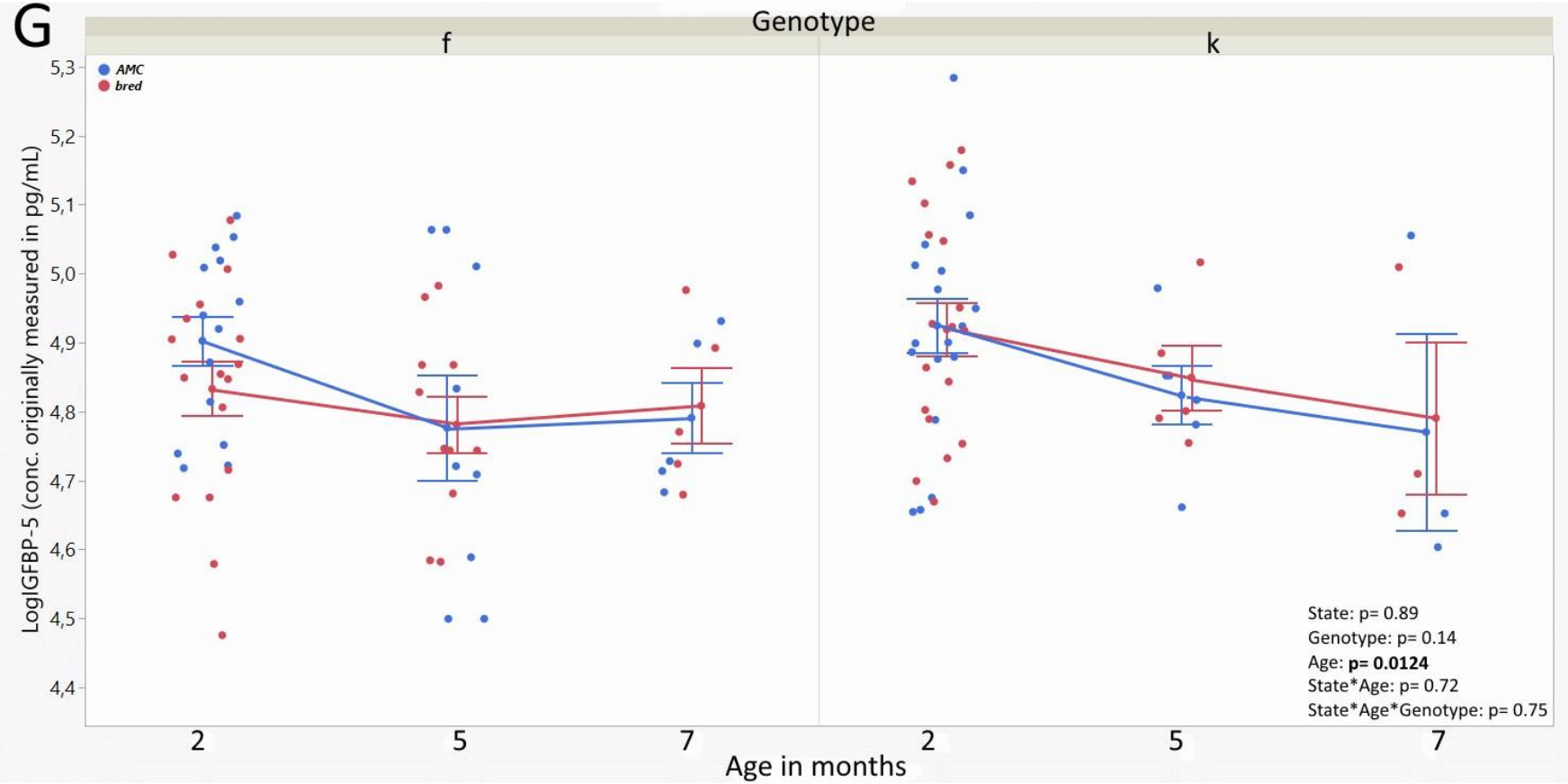

Figure 9G:The effect of age on recovery of serum logIGFBP-5. *Presented are mice at the ages of 2 months, 5 months and 7 months who have been given a time of three weeks for recovery after lactation (n= 111; 64 mice at the age of 2 months (17 mice were knockouts and AMCs, 17 were knockouts and bred, 14 mice were flox and AMC, 16 mice were flox and bred), 20 mice at the age of 5 months (3 mice were flox and AMCs, 7 mice where knockout and AMCs, 7 mice were knockout and bred, 3 mice were flox and bred), 26 at 7 months old (with 6 mice being flox and AMC, 6 mice being flox and bred, 7 mice being knockout and AMC and 7 mice being knockout and bred).P-values for state, genotype, age and state* age and state*age*genotype test whether there is an effect of stategenotype and age and whether the effect of state differs between age and genotype, respectively.*

3.5. How does lactation, independent of pregnancy, affect bone?

To investigate whether effects of reproductive state were due to lactation or pregnancy, I examined females who gave birth to a litter but did not lactate because their litter did not survive longer than 1 day. These females were collected 3 weeks after the birth of their litter (i.e., the time when pups would have been weaned) and each female that lost a litter was matched with 2 bred females and 2 AMC females of the same age and genotype collected at wean in the same cohort (Table 5). There were therefore three different groups: Successfully bred mice, their AMCs, and bred mice of the same age who had lost their litters right after birth, so that no lactation has taken place.

Trabecular bone volume fraction differed significantly between groups, although post hoc tests (between pairs of groups) did not reveal any pair-wise differences (Table 5). Mice that lost their litters had the lowest trabecular fraction and AMCs had the highest. Trabecular spacing also differed between groups, with mice that lactated having significantly lower spacing than mice that lost their litter (Table 5). Cortical bone area fraction as well as thickness were significantly different between the groups (Table 5), with mice that lactated having significantly lower values than AMC and mice that lost their litters, reflecting an effect of lactation rather than pregnancy.

Table 5:The effect of lactation on bone independent of pregnancy. *Presented are bred mice at 2 and 5 months of age that were collected at wean or who lost their litters (n=33, 19 of these were 2 months old (1 mouse flox + AMC; 2 mice flox and successfully bred; 1 mouse flox and lost litter; 6 mice were ko + AMC, 6 mice were ko and successfully bred, 3 mice were flox and lost their litter) and 14 mice were 5 months old (3 mice were flox + AMC, 4 mice flox and successfully bred, 2 mice were flox and lost their litter; 2 mice were ko + AMC, 2 mice were ko and successfully bred and 1 mouse was ko and lost their litter)). P-values for group reflect the test of variation among all 3 groups. Pair-wise tests between groups were performed using Tukey HSD analysis; means sharing a superscript letter are not significantly different.*

