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Osteoporosis: comparison of genetic and environmental effects determining bone mineral density

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OSTEOPOROSIS: COMPARISON OF GENETIC AND ENVIRONMENTAL EFFECTS DETERMINING BONE MINERAL DENSITY

by

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DEDICATION

I would like to dedicate this work to my parents, grandmother, brother, sister-in-law, and nephews. Without their endless support and sacrifices, I would not have had this opportunity to pursue higher education and my dreams.

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OSTEOPOROSIS: COMPARISON OF GENETIC AND ENVIRONMENTAL EFFECTS DETERMINING BONE MINERAL DENSITY

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ABSTRACT

Osteoporosis has affected millions of patients worldwide and is a continuing concern for the increasing ageing population. It is a skeletal disease due to abnormal bone growth and resorption, characterized by low bone mass. Onset could be due to genetics and family history or acquired risk factors like lifestyle, diet, and exercise. As a result, it is important to try to understand the mechanisms of the onset of low bone mineral density so that effective treatment plans and prevention methods can be determined.

The purpose of this study is to compare how the genetic makeup of an individual interacts with one's environment in the determination of bone mineral density (BMD) and the onset of osteoporosis. This study focused on comparing biochemical markers of osteogenic cells and their mineralization potential between primary and secondary cultures of mesenchymal stem cells (MSC) that were harvested from pelvic bone-marrow remains of patients undergoing hip replacement. We hypothesized that the primary cultures grown out directly after being acquired; should be impacted more directly by comorbidities present at the time of collection while the secondary cultures expanded from the initial marrow stromal cells should show less impact of comorbidities and reflect more closely genetic aspects that affect BMD. It was found that while the secondary cultures overall produced greater values for DNA, ALP, calcium,

hydroxyproline, and protein, when samples were normalized the values between the primary and secondary cultures did not show significant differences. This data appeared however to validate our hypothesis since the overall increased growth and mineralization of the secondary cultures showed a loss in their overall correlation to the environmental impacts of smoking and BMI that were observed in the primary cultures.

The second hypothesis explored the specific correlations between the biochemical markers as indices of osteogenic potential of the cultures (DNA, ALP, calcium, hydroxyproline, and total protein) in relation to each other. The hypothesis was mostly supported with positive correlations, between all the features except for that between hydroxyproline and calcium which showed a negative correlation.

Overall, this study demonstrates that although the normalized values between the primary and secondary cultures did not show significant different osteogenic features; it did show the correlations to comorbidities identified in primary cultures were lost upon expansion following sub cultivation. Further studies with larger sample sizes are needed which will provide a more statistically significant conclusion, allowing for further analysis when comparing genetic and environmental effects.

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LIST OF ABBREVIATIONS

ACF.....Animal Component Free

ALP ..... Alkaline Phosphatase

α-MEM .....Minimum Essential Medium-alpha

Anti-anti ..... Antibiotic-antimycotic

ARS.....Alizarin Red S

BMD ..... Bone Mineral Density

DI ..... Deionized

DMAB..... 4-(Dimethylamino) benzaldehyde

DPBS..... Dulbecco's-Phosphate Buffered Saline

FBS .....Fetal Bovine Serum

MSC ..... Mesenchymal Stem Cell

PFA.....Paraformaldehyde

pNPP ..... P-Nitrophenol Phosphate

RANKL.....Receptor Activator of Nuclear factor Kappa-B Ligand

INTRODUCTION

Osteoporosis is a disorder characterized by the decrease in bone mass, microarchitectural deterioration, and increased likelihood of fractures. (Lane, Russel, & Khan, 2000). It affects approximately slightly more than 10 million Americans today and is projected to affect more than 14 million by 2020 (Lane, 2006). As the aging population increases, there will be an increase in incidence rates as older patients are more susceptible to fractures (Ettinger, 2003).