	AMC	Bred but lost litter	Bred and lactated	Groups
	Mean + Std.Err	Mean + Std.Err	Mean + Std.Err	P
Trabecular				
BV/TV (%)	0.066 ± 0.005^{A}	0.041 ± 0.006^{A}	0.055 ± 0.005^{A}	**0.0245**
Tb.Th (mm)	0.032 ± 0.001^{A}	0.029 ± 0.002^{A}	0.029 ± 0.001^{A}	0.209
Tb.Sp (mm)	$0.570 \pm 0.052^{A,B}$	0.761 ± 0.059^{A}	0.541 ± 0.043^{B}	**0.0161**
Cortical				
Ct.Ar/Tt.Ar (%)	0.493 ± 0.010^{A}	0.485 ± 0.012^{A}	0.360 ± 0.009^{B}	**<0.0001**
Ct.Th (mm)	0.194 ± 0.004^{A}	0.195 ± 0.005^{A}	0.138 ± 0.004^{B}	**<0.0001**

Ct.Po (%)	0.049 ± 0.025^{A}	0.111 ± 0.029^{A}	0.080 ± 0.021^{A}	0.288
logIGF-1 Conc.	3.955 ± 0.089^{A}	4.156 ± 0.167^{A}	4.061 ± 0.061^{A}	0.486
logIGFBP5 Conc.	4.913 ± 0.063^{A}	5.015 ± 0.126^{A}	4.873 ± 0.061^{A}	0.599

Chapter 4. **Discussion**

4.1. Traits affected by breeding

Lactation can lead to dramatic changes in bone microarchitecture, with women losing up to 7% of their bone mass within the first 6 months of lactation, and mice losing between 20 to 30% of bone mass during lactation due to the increased number of offspring (Liu, Ardeshirpour, Vanhouten, Shane, & Wysolmerski, 2012). First, I sought to identify traits affected by breeding and lactation, including changes in the microarchitecture of the cortical and trabecular portion of the femurs, as well as the serum concentrations of IGF-1 and IGFBP-5. To facilitate comparison with other literature, I first analysed 2 month old flox-mice, which are comparable with young, wild-type mice. Bred mice showed a lower trabecular BV/TV and trabecular thickness than their non-bred sisters, but showed no signs of recovery after three weeks. While we observed significant differences in mice when comparing between their reproductive state (bred vs. non-bred), and their recovery time (at wean vs. three weeks after), we found no interaction between both reproductive state and recovery time for trabecular bone values, i.e., the effect of breeding on trabecular bone did not diminish after 3 weeks. This reduction in femoral trabecular BV/TV for 2 month old mice has also been found by Bornstein et al. (2014), who reported that total femoral BV/TV was lower in lactating mice versus non-bred sisters no matter if collected seven days into lactation, at wean or three weeks later. Seven days into lactation, they found that trabecular number had already decreased for bred mice, and noted an increase in trabecular spacing as well as thinner trabeculae (Bornstein et al., 2014). In both my results and those of Bornstein, trabecular bone was reduced by lactation and had not recovered after three weeks. The study of Liu et al. used different mice (CD-1 mice instead of C57BL/6 mice), with the addition of two more skeletal sites (tibia and L3 vertebra). Mice were bred at the age of 10-13 weeks, had nulliparous controls, and were collected either at day 12 of lactation, or 28 days after lactation. This study found a similar negative effect of breeding on

trabecular femoral bone. Lactating mice showed compromised bone architecture and lowered trabecular BV/TV than in nulliparous controls. At the distal femur, bred mice showed a decrease of 29% in rod bone volume fraction, but no difference in trabecular number. Trabecular thickness was also reduced (Liu et al.2012). The only parameter that recovered after 28 days was femoral trabecular thickness, which was comparable to nulliparous mice.

In mice bred at the age of two months, I found that most cortical bone parameters (such as cortical area, Ct.Ar/Tt.Ar and cortical thickness) were lower in bred mice than in unbred controls. One trait that did not show recovery was cortical porosity, which was higher in bred mice collected at wean, but not three weeks later (Bornstein et al., 2014). However, some recovery took place over the span of three weeks in cortical BA/TA and cortical thickness. Similarly, in Bornstein et al.'s study, cortical thickness was significantly lower in bred mice that were collected at wean, but not in mice collected three weeks later. Cortical area fraction was significantly lower in bred mice but showed no recovery. Similarly, Liu et al. showed that cortical thickness was lower in lactating mice than in nulliparous controls. Recovered mice never quite reached the level of nulliparous mice in terms of cortical thickness, but had higher cortical thickness than mice collected at wean (Liu, Ardeshirpour, Vanhouten, et al., 2012). Cortical bone area and cortical thickness were decreased by about 30% in lactating mice, while cortical porosity was 36% higher in lactating than in nulliparous mice, but following 4 weeks of recovery, cortical area and thickness were 18% lower and cortical porosity 16% higher than in nulliparous mice (Liu, Ardeshirpour, Vanhouten, et al., 2012). This is consistent with my results; even though some recovery has taken place after three weeks, the bred mice did not reach the same level as the AMCs.

IGF-1 concentrations are expected to be lower in *Pappa2* knockout mice than in flox mice, because more IGF-1 will be bound to IGFBP-5 (as it was not cleaved by PAPP-A2). An increase in IGF-1 is hypothesized to contribute to the recovery of BMD after lactation (Bornstein et al., 2014). I found that bred mice at the age

of 2 months have lower total IGF-1 serum concentrations than their AMC. Bornstein found that bred mice at the seventh day of lactation have a lowered IGF-1 concentration than their 12 week old controls (Bornstein et al., 2014). In my study, mice given a recovery time of three weeks did not show a significant change in their total IGF-1 concentrations. In contrast, Bornstein et al. showed that IGF-1 concentrations at wean were lower (445.9 ng/mL) than 3 weeks after wean (646.2 ng/mL) (Bornstein et al., 2014). In human subjects, it has been found that longer lactation time reduces IGF-1 concentrations, with women that lactated for more than four months having markedly reduced levels (Møller et al., 2013) that increase once lactation ceases.

IGFBP-5 needs to be cleaved in order to make IGF-1 biologically available (Duan & Allard, 2020) for the communication between osteoclasts and osteoblasts. Though there is no significant effect of reproductive state, the timepoint of collection and their interaction, a trend can still be seen whereby nulliparous mice have a higher IGFBP5-concentration than bred mice, while mice collected at wean don't show a significantly different concentration than those collected after 3 weeks. The non-significant results here may indicate that other IGFBPs are involved to a greater degree in bone remodelling after lactation, or that IGFBPs in general aren't important for the recovery of bone after lactation. IGFBP-3 is another binding protein that can bind IGF-1 and is also prominently found in bones. IGFBP-3 is a major actor in the activation of osteoblasts and thus bone formation (Locatelli & Bianchi, 2014).

4.2. The effects of genotype on recovery after lactation

One of my goals was to test whether PAPP-A2 is involved in the process of bone recovery, as its main role is the cleaving of IGFBP-5 in order to make IGF-1 available. Given that IGF-1 is important in the communication between osteoblasts and osteoclasts, PAPP-A2 could be involved in post-lactation bone

remodeling. I found that trabecular bone (especially in BV/TV and trabecular thickness) showed an effect of breeding, but no recovery. In contrast, cortical bone (CtAr/TtAr and cortical thickness) showed an effect of breeding as well as partial recovery, which is why I focused on mainly these four traits in the analyses of genotype.

Overall, the lack of interactions between genotype, collection date and reproductive state indicates that the deletion of *Pappa2* did not affect recovery in trabecular bones, although trabecular bones didn't show any recovery in the wild type. As in the analyses of flox mice only, AMCs in general had a higher trabecular BMD than their bred counterparts, and mice that had been collected 3 weeks after weaning had a trend toward lowered trabecular thickness than mice that were collected at wean, thus indicating that mice do lose some trabecular BMD due to breeding and are unable to recover to the level of AMCs. This is consistent with previous findings that trabecular bone is the site most affected by bone resorption, although this isn't specific to breeding (Glatt et al., 2007).

While no significant effects were observed for IGFBP-5, the serum concentration of IGF-1 was significantly affected by both collection date and genotype. I expected that our *Pappa2* knockout mice would have a lowered overall concentration of IGF-1. Our results confirm this, as knockout mice indeed show an overall lower concentration of IGF-1 than flox mice. Furthermore, mice collected 3 weeks after lactation had a lower concentration of IGF-1 than at wean. This is the opposite to what was observed by Bornstein, who noted that IGF-1 concentrations were lower at wean that in mice collected 3 weeks after wean (Bornstein et al., 2014). During lactation, bone resorption is very active, which means an increased activity of osteoclasts over osteoblasts. This necessitates an increase in osteoclastogenesis, which is regulated by IGF-1 by promoting the differentiation of osteoclasts (Wang et al., 2006). Thus it is expected that mice collected at wean would show a higher concentration of IGF-1 due to the increased bone resorption necessary for lactation.

4.3. The effects of age on recovery after lactation

There have been few studies of whether age has any effect on recovery after lactation, even though this is important in the light of the increasing age at which first reproduction and lactation occurs, with more women increasingly having children at older ages (Stein & Susser, 2000). Lactation is the cause of fundamental changes in bone microarchitecture, and I hypothesized that the ability to recover from these changes would be affected by age. 90% of peak BMD in human women is reached by the age of 18 years (National Institutes of Health, 2015), and this maximum BMD is retained until the age of 50. However, during their lifetime, women will lose a substantial amount of bone by their late thirties or early forties, when many of them will lactate for the first time due to childbirth.

Earlier studies (Bornstein et al., 2014; Liu, Ardeshirpour, VanHouten, et al., 2012) looked at the effects of lactation on bone microarchitecture, but only measured mice around the age of 8 weeks old (in the case of Bornstein) and 10-13 weeks old (in the case of Liu). I therefore looked at the effects of age on lactating mice by comparing three different age groups, breeding them at the ages of two months, five months and seven months. In mice, peak BMD is achieved at an age of 4 months (Beamer et al., 1996), though trabecular bone peaks at an age of 2 months and declines further with age (Glatt et al., 2007). This means that mice bred at an age of 2 months are young breeders who haven't achieved their peak BMD. In humans, 90% of peak BMD is reached at an age of approximately 18 years, while BMD starts to decline around the age of 40 (Berger et al., 2008). Mice bred at 5 months have reached their peak BMD, though show some loss in their trabecular bones, while 7-month-old mice show a further decline in BMD and are reaching the end of their reproductive capabilities. Thus, two month old mice are comparable to young teenage mothers, 5 month olds to women in their mid-twenties, and 7 month old mice are comparable to women over the age of 35.

Mice grow with age and this can lead to an increase in cortical thickness (Fig. 8D). Trabecular spacing was the lowest for two month old mice, with bred mice having a slightly higher trabecular spacing than their AMCs. At five months, the trabecular spacing increased for both groups, with bred mice having increased trabecular spacing than AMCs. Trabecular BMD in AMC mice is higher than in bred mice at 2 months, but not at 5 or 7 months, suggesting either improved recovery at older ages, or that trabecular spacing has been reduced by age in such a manner that it cannot be further reduced by the effects of lactation. Trabecular bone peaks at an early age, sometime before the age of 2 months, after which is steadily declines. This age-related decline in trabecular BV/TV has been shown to be caused by a decrease in trabecular number and an increase in trabecular thickness, found especially in long bones such as the femur (Halloran et al., 2002). In general, trabecular bone deteriorates faster within the femur than in bones of the vertebral body in mice, which makes the femur a good target if one wishes to see more marked changes, especially in female mice, which show greater trabecular decline than males (Glatt et al., 2007).

Cortical bone area fraction was consistently lower in bred mice than their AMCs at all ages, with no effect of age on recovery. In contrast, cortical thickness showed impaired recovery at 5 months compared with 2 and 7 months.

I found that a recovery time of three weeks was not sufficient for bred mice to reach the levels of trabecular and cortical BMD seen in their never-bred sisters. In general, it has been found that time of approximately 2 to 4 weeks is required by bred mice to acheive a full recovery in bone volumes and mineral content (Bornstein et al., 2014; Liu, Ardeshirpour, VanHouten, et al., 2012). My results indicate that at the age of 2 months, 3 weeks is not sufficient for recovery.

In the literature, IGF-1 concentrations are often studied in relation to longevity and fitness, and it has been noted that the reduction of IGF-1 leads to an increased lifespan in yeast, worms and flies (Altintas, Park, & Lee, 2016). In humans, a gradual fall in plasma IGF-1 concentrations occurs with age, which is

known as somatopause (Lombardi et al., 2005). In rodents, such as mice, serum IGF-1 levels have been shown to be high throughout their life, with a forced, late-life reduction of serum IGF-1 levels leading to increased liver inflammation and accelerated bone loss (Gong et al., 2014). My findings showed that IGF-1 concentrations increased with age, both for bred mice and their AMCs, with *Pappa2* knockout mice consistently showing lower IGF-1 levels than controls, which is similar to studies stating that mice do not experience somatopause and have high IGF-1 levels throughout their lives (Gong et al., 2014). In one longevity study, mice with a reduced level of IGF-1 were created, and showed a significant increase in maximum lifespan (Lorenzini et al., 2014). There is currently no study on whether *Pappa2* knockout mice would show a similar increase in maximum lifespan connected to their lowered total IGF-1 concentrations.

4.4. The effect of lactation on bone independent of pregnancy

Changes in the skeleton of bred mice compared with AMC could be due to pregnancy and/or lactation. To distinguish between these possibilities, I compared three different groups of mice: (a) AMC mice that were never pregnant and that never lactated, (b) bred mice that lost their litters soon after birth and so were only pregnant but never lactated, and (c) mice that successfully bred and lactated for a period of 21 days. These groups spanned mice from the ages of 2 months and 5 months (Table 5).

Mice who lost their litters have the lowest trabecular bone volume fraction and highest spacing, which may have contributed to the loss of their litters, e.g., because they were unable to produce sufficient milk. Unnoticed sickness, aberrant behavior and less physical activity could have been contributing factors that led to the loss of pregnancy in these cases.