Bone Physiology

Osteoblasts, Osteoclasts, and Osteocytes Bone is a specialized type of connective tissue composed of three major cells, osteocytes, osteoblasts, and osteoclasts. These cells interact together to remodel the bone throughout life, as they find balance between bone resorption and bone formation. Osteoblasts are cells that create the bone matrix (Mescher, 2016). Osteoblasts are derived from and maintained by osteoprogenitor cells, which arise from multipotent stem cells within the bone marrow called mesenchymal stem cells. Different from hematopoietic stem cells, mesenchymal stem cells are what give rise to bone, cartilage, fat, and fibrous connective tissue. Once fully differentiated, they secrete type I collagen and other proteins that are necessary for bone formation (Clarke, 2008). Osteoclasts are multinucleated cells that are derived from the myeloid lineage and break down, remove, and remodel calcified bone tissue and matrix (Mescher, 2016). There are two cytokines that are important for osteoclast formation, which are RANKL and macrophage CSF. They are produced mainly by marrow stromal cells, osteoblasts and osteocytes. For bone resorption to occur properly, it depends on

osteoclasts to secrete hydrogen ions and cathepsin K enzyme, as the hydrogen ions acidify the resorption pit under the osteoclast, which is in the process of dissolving and reabsorbing the mineral components of the bone matrix. The cathepsin K activates proteases which in turn activate collagenases that digest the type I collagen component of the matrix (Clarke, 2008). Osteocytes are terminally differentiated osteoblasts that have become surrounded by the mineralized matrix that they have synthesized exists within lacunae (Mescher, 2016). These cells are the most abundant cells of bone tissue and they support the structure of bone and its metabolism (Clarke, 2008). They also are the primary mediators of both mechanical signals that control bone structure and produce the major regulators that control osteoclastogenesis (Pajevic, 2019).

Osteogenesis Osteogenesis is the process of bone development, which can occur by two processes. Intramembranous ossification is the process by which differentiated osteoblasts from mesenchyme start to secrete osteoid (Mescher, 2016). Through this process, flat bones of the skull form. It begins with neural crest-derived mesenchymal cells proliferating and condensing into compact nodules. Osteoblasts then start to secrete collagen into the matrix that allows calcium salts to bind and calcify. As the osteoblasts continue to secrete matrix and it continues to mineralize, they become encased in their matrix and become osteocytes. As the calcified areas become surrounded by other mesenchymal cells, it begins to form the periosteum, which is a membrane that surrounds the bone. For the cells on the inner side of the periosteum, they differentiate into osteoblasts and also lay down the matrix, thus creating more layers of the bone (Gilbert, 2000). Endochondral ossification is a bone forming process in which nascent skeletal

organs are first modeled in cartilage. Mesenchymal cells surround the central regions of a cartilage model then differentiate into osteoblasts and follow the same progression of development as seen in the calvaria. Bone tissues form a bony color around the central regions of the cartilage models. This region is then perforated with a feeding blood vessel bringing in osteoblasts that invade the existing cartilage templates of bone, leading to the resorption of the cartilage. The osteoid production in this space forms trabecular bone, which will eventually form in the marrow spaces of most appendicular and axial bones.

When bone is formed, it first creates a woven structure that is then replaced by a stronger and lamellar bone (Mescher, 2016). Endochondral ossification can be broken down into five stages. In the first stage, the mesenchymal cells are committed to becoming cartilage cells due to paracrine factors from mesodermal cells that cause two transcription factors to be expressed, Pax1 and Scleraxis. Next, the committed mesenchyme cells differentiates toward chondrocytes which then forms a model of bone by secreting cartilage into the extracellular matrix. In the fourth part of this process, the chondrocytes then stop proliferating and progress to hypertrophic chondrocytes. These are chondrocytes that produce collagen and fibronectin that allow the matrix to become mineralized. The final stage of endochondral ossification is blood vessels invading the cartilage, which then leads to the hypertrophic chondrocytes to apoptosis and creating a space called the bone marrow (Gilbert, 2000).