Cortical bone area fraction and cortical thickness of the lost litter group are comparable to the AMCs, whereas mice that lactated have lower values. This confirms that changes in cortical bones are attributable to the effects of lactation rather than pregnancy.

4.5. Conclusions and Future Research

Previous work (Bornstein et al. 2014; Liu et al. 2012) has shown that cortical bones are negatively affected by lactation, and require approximately three weeks to recover. The hypothesis that PAPP-A2 affects the process of bone recovery after lactation was not supported, as my results show that regardless of genotype, bred mice in general have a lower cortical area fraction compared to their AMCs after a recovery time of three weeks. Consistent with similar experiments (Liu, Ardeshirpour, VanHouten, et al., 2012), trabecular bone doesn't recover, but steadily declines after the age of 2 months. Wild-type mice have a higher concentration of serum IGF-1 than the PAPP-A2 knockout mice, as was expected due to the normal function of PAPP-A2 as the cleaving protein of IGFBP-5 (Cheryl A. Conover et al., 2011). Overall, IGF-1 concentrations showed an increase with age. However, no changes in IGFBP-5 were found, which does not support a role in the recovery of bone after lactation despite the abundance of IGFBP-5 in bone.

This study was the first that took age into account when looking at bone recovery after lactation. One limitation however was fewer mice were collected at 7 months, as at this age, they are more prone to reproductive failure due to their reproductive senescence. It might still be worthwhile to try and adjust the experimental settings so that more breeders at the age of 7 months successfully lactate. This could be achieved by giving mice that had just given birth, but had deceased pups other pups from another litter as soon as possible to ensure continued lactation. If the failure of breeding was due to psychological distress, a

pregnant female could be housed together with a non-pregnant female long before the impending birth. Other possibilities would need to include perhaps the measurement of IGF-1 and IGFBP-5 directly in sliced bone samples through the use of immunohistochemistry to see if more local effects of these hormones were previously masked by only focusing on the measurement of their concentrations in total blood serum. The ELISA kit that was used was measuring the total IGF-1 concentration, which includes IGF-1 that was also bound to IGFBP-5. It would be advantageous to use an ELISA that only measures free and bioavailable IGF-1 (i.e., not bound by IGFBP-5).

In conclusion, the present study shows that PAPP-A2 has no effects on the recovery of bone after lactation. Lactation itself affects trabecular BV/TV and trabecular thickness in mouse femurs negatively, while cortical bone shows partial recovery after three weeks of recovery time. Age affected the recovery of cortical thickness, which highlights the importance of age for the evaluation of bone recovery after lactation.

REFERENCES

Abu-Amer, Y. (2013). NF-κB signaling and bone resorption. *Osteoporosis International : A Journal Established as Result of Cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*, *24*(9), 2377–2386. https://doi.org/10.1007/s00198-013-2313-x

Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M., & Berns, A. (1997). Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Research*, *25*(9), 1766–1773. https://doi.org/10.1093/nar/25.9.1766

Altintas, O., Park, S., & Lee, S.-J. V. (2016). The role of insulin/IGF-1 signaling in the longevity of model invertebrates, C. elegans and D. melanogaster. *BMB Reports*, *49*(2), 81–92. https://doi.org/10.5483/bmbrep.2016.49.2.261

Amiri, N., & Christians, J. K. (2015). PAPP-A2 expression by osteoblasts is required for normal postnatal growth in mice. *Growth Hormone and IGF Research*, *25*(6), 274–280. https://doi.org/10.1016/j.ghir.2015.09.003

Andrew, M., Liao, L., Fujimoto, M., Khoury, J., Hwa, V., & Dauber, A. (2018). PAPPA2 as a Therapeutic Modulator of IGF-I Bioavailability: in Vivo and in Vitro Evidence. *Journal of the Endocrine Society*, *2*(7), 646–656. https://doi.org/10.1210/js.2018-00106

Archer, D. F., Zeleznik, A. J., & Rockette, H. E. (1988). Ovarian follicular maturation in women. II. Reversal of estrogen inhibited ovarian folliculogenesis by human gonadotropin. *Fertility and Sterility*, *50*(4), 555–561.

Ardeshirpour, L., Dann, P., Adams, D. J., Nelson, T., VanHouten, J., Horowitz, M. C., & Wysolmerski, J. J. (2007). Weaning triggers a decrease in receptor activator of nuclear factor-kappaB ligand expression, widespread osteoclast

apoptosis, and rapid recovery of bone mass after lactation in mice. *Endocrinology*, *148*(8), 3875–3886. https://doi.org/10.1210/en.2006-1467

Asagiri, M., & Takayanagi, H. (2007). The molecular understanding of osteoclast differentiation. *Bone*, *40*(2), 251–264. https://doi.org/10.1016/j.bone.2006.09.023

Badger, M. R., & Price, G. D. (1994). The Role of Carbonic Anhydrase in Photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology*, *45*(1), 369–392. https://doi.org/10.1146/annurev.pp.45.060194.002101

Bain, S. D., Bailey, M. C., Celino, D. L., Lantry, M. M., & Edwards, M. W. (1993). High-dose estrogen inhibits bone resorption and stimulates bone formation in the ovariectomized mouse. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, *8*(4), 435–442. https://doi.org/10.1002/jbmr.5650080407

Baylink. (2003). Insulin-Like Growth Factor Regulates Peak Bone Mineral Density in Mice by Both Growth Hormone-Dependent and -Independent Mechanisms. *Endocrinology.*, *144(3)*(6), 929–936. https://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Beamer, W. G., Donahue, L. R., Rosen, I. C. J., & Baylink, D. J. (1996). Genetic Variability in Adult Bone Density Among Inbred Strains of Mice. *Bone*, *18*(5), 397–3. https://doi.org/10.1016/8756-3282(96)00047-6

Bentov, I., & Werner, H. (2013). *Insulin-like Growth Factor-1*. *Handbook of Biologically Active Peptides* (Second Edi). Elsevier Inc. https://doi.org/10.1016/B978-0-12-385095-9.00222-0

Berger, C., Goltzman, D., Langsetmo, L., Joseph, L., Jackson, S., Kreiger, N., … Vigna, Y. (2010). Peak bone mass from longitudinal data: Implications for the prevalence, pathophysiology, and diagnosis of osteoporosis. *Journal of*

Bone and Mineral Research, *25*(9), 1948–1957. https://doi.org/10.1002/jbmr.95

Berger, C., Langsetmo, L., Joseph, L., Hanley, D. A., Davison, K. S., Josse, R., … Tenenhouse, A. (2008). Change in bone mineral density as a function of age in women and men and association with the use of antiresorptive agents. *CMAJ*, *178*(13), 0–8.

Blair, H. C., Robinson, L. J., Huang, C. L.-H., Sun, L., Friedman, P. A., Schlesinger, P. H., & Zaidi, M. (2011). Calcium and bone disease. *BioFactors (Oxford, England)*, *37*(3), 159–167. https://doi.org/10.1002/biof.143

Boass, A., Toverud, S. U., Pike, J. W., & Haussler, M. R. (1981). Calcium metabolism during lactation: enhanced intestinal calcium absorption in vitamin D-deprived, hypocalcemic rats. *Endocrinology*, *109*(3), 900–907. https://doi.org/10.1210/endo-109-3-900

Bornstein, S., Brown, S. A., Le, P. T., Wang, X., DeMambro, V., Horowitz, M. C., … Rosen, C. J. (2014). FGF-21 and skeletal remodeling during and after lactation in C57BL/6J mice. *Endocrinology*, *155*(9), 3516–3526. https://doi.org/10.1210/en.2014-1083

Boskey, A. L., & Coleman, R. (2010). Aging and bone. *Journal of Dental Research*, *89*(12), 1333–1348. https://doi.org/10.1177/0022034510377791

Bouxsein, M. L., Boyd, S. K., Christiansen, B. A., Guldberg, R. E., Jepsen, K. J., & Mu, R. (2010). Guidelines for Assessment of Bone Microstructure in Rodents Using Micro – Computed Tomography. *Journal of Bone and Mineral Research*, *25*(7), 1468–1486. https://doi.org/10.1002/jbmr.141

Boyce, B. F., & Xing, L. (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Archives of Biochemistry and Biophysics*, *473*(2), 139–146. https://doi.org/10.1016/j.abb.2008.03.018

Boyce, B. F., Xiu, Y., Li, J., Xing, L., & Yao, Z. (2015). NF-κB-Mediated Regulation of Osteoclastogenesis. *Endocrinology and Metabolism (Seoul, Korea)*, *30*(1), 35–44. https://doi.org/10.3803/EnM.2015.30.1.35

Caetano-Lopes, J., Canhão, H., & Fonseca, J. E. (2007). Osteoblasts and bone formation. *Acta Reumatológica Portuguesa*. https://doi.org/10.1016/S1569-2590(08)60130-5

Cao, J., Venton, L., Sakata, T., & Halloran, B. P. (2003). Expression of RANKL and OPG correlates with age-related bone loss in male C57BL/6 mice. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, *18*(2), 270–277. https://doi.org/10.1359/jbmr.2003.18.2.270

Cao, X. (2011). Targeting osteoclast-osteoblast communication. *Nature Medicine*, *17*(11), 1344–1346. https://doi.org/10.1038/nm.2499

Chantry, C. J., Auinger, P., & Byrd, R. S. (2004). Lactation among adolescent mothers and subsequent bone mineral density. *Archives of Pediatrics and Adolescent Medicine*, *158*(7), 650–656. https://doi.org/10.1001/archpedi.158.7.650

Cho, G. J., Shin, J.-H., Yi, K. W., Park, H. T., Kim, T., Hur, J. Y., & Kim, S. H. (2012). Adolescent pregnancy is associated with osteoporosis in postmenopausal women. *Menopause*, *19*(4). Retrieved from https://journals.lww.com/menopausejournal/Fulltext/2012/04000/Adolescent_pregnancy_is_associated_with.14.aspx

Christians, J., Hoeflich, A., & Keightley, P. (2006). PAPPA2, an enzyme that cleaves an insulin-like growth-factor-binding protein, is a candidate gene for a quantitative trait locus affecting body size in mice. *Genetics*, *173*(3), 1547–1553. https://doi.org/10.1534/genetics.106.057513

Christians, Julian K, Amiri, N., Schipilow, J. D., Zhang, S. W., & May-Rashke, K.

I. (2019). Pappa2 deletion has sex- and age-specific effects on bone in mice. *Growth Hormone & IGF Research : Official Journal of the Growth Hormone Research Society and the International IGF Research Society*, *44*, 6–10. https://doi.org/10.1016/j.ghir.2018.10.006

Christians, Julian K, Bath, A. K., & Amiri, N. (2015). Pappa2 deletion alters IGFBPs but has little effect on glucose disposal or adiposity. *Growth Hormone & IGF Research : Official Journal of the Growth Hormone Research Society and the International IGF Research Society*, *25*(5), 232–239. https://doi.org/10.1016/j.ghir.2015.07.001

Christians, Julian Kenneth, de Zwaan, D. R., & Fung, S. H. Y. (2013). Pregnancy Associated Plasma Protein A2 (PAPP-A2) Affects Bone Size and Shape and Contributes to Natural Variation in Postnatal Growth in Mice. *PLoS ONE*, *8*(2), e56260. https://doi.org/10.1371/journal.pone.0056260

Chung, P.-L., Zhou, S., Eslami, B., Shen, L., LeBoff, M. S., & Glowacki, J. (2014). Effect of age on regulation of human osteoclast differentiation. *Journal of Cellular Biochemistry*, *115*(8), 1412–1419. https://doi.org/10.1002/jcb.24792

Confavreux, C. (2011). Bone: From a reservoir of minerals to a regulator of energy metabolism. *Kidney International. Supplement*, *79*, S14-9. https://doi.org/10.1038/ki.2011.25

Conover, C A, Kiefer, M. C., & Zapf, J. (1993). Posttranslational regulation of insulin-like growth factor binding protein-4 in normal and transformed human fibroblasts. Insulin-like growth factor dependence and biological studies. *The Journal of Clinical Investigation*, *91*(3), 1129–1137. https://doi.org/10.1172/JCI116272

Conover, Cheryl A., Boldt, H. B., Bale, L. K., Clifton, K. B., Grell, J. A., Mader, J. R., … Powell, D. R. (2011). Pregnancy-Associated Plasma Protein-A2 (PAPP-A2): Tissue expression and biological consequences of gene knockout in mice. *Endocrinology*, *152*(7), 2837–2844.

https://doi.org/10.1210/en.2011-0036

Courtland, H. W., Kennedy, O. D., Wu, Y., Gao, Y., Sun, H., Schaffler, M. B., & Yakar, S. (2013). Low levels of plasma IGF-1 inhibit intracortical bone remodeling during aging. *Age*, *35*(5), 1691–1703. https://doi.org/10.1007/s11357-012-9469-8

Dauber, A., Muñoz-Calvo, M. T., Barrios, V., Domené, H. M., Kloverpris, S., Serra-Juhé, C., … Argente, J. (2016). Mutations in pregnancy-associated plasma protein A2 cause short stature due to low IGF-I availability. *EMBO Molecular Medicine*, *8*(4), 363–374. https://doi.org/10.15252/emmm.201506106

Downey, P. A., & Siegel, M. I. (2006). Bone biology and the clinical implications for osteoporosis. *Physical Therapy*, *86*(1), 77–91. https://doi.org/10.1093/ptj/86.1.77

Duan, C., & Allard, J. B. (2020). Insulin-Like Growth Factor Binding Protein-5 in Physiology and Disease. *Frontiers in Endocrinology*, *11*, 100. https://doi.org/10.3389/fendo.2020.00100

Florencio-Silva, R., Sasso, G. R. D. S., Sasso-Cerri, E., Simões, M. J., & Cerri, P. S. (2015a). Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/421746