Biochemical Markers of Bone Formation and Turnover The bone is made of mineral, organic matrix, water, and lipids. Hydroxyapatite, a crystalline calcium phosphate salt is the primary mineralized portion of the bone matrix (Clarke, 2008).

Important determinants of bone mineralization are serum calcium, inorganic phosphate, and having a collagen-rich extracellular matrix (Murshed, 2018). Important to the mineralization process is ionic calcium and inorganic phosphate concentrations. Lower levels of these minerals can lead to greatly decreased amounts of mineralization, which could be affected by the levels of vitamin D. Vitamin D is an important factor that allows for absorption of ionic calcium (Murshed, 2018). Its major function is to optimize the absorption of intestinal calcium and phosphorus that are vital for the formation of the bone mineral matrix. Vitamin D-dependent calcium transport proteins that regulate this calcium absorption through the intestine includes the transient receptor potential cation channel, subfamily V, member 6 (TRPV6). It is this channel that allows calcium to enter the enterocytes of the intestines and is highly regulated by 1,25(OH)2D. As women age, the TRPV6 levels decline which contributes to the decrease in calcium absorption. Therefore, maintaining optimal vitamin D levels is so important so that calcium could be efficiently absorbed. The presence of vitamin D allows for intestinal calcium to be absorbed at a 30%-40% rate, while the absence only allows for 10%-15% absorption. Through studies with rodent models, it was found that mice without the vitamin D receptor eventually developed osteomalacia. This finding suggested that vitamin D is vital to increase calcium reabsorption through the intestine allowing for bone mineralization to properly happen (Khazai, 2008). In the process of bone formation and mineralization, osteoblasts release alkaline phosphatase and other proteins are expressed (Clarke, 2008). The alkaline phosphatase is attached to the osteoblast cell membrane, and can cleave inorganic pyrophosphate, which is a strong mineralization inhibitor, releasing

free inorganic phosphate ions thus allowing mineralization to occur (Murshed, 2018). During bone turnover, also known as bone resorption, hydroxyproline is released when type I collagen is broken down. However, it is also understood that hydroxyproline is associated with osteoblast activity, as some newly created collagen chains are degraded before they are secreted by osteoblasts (Schönau and Frank, 2003). These biochemical markers can then be used to predict the rate of both bone loss in patients and their risk for developing osteoporosis (Swaminathan, 2001).

Types of Bones Throughout the body, there are several types of bones. The woven bone is a newly calcified bone that is found in developing and growing bones or when a hard callus forms during bone fracture healing. It is also known as an immature bone. The lamellar bone is a type of remodeled woven bone that is found throughout adult bones. It is also known as a mature bone. The compact bone, which comprises about 80% of lamellar bones, are the outer region of bones, also known as the cortical bone. The cancellous bone is what makes up the other 20% of lamellar bones. It is the inner region of bones, which are next to the marrow cavities and is also called the spongy bone (Mescher, 2016).

Osteoporosis

Pathogenesis Osteoporosis is a skeletal disorder due to an imbalance in bone remodeling, leading to increased susceptibility of fractures as well as reduced strength in bones. The two factors that lead to diagnosis of osteoporosis are failure of the bone to reach peak mass and having excessive amounts of bone resorption or a decrease in bone formation during bone remodeling. Not reaching peak bone mass can lead to osteoporosis