Forbes, B. E., McCarthy, P., & Norton, R. S. (2012). Insulin-like growth factor binding proteins: a structural perspective. *Frontiers in Endocrinology*, *3*, 38. https://doi.org/10.3389/fendo.2012.00038

Fujimoto, M., Andrew, M., Liao, L., Zhang, D., Yildirim, G., Sluss, P., … Dauber, A. (2019). Low IGF-I Bioavailability Impairs Growth and Glucose Metabolism in a Mouse Model of Human PAPPA2 p.Ala1033Val Mutation. *Endocrinology*, *160*(6), 1363–1376. https://doi.org/10.1210/en.2018-00755

Fujimoto, M., Khoury, J. C., Khoury, P. R., Kalra, B., Kumar, A., Sluss, P., … Dauber, A. (2020). Anthropometric and biochemical correlates of PAPP-A2, free IGF-I, and IGFBP-3 in childhood. *European Journal of Endocrinology*, *182*(3), 363–374. https://doi.org/10.1530/EJE-19-0859

Glatt, V., Canalis, E., Stadmeyer, L., & Bouxsein, M. L. (2007). Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. *Journal of Bone and Mineral Research*, *22*(8), 1197–1207. https://doi.org/10.1359/jbmr.070507

Gong, Z., Kennedy, O., Sun, H., Wu, Y., Williams, G. A., Klein, L., … Yakar, S. (2014). Reductions in serum IGF-1 during aging impair health span. *Aging Cell*, *13*(3), 408–418. https://doi.org/10.1111/acel.12188

Govoni, K. E., Baylink, D. J., & Mohan, S. (2005). The multi-functional role of insulin-like growth factor binding proteins in bone. *Pediatric Nephrology (Berlin, Germany)*, *20*(3), 261–268. https://doi.org/10.1007/s00467-004-1658-y

Grabowski, P. (2009). Physiology of bone. *Endocrine Development*, *16*, 32–48. https://doi.org/10.1159/000223687

Guntur, A. R., & Rosen, C. J. (2013). IGF-1 regulation of key signaling pathways in bone. *BoneKEy Reports*, *2*(May), 1–6. https://doi.org/10.1038/bonekey.2013.171

Haffner-Luntzer, M., Kovtun, A., Rapp, A. E., & Ignatius, A. (2016). Mouse Models in Bone Fracture Healing Research. *Current Molecular Biology Reports*, *2*(2), 101–111. https://doi.org/10.1007/s40610-016-0037-3

Halloran, B. P., Ferguson, V. L., Simske, S. J., Burghardt, A., Venton, L. L., & Majumdar, S. (2002). Changes in Bone Structure and Mass With Advancing Age in the Male C57BL/6J Mouse. *Journal of Bone and Mineral Research*, *17*(6), 1044–1050.

https://doi.org/https://doi.org/10.1359/jbmr.2002.17.6.1044

Han, Y., You, X., Xing, W., Zhang, Z., & Zou, W. (2018). Paracrine and endocrine actions of bone—the functions of secretory proteins from osteoblasts, osteocytes, and osteoclasts. *Bone Research*, *6*(1), 16. https://doi.org/10.1038/s41413-018-0019-6

Hawkins-Carranza, F. G., Muñoz-Calvo, M. T., Martos-Moreno, G. Á., Allo-Miguel, G., Del Río, L., Pozo, J., … Argente, J. (2018). rhIGF-1 Treatment Increases Bone Mineral Density and Trabecular Bone Structure in Children with PAPP-A2 Deficiency. *Hormone Research in Paediatrics*, *89*(3), 200–204. https://doi.org/10.1159/000486336

Hopkinson, J. M., Butte, N. F., Ellis, K., & Smith, E. O. (2000). Lactation delays postpartum bone mineral accretion and temporarily alters its regional distribution in women. *The Journal of Nutrition*, *130*(November 1999), 777–783.

Hunscher, H. A. (1930). METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE: II. CALCIUM AND PHOSPHORUS UTILIZATION IN TWO SUCCESSIVE LACTATION PERIODS. *Journal of Biological Chemistry*, *86*(1), 37–57. https://doi.org/https://doi.org/10.1016/S0021-9258(18)76903-2

Jasienska, G. (2009). Reproduction and lifespan: Trade-offs, overall energy budgets, intergenerational costs, and costs neglected by research. *American Journal of Human Biology : The Official Journal of the Human Biology Council*, *21*(4), 524–532. https://doi.org/10.1002/ajhb.20931

Jee, Y. H., Baron, J., & Nilsson, O. (2018). New developments in the genetic diagnosis of short stature. *Current Opinion in Pediatrics*, *30*(4), 541–547. https://doi.org/10.1097/MOP.0000000000000653

Jehle, P. M., Schulten, K., Schulz, W., Jehle, D. R., Stracke, S., Manfras, B., …

Mohan, S. (2003). Serum levels of insulin-like growth factor (IGF)-I and IGF binding protein (IGFBP)-1 to -6 and their relationship to bone metabolism in osteoporosis patients. *European Journal of Internal Medicine*, *14*(1), 32–38. https://doi.org/10.1016/S0953-6205(02)00183-8

Kavukcuoglu, N. B., Denhardt, D. T., Guzelsu, N., & Mann, A. B. (2007). Osteopontin deficiency and aging on nanomechanics of mouse bone. *Journal of Biomedical Materials Research. Part A*, *83*(1), 136–144. https://doi.org/10.1002/jbm.a.31081

Khosla, S., Oursler, M. J., & Monroe, D. G. (2012). Estrogen and the skeleton. *Trends in Endocrinology and Metabolism: TEM*, *23*(11), 576–581. https://doi.org/10.1016/j.tem.2012.03.008

Knothe Tate, M. L., Adamson, J. R., Tami, A. E., & Bauer, T. W. (2004). The osteocyte. *The International Journal of Biochemistry & Cell Biology*, *36*(1), 1–8. https://doi.org/10.1016/s1357-2725(03)00241-3

Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., … Penninger, J. M. (1999). OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*, *397*(6717), 315–323. https://doi.org/10.1038/16852

Kouvelioti, R., Kurgan, N., Falk, B., Ward, W. E., Josse, A. R., & Klentrou, P. (2018). Response of Sclerostin and Bone Turnover Markers to High Intensity Interval Exercise in Young Women: Does Impact Matter? *BioMed Research International*, *2018*, 4864952. https://doi.org/10.1155/2018/4864952

Kovacs, C. S. (2016). Maternal Mineral and Bone Metabolism During Pregnancy, Lactation, and Post-Weaning Recovery. *Physiological Reviews*, *96*(2), 449–547. https://doi.org/10.1152/physrev.00027.2015

Kovacs, C. S. (2017). The Skeleton Is a Storehouse of Mineral That Is Plundered During Lactation and (Fully?) Replenished Afterwards. *Journal of Bone and*

Mineral Research. https://doi.org/10.1002/jbmr.3090

Lampinen, R., Vehviläinen-Julkunen, K., & Kankkunen, P. (2009). A review of pregnancy in women over 35 years of age. *Open Nursing Journal*, *3*, 33–38. https://doi.org/http://dx.doi.org/10.2174/1874434600903010033

Laron, Z. (1999). Somatomedin-1 (recombinant insulin-like growth factor-1): clinical pharmacology and potential treatment of endocrine and metabolic disorders. *BioDrugs : Clinical Immunotherapeutics, Biopharmaceuticals and Gene Therapy*, *11*(1), 55–70. https://doi.org/10.2165/00063030-199911010-00006

Laron, Z., Pertzelan, A., Karp, M., Kowadlo-Silbergeld, A., & Daughaday, W. H. (1971). Administration of growth hormone to patients with familial dwarfism with high plasma immunoreactive growth hormone: measurement of sulfation factor, metabolic and linear growth responses. *The Journal of Clinical Endocrinology and Metabolism*, *33*(2), 332–342. https://doi.org/10.1210/jcem-33-2-332

Laursen, L. S., Overgaard, M. T., Søe, R., Boldt, H. B., Sottrup-Jensen, L., Giudice, L. C., … Oxvig, C. (2001). Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A. *FEBS Letters*, *504*(1–2), 36–40. https://doi.org/10.1016/s0014-5793(01)02760-0

Le, A. R. T. I. C., Cabrera-salcedo, C., Mizuno, T., Tyzinski, L., Andrew, M., Vinks, A. A., … Dauber, A. (2017). Pharmacokinetics of IGF-1 in PAPP-A2-Deficient Patients, Growth Response, and Effects on Glucose and Bone Density. *J Clin Endocrinol Metab.*, *102*(September), 4568–4577. https://doi.org/10.1210/jc.2017-01411

Li, J., Bao, Q., Chen, S., Liu, H., Feng, J., Qin, H., … Zong, Z. (2017). Different bone remodeling levels of trabecular and cortical bone in response to

changes in Wnt/β-catenin signaling in mice. *Journal of Orthopaedic Research : Official Publication of the Orthopaedic Research Society*, *35*(4), 812–819. https://doi.org/10.1002/jor.23339

Liu, X. S., Ardeshirpour, L., VanHouten, J. N., Shane, E., & Wysolmerski, J. J. (2012). Site-specific changes in bone microarchitecture, mineralization, and stiffness during lactation and after weaning in mice. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, *27*(4), 865–875. https://doi.org/10.1002/jbmr.1503

Locatelli, V., & Bianchi, V. E. (2014). Effect of GH/IGF-1 on Bone Metabolism and Osteoporsosis. *International Journal of Endocrinology*, *2014*, 235060. https://doi.org/10.1155/2014/235060

Lombardi, G., Tauchmanova, L., Di Somma, C., Musella, T., Rota, F., Savanelli, M. C., & Colao, A. (2005). Somatopause: dismetabolic and bone effects. *Journal of Endocrinological Investigation*, *28*(10 Suppl), 36–42.

Long, F. (2011). Building strong bones: molecular regulation of the osteoblast lineage. *Nature Reviews. Molecular Cell Biology*, *13*(1), 27–38. https://doi.org/10.1038/nrm3254

Lorenzini, A., Salmon, A. B., Lerner, C., Torres, C., Ikeno, Y., Motch, S., … Sell, C. (2014). Mice producing reduced levels of insulin-like growth factor type 1 display an increase in maximum, but not mean, life span. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *69*(4), 410–419. https://doi.org/10.1093/gerona/glt108

Manigrasso, M. B., & O'Connor, J. P. (2008). Comparison of fracture healing among different inbred mouse strains. *Calcified Tissue International*, *82*(6), 465–474. https://doi.org/10.1007/s00223-008-9144-3

Mastrangelo, A., Martos-Moreno, G. Á., Rupérez, F. J., Chowen, J. A., Barbas, C., & Argente, J. (2018). Metabolomics changes in patients with PAPP-A2

deficiency in response to rhIGF1 treatment. *Growth Hormone & IGF Research : Official Journal of the Growth Hormone Research Society and the International IGF Research Society*, *42–43*, 28–31. https://doi.org/10.1016/j.ghir.2018.08.002

Matsuo, K., & Irie, N. (2008). Osteoclast-osteoblast communication. *Archives of Biochemistry and Biophysics*, *473*(2), 201–209. https://doi.org/10.1016/j.abb.2008.03.027

McClung, M. (2007). Role of RANKL inhibition in osteoporosis. *Arthritis Research and Therapy*, *9*(SUPPL.1), 1–6. https://doi.org/10.1186/ar2167

Mohan, S., Nakao, Y., Honda, Y., Landale, E., Leser, U., Dony, C., … Baylink, D. J. (1995). Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. *The Journal of Biological Chemistry*, *270*(35), 20424–20431. https://doi.org/10.1074/jbc.270.35.20424

Møller, U. K., Streym, S., Mosekilde, L., Heickendorff, L., Flyvbjerg, A., Frystyk, J., … Rejnmark, L. (2013). Changes in calcitropic hormones, bone markers and insulin-like growth factor I (IGF-I) during pregnancy and postpartum: a controlled cohort study. *Osteoporosis International : A Journal Established as Result of Cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*, *24*(4), 1307–1320. https://doi.org/10.1007/s00198-012-2062-2

Nagaraja, S., Lin, A. S. P., & Guldberg, R. E. (2007). Age-related changes in trabecular bone microdamage initiation. *Bone*, *40*(4), 973–980. https://doi.org/10.1016/j.bone.2006.10.028

Nakamura, T., Imai, Y., Matsumoto, T., Sato, S., Takeuchi, K., Igarashi, K., … Kato, S. (2007). Estrogen Prevents Bone Loss via Estrogen Receptor α and Induction of Fas Ligand in Osteoclasts. *Cell*, *130*(5), 811–823. https://doi.org/10.1016/j.cell.2007.07.025

National Institutes of Health. (2015). Osteoporosis : Peak Bone Mass in Women. *National Institute of Health*, (Juni), 1–2. Retrieved from www.nih.gov

Oftadeh, R., Perez-Viloria, M., Villa-Camacho, J. C., Vaziri, A., & Nazarian, A. (2015). Biomechanics and mechanobiology of trabecular bone: a review. *Journal of Biomechanical Engineering*, *137*(1), 108021–1080215. https://doi.org/10.1115/1.4029176

Ohlsson, C., Mohan, S., Sjögren, K., Tivesten, A., Isgaard, J., Isaksson, O., … Svensson, J. (2009). The role of liver-derived insulin-like growth factor-I. *Endocrine Reviews*, *30*(5), 494–535. https://doi.org/10.1210/er.2009-0010

Ott, S. M. (2018). Cortical or Trabecular Bone: What's the Difference? *American Journal of Nephrology*, *47*(6), 373–375. https://doi.org/10.1159/000489672

Overgaard, M. T., Boldt, H. B., Laursen, L. S., Sottrup-Jensen, L., Conover, C. A., & Oxvig, C. (2001). Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase. *The Journal of Biological Chemistry*, *276*(24), 21849–21853. https://doi.org/10.1074/jbc.M102191200

Peng, T. C., Garner, S. C., Kusy, R. P., & Hirsch, P. F. (1988). Effect of number of suckling pups and dietary calcium on bone mineral content and mechanical properties of femurs of lactating rats. *Bone and Mineral*, *3*(4), 293–304.