and fractures, which are most impacted by genetic factors. Some of the genetic variants that regulate bone mass are lipoprotein receptor-related protein 5, sclerostin, osteoprotegerin, oestrogen receptor 1, and receptor activator of RANK pathway genes (Sandhu, 2011). Furthermore, having an imbalance in bone remodeling can create damage to the integrity of the bone structure. The most important regulators and mediators of osteoblast activity is LRP5, while the most important mediator for osteoclast activity is OPG/RANK and its ligand RANKL, as the OPG/RANKL ratio is vital to maintain normal bone mass and strength, growth hormones, cytokines, and drugs affect their expressions. Hormones like estrogen affect bone remodeling and with a deficiency, it can lead to abnormal bone turnover and lead to osteoporosis (Sandhu, 2011). Women who are older and post-menopausal tend to have abnormalities in bone remodeling processes that increase the likelihood of fractures. This is due to the estrogen deficiency caused after menopause which accelerates bone loss, increasing the risk of fractures. A decrease in estrogen levels leads to having greater bone resorption and less bone building. (Lane, 2006). Age also plays a large role in increased resorption and decreased bone formation, leading to bone loss and fractures. This is thought to be due to the lack of osteoprogenitor cells differentiating into osteoblasts with increasing age, as they tend to differentiate into adipocytes (Sandhu, 2011).

Diagnosis When diagnosing osteoporosis, it is asymptomatic and difficult to diagnose until the bone fails when under physical stress (Dobbs, 1999). However, when detected, a way to diagnose osteoporosis is to measure bone mineral density (BMD) by using a dual X-ray absorptiometry. To measure BMD, the spine and hip are usually

analyzed; however, if unable to reach the spine and hip, the forearm can also be used to measure BMD (Sandhu, 2011). Being able to measure BMD and finding low bone mass is one of the most accurate predictors for increased fracture risk (Smith, 2000). According to the NIH, the BMD test results give the patient a T-score and compared to a healthy adult. A normal T-score is between +1 and -1. When diagnosed with low bone mass, it is between -1 to -2.5. To be diagnosed with osteoporosis, a patient will receive a T-score of -2.5 or lower. A case of severe osteoporosis will however score a greater negative score than -2.5. This means the patient has had one or more osteoporotic fractures previously (NIH,2018). Another method of diagnosis is by going over a patient's history of fragility fracture, since prior fractures can also lead to a diagnosis of osteoporosis (Sandhu, 2011). Further evaluation should also note whether they have secondary osteoporosis due to other diseases like hyperthyroidism, Cushing's disease, or drugs and treatments that may lead to osteoporosis and lowered bone mineral density (Dobbs, 1999).

Risk Factors Risk factors that are associated with inducing osteoporotic fractures are hormonal factors, use of certain drugs, low physical activity, low levels of calcium and vitamin D, race, body size, and familial history of fracture incidences (Lane, 2006). Behavioral environmental factors that impact peak bone mass are smoking and alcohol consumption (Sandhu, 2011). It has further been found that the effects of smoking on bone metabolism are exacerbated by alcohol consumption. Large amounts of alcohol consumption alone result in increased risk of fractures for both men and women and bone histological examinations have shown physical changes to bone structure of those who

consume large amounts of alcohol. Alcohol is known to suppress the function of osteoblasts, suppressing and decreasing bone mineralization. As a result, it can cause calcium to be excreted in urine causing hypercalciuria and lowered serum levels of calcium, causing hypocalcaemia and reduced BMD. Effects on bone metabolism due to alcohol can result from the effects alcohol has on other organs such as liver damage, hypogonadism and nutritional deficiencies (Metcalfe, 2008).

Low levels of hormones like estrogen in women and testosterone in men can lead to increased risk for osteoporosis. Low estrogen levels in women can occur after menopause or in abnormal cases of extremely low levels of estrogen pre-menopause. For men, as testosterone gradually decreases with age, it may not play as large of a role in decreases of BMD (NIH, 2019). Certain drugs and medications like glucocorticoid steroids and cancer medications may increase the risk of osteoporosis. Other drugs like Thiazolidinediones and selective serotonin reuptake inhibitors may also contribute to the decrease in BMD and risk for osteoporosis (NIH, 2019).

Ethnicity and race play a large role as well. Women who are Asian and non-Hispanic are more at risk than African American and Hispanic women. Overall, the demographics of patients who are more likely to be affected by osteoporosis are generally the older population, especially women a few years before menopause. For men, osteoporosis is more common among non-Hispanic whites (NIH, 2019). A patient's body size also plays a role in the risk of osteoporosis. Women and men who are thinner-boned and slim are at greater risk than those who are thicker-boned. It has also been shown that

patients with parents who have a history of osteoporosis or hip fractures are more likely to be at risk for osteoporosis (NIH, 2019).

Common Osteoporotic Fractures About 51% of women and 24% of men have fractures as a result of osteoporosis. 250,000 of these cases are hip fractures (Metcalfe, 2008). Hip fractures are one of the most frequently occurring osteoporotic fractures, with a 20% occurrence rate (Pouresmaeli, 2018). The hip is a multiaxial ball-and-socket synovial joint where the head of the femur articulates with the concave acetabulum of the pelvis. The joint is covered by a dense layer of articular hyaline cartilage and the primary function is to sustain body weight when moving and static. The femoral head is supported by a thin femoral neck, which is prone to fractures. It is more prone when a patient suffers from bone disorders such as osteomalacia, osteogenesis imperfecta, and metabolic bone diseases. However, most patients who fracture the femoral neck suffer from osteoporosis (Metcalfe, 2008). In addition to physical and economic burden, hip fractures cause patients to be dependent on others. About 50% of patients with hip fractures require assistance when walking and then another 25% requiring domiciliary care after (Metcalfe, 2008). In addition, fracture of the proximal femur is associated with an 8%-36% mortality rate, with a higher rate among men than women. This rate is only increasing as the ageing population continues to increase every year (Pouresmaeli, 2018). Another common osteoporotic fracture is the fracture of the distal forearm. Around 18% of patients who were over the age of 65 had osteoporotic fractures of the forearms, while those who fractured their forearms, 75% of them were a result of osteoporosis

(Pouresmaeili, 2018). Other types of common osteoporotic fractures include vertebral, pelvic, and closed fractures of the humerus, radius, and ulna (Warriner, 2010).

Types of Osteoporosis There are presently two categories of osteoporosis. Primary osteoporosis is the more common form which includes osteoporosis occuring in post-menopausal women. Secondary osteoporosis is a result from medical disorders that affects a patient's bone mineral density as well as senile osteoporosis. Senile osteoporosis is due to a clear and definable etiologic mechanism, while primary (type I) is associated with reduced hormones leading to increased bone resorption. Furthermore, secondary osteoporosis (type II) occurs with gradual aging where there is a loss of stem-cell precursors, therefore a loss of bone growth and loss of bone (Dobbs, 1999). Some disorders include gastrointestinal diseases, hematologic disorders, and even hypogonadal states. Another factor could be a result of the medications taken when treating these diseases, such as glucocorticoids. Glucocorticoids affect the quantity and quality of bone, which could increase the risk of fractures in patients as a result of decreased bone mineral density (Lane, 2006).

Prevention and Treatment As it is increasingly affecting the ageing population, it is important to find ways in preventing and treating osteoporosis. Some treatments include preventing fractures and modifying general lifestyles that can reduce susceptibility to fractures. For example, optimizing the intake of calcium and vitamin D (Sandhu, 2011). It is recommended to eat a healthy diet of foods like fruits and vegetables that are rich in calcium, vitamin D, and protein. Practicing a healthy and balanced diet can help maintain not only overall health of a patient, but also minimize the

loss in bone and decrease of BMD (NIH, 2019). Foods that are rich in vitamin D include low-fat dairy products, dark green leafy vegetables, broccoli, and salmon. Vitamin D is important for minimizing loss in bone due to its role in absorbing calcium from the intestine (NIH, 2019). Furthermore, according to the NIH, steps and precautions that can be taken to prevent the onset of osteoporosis and fractures is by staying physically active, drinking alcohol in moderation, not smoking, and eating foods rich in calcium and vitamin D (NIH, 2019). Other treatment options to maintain and sustain osteoporotic fractures include encouraging weight-bearing exercise, intaking adequate calcium levels, exercise, and frequent physical examinations. It is recommended for patients 65 years and older to intake 400-800 IU/day of Vitamin D. For calcium, it is recommended that patients who are 4 years and older have an intake of 1000-15000 mg/day. For patients who are estrogen deficient, it is recommended to also have an estrogen intake of 0.625 mg/day or transdermal estradiol of 0.05 mg/day (Dobbs, 1999).

Present Study

This study investigates how genetic disposition and preexisting environmental comorbidities affects osteogenic bone cell growth and metabolic activity in vitro. It assesses the relationship between primary and secondary growth of bone marrow osteogenic cells in culture to the expression of osteogenic functions. The factors that are investigated relative in vitro osteogenic growth and activity are sex, age, race, BMI, vitamin D level, renal disease status, and smoker status.

Hypothesis: The first hypothesis is that comorbidities present at the time of marrow collection will have a greater impact on the primary cultures of a patient's

mesenchymal stem cells osteogenic ability than seen in sub cultivated cultures. The second hypothesis is that there will be a greater positive correlation between specific biochemical markers within the secondary cultures of marrow stromal cells as the osteogenic cell population becomes more homogeneous.

This study will provide more insight and understanding of osteoporosis by analyzing the biochemical markers of osteogenic bone cell growth and activity while considering various osteoporosis risk factors. It will shed light on those environmental comorbidities that have greatest effects on a patient's MSCS osteogenic abilities compared to underlying genetic factors.

METHODS

Cell Culture Procedure

MSC Culture Methods For each patient two vials containing 2 x 10 frozen unfractionated total marrow cells were used for initial culture plating per one 100 mm cell culture plate. Each 100 mm plate was evenly coated with 6.5 mL Animal Component-Free (ACF) Cell Attachment Substrate (Stem Cell Technologies Inc Cat #07130). The plates were then incubated for at least 2 hours at room temperature under UV lights in a Biocontainment tissue culture hood. The substrate was then aspirated off and the plate was washed with 10 mL of Dulbecco's phosphate-buffered saline (DPBS).

Two vials per patient frozen stocks of marrow were thawed in a 37°C water bath. Once they were thawed cell solutions were placed into 10 mL of artificial media, Mesencult-ACF Plus Medium (Stem Cell Technologies Inc Cat #05445) made 1X with Mesencult-ACF Plus 500X Supplement (Stem cell Technologies Inc Cat #05447). This media was then made to a final of 2 mM L-Glutamine and 1X with an antibiotic-antimycotic solution (Life Sciences Inc.). The cell suspensions were then thoroughly dispersed by vortex mixing for 10 second and pelleted by centrifugation at 1150 RPM for 5 minutes. The artificial media was aspirated off carefully and the cells were suspended in a new 10 mL of the above media.

Each patient sample was seeded in one 100mm culture plate that had been coated with attachment substrate as described above. The plates were then grown in a humidified incubator at 37°C 5% CO. After 4-5 days, a half media change was performed by removing half the volume of media and replacing it with fresh stem cell culturing media.

A full media change was done one week after plating. Every 2-3 days thereafter the media was changed for 2 weeks. After 3 weeks of growth the cells were trypsinized and plated into 12-well plates and grown in α-MEM + 10% fetal bovine serum (FBS) + 1% Pen-Strep for 2 weeks. At this time, they were trypsinized and replated at 50,000 cells per/ 4 cm^2 well of the 12-well plates. The growth media was then switched to osteoinductive media-MEM + 10% FBS + 1% Pen-Strep supplemented to a final concentration of with 8mM β-glycerophosphate, Dexamethasone 1x10M and 12.5µg/ml ascorbate. After 21 days, the cells were harvested for sequential assays. The Alkaline Phosphatase (ALP) Assay and (Alizarin Red S) ARS Quantification Assays were performed directly on the wells in the dish on day 21. The ARS Quantification Assay was done directly on the 12 well of the plates.

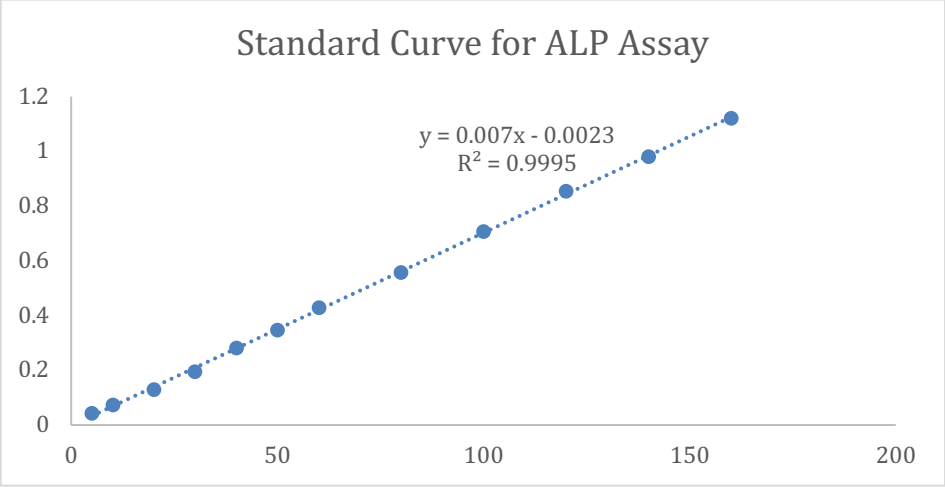
Biochemical Assays

All plate assays were read in a BioTek Cytation 1 plate reader. Individual plate characteristics and background assays were predetermined for all assays.

Alkaline Phosphatase Assay To run the assay, ALP assay buffer and ALP substrate were first prepared. The ALP assay buffer consisted of 0.01M glycine, 1mMgCl made pH 10.5 using NaOH. The buffer was filtered and ready to use or stored at 4° C for up to 6 months. The ALP substrate was made on the day of the assay by dissolving 20 mg p-nitrophenol phosphate disodium salt (Sigma #4876-1gm) per ml deionized (DI) water. 450µL of buffer and 50 microliters of substrate were added per well incubated in the dark for 30 minutes at room temperature. During incubation, a 96-well plate was set up to read for each set of ALP samples. Each plate was prepared containing a standard curve which

was prepared by diluting 10mM p-nitrophenol stock solution with the reaction buffer. Once 30 minutes of incubation was complete samples from each well were transferred to the 96-well plate containing the standards for assay (See Figure 1). To each well of the 96 well-plate 500µl of 0.2M NaOH was added to stop the reaction. The absorbance was then read at 410nm.

Figure 1- Standard Curve for ALP Assay. Representative image of ALP assay standard curve (Patient 231, 237)

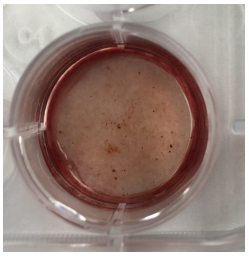
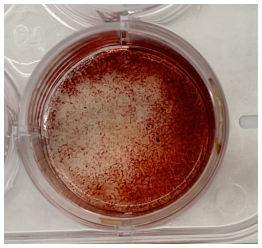
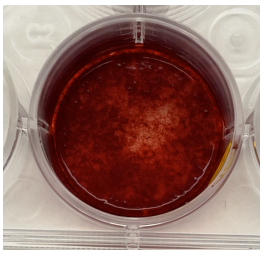


Cell Layer Extractions After removing the p-nitrophenyl phosphate, disodium salt (pNPP) solution from the sample plates, the plates were washed three times with 1mL/well D-PBS to remove any residual pNPP solution. Prior to harvesting the wells, one of the wells was set aside overlaid with D-PBS to run the Alizarin Red S Assay. To make 100mL of the extraction buffer, 38.17g of 4M Guanidine-HCl, 1mL of 1% Triton X-100 lysis buffer, 5 mL of 1x TE buffer pH 7.4 (20x solution, 200mM Tris-HCl, and 20mM EDTA) were dissolved in 100mL of DI water. To each sample well, 100-microliters of the extraction buffer was added and placed on a shaker for 30 minutes. While the plates were on the shaker, 2mL pressure-tight micro-centrifuge were labeled

with patient number and individual well numbers with 100-L of ultrapure H.O. After 30 minutes, a mini cell scraper was used to scrape the sample well plates. The insoluble material and extraction buffer were then transferred to 2mL pressure-tight microcentrifuge tubes. They were then stored in -80˚C freezer until ready to proceed to the subsequent assays.

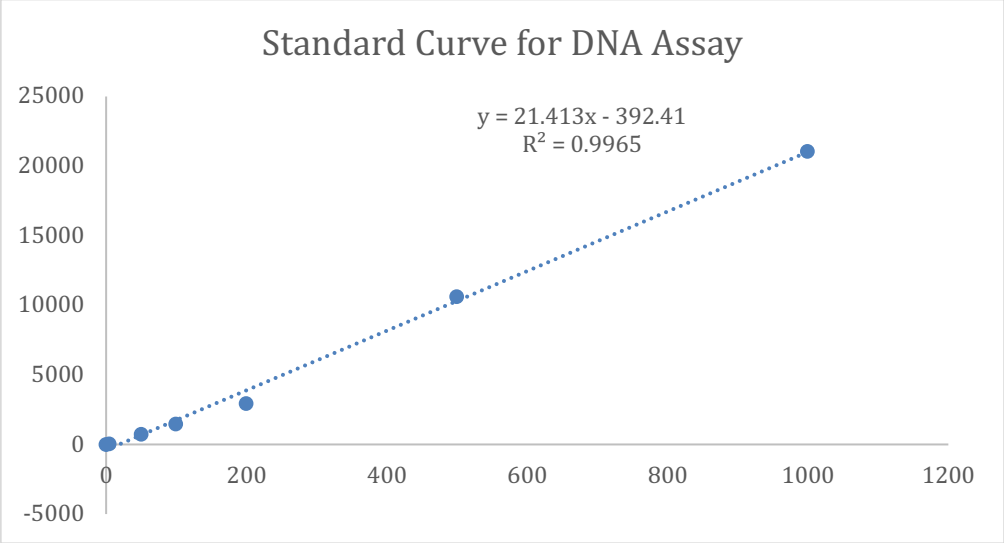
Alizarin Red S Quantification Assay To prepare the Alizarin Red Solution (2%), 2g Alizarin Red S (Acros Organics, Fisher #AC400481000) was dissolved in DI water per 100mL. The pH was then adjusted to 4 using 1% ammonium hydroxide solution and filtered. Fresh solutions were made every 2 weeks and stored at 4˚C. For the one well that was not harvested and side aside, the D-PBS was aspirated off. Then 2mL of 2% paraformaldehyde (PFA) is added to each well for 20 minutes. Afterwards, the 2% PFA was aspirated off and washed with D-PBS 3x. 2mL of 2% Alizarin Red Solution was then added to each well and left for 30 minutes. The solution was then aspirated off and washed 3x with D-PBS and 1x with DI water. Images of the nodules were taken and scored based on evidence of mineralization (See Figure 2).

Figure 2- Representative Qualitative Alizarin Red Nodule Assay Staining Scale Each stain was scored between 0 and 2. 0= no mineralization, 1= partial well mineralization, 2= uniform and complete mineralization (a) Patient 226, Score: 1 (b) Patient 232, Score: 1 (c) Patient 240, Score: 2

(a)  (b)  (c) 

DNA Assay The DNA assay was run first to minimize the effects of DNAses and since these assays required the smallest amount of sample. For these fluorescent assays black 96-well microplates were used. Prior to running the DNA Assay, the samples stored in -80˚C were retrieved and thawed on ice. Once the samples were thawed, they were spun