Peruzzi, F., Prisco, M., Dews, M., Salomoni, P., Grassilli, E., Romano, G., … Baserga, R. (1999). Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. *Molecular and Cellular Biology*, *19*(10), 7203–7215. https://doi.org/10.1128/mcb.19.10.7203

Ren, S., Nie, Y., & Wang, A. (2016). Effects of recombinant human growth hormone in the treatment of dwarfism and relationship between IGF-1, IGFBP-3 and thyroid hormone. *Exp Ther Med*, *12*(6), 3579–3582.

https://doi.org/10.3892/etm.2016.3831

Rinderknecht, E., & Humbel, R. E. (1978). The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *The Journal of Biological Chemistry*, *253*(8), 2769–2776.

Salari, P., Abdollahi, M., & D, P. (2014). The I nfluence of P regnancy and L actation on M aternal B one H ealth : A S ystematic R eview, *8*(4), 135–148.

Salih, D. a M., Mohan, S., Kasukawa, Y., Tripathi, G., Lovett, F. A., Anderson, N. F., … Pell, J. M. (2005). Insulin-Like Growth Factor-Binding Protein-5 Induces a Gender- Related Decrease in Bone Mineral Density in Transgenic Mice. *Endocrinology*, *146*(2), 931–940. https://doi.org/10.1210/en.2004-0816.Insulin-Like

Sarko, J. (2005). Bone and mineral metabolism. *Emergency Medicine Clinics of North America*, *23*(3), 703–721, viii. https://doi.org/10.1016/j.emc.2005.03.017

Schmiedel, B. J., Scheible, C. A., Nuebling, T., Kopp, H.-G., Wirths, S., Azuma, M., … Salih, H. R. (2013). RANKL expression, function, and therapeutic targeting in multiple myeloma and chronic lymphocytic leukemia. *Cancer Research*, *73*(2), 683–694. https://doi.org/10.1158/0008-5472.CAN-12-2280

Schnatz, P. F., Barker, K. G., Marakovits, K. A., & O'Sullivan, D. M. (2010). Effects of age at first pregnancy and breast-feeding on the development of postmenopausal osteoporosis. *Menopause*, *17*(6). Retrieved from https://journals.lww.com/menopausejournal/Fulltext/2010/17060/Effects_of_age_at_first_pregnancy_and.14.aspx

Seeman, E. (2013). Age- and menopause-related bone loss compromise cortical and trabecular microstructure. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *68*(10), 1218–1225. https://doi.org/10.1093/gerona/glt071

Silbermann, M., Weiss, A., Reznick, A. Z., Eilam, Y., Szydel, N., & Gershon, D. (1987). Age-related trend for osteopenia in femurs of female C57BL/6 mice. *Comprehensive Gerontology. Section A, Clinical and Laboratory Sciences*, *1*(1), 45–51.

Stein, Z., & Susser, M. (2000, November). The risks of having children in later life. *The Western Journal of Medicine*. https://doi.org/10.1136/ewjm.173.5.295

Steinbrecher, A., Janke, J., Poy, M. N., Oxvig, C., & Pischon, T. (2017). Pregnancy-Associated Plasma Protein-A2 and Anthropometry, Lifestyle, and Biochemical Factors in a Human Adult Population. *Scientific Reports*, *7*(1), 10455. https://doi.org/10.1038/s41598-017-10629-y

Streicher, C., Heyny, A., Andrukhova, O., Haigl, B., Slavic, S., Schüler, C., … Erben, R. G. (2017). Estrogen Regulates Bone Turnover by Targeting RANKL Expression in Bone Lining Cells. *Scientific Reports*, *7*(1), 6460. https://doi.org/10.1038/s41598-017-06614-0

Tanner, S. J., Hefferan, T. E., Rosen, C. J., & Conover, C. A. (2008). Impact of pregnancy-associated plasma protein-a deletion on the adult murine skeleton. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, *23*(5), 655–662. https://doi.org/10.1359/jbmr.071210

Teitelbaum, S. L. (2000). Bone Resorption by Osteoclasts. *Science*, *289*(5484), 1504 LP – 1508. Retrieved from http://science.sciencemag.org/content/289/5484/1504.abstract

Teitelbaum, S. L. (2007). Osteoclasts: What do they do and how do they do it? *American Journal of Pathology*, *170*(2), 427–435. https://doi.org/10.2353/ajpath.2007.060834

Thompson, D. D., Simmons, H. A., Pirie, C. M., & Ke, H. Z. (1995). FDA

Guidelines and animal models for osteoporosis. *Bone*, *17*(4 Suppl), 125S-133S. https://doi.org/10.1016/8756-3282(95)00285-l

Tommasini, S. M., Nasser, P., & Jepsen, K. J. (2007). Sexual dimorphism affects tibia size and shape but not tissue-level mechanical properties. *Bone*, *40*(2), 498–505. https://doi.org/10.1016/j.bone.2006.08.012

Wang, Y., Nishida, S., Elalieh, H. Z., Long, R. K., Halloran, B. P., & Bikle, D. D. (2006). Role of IGF-I signaling in regulating osteoclastogenesis. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, *21*(9), 1350–1358. https://doi.org/10.1359/jbmr.060610

Weiss, A., Arbell, I., Steinhagen-Thiessen, E., & Silbermann, M. (1991). Structural changes in aging bone: osteopenia in the proximal femurs of female mice. *Bone*, *12*(3), 165–172. https://doi.org/10.1016/8756-3282(91)90039-l

Xian, L., Wu, X., Pang, L., Lou, M., Rosen, C., Qiu, T., … Cao, X. (2013). Matrix IGF-1 regulates bone mass by activation of mTOR in mesenchymal stem cells. *Nat Med.*, *18*(7), 1095–1101. https://doi.org/10.1038/nm.2793.Matrix

Yakar, S., Rosen, C. J., Beamer, W. G., Ackert-Bicknell, C. L., Wu, Y., Liu, J.-L., … LeRoith, D. (2002). Circulating levels of IGF-1 directly regulate bone growth and density. *The Journal of Clinical Investigation*, *110*(6), 771–781. https://doi.org/10.1172/JCI15463

Yates, L. B., Karasik, D., Beck, T. J., Cupples, L. A., & Kiel, D. P. (2007). Hip structural geometry in old and old-old age: similarities and differences between men and women. *Bone*, *41*(4), 722–732. https://doi.org/10.1016/j.bone.2007.06.001

Ziomkiewicz, A., Sancilio, A., Galbarczyk, A., & Klimek, M. (2016). Evidence for the Cost of Reproduction in Humans : High Lifetime Reproductive Effort Is

Associated with Greater Oxidative Stress in Post-Menopausal Women, 1–14. https://doi.org/10.1371/journal.pone.0145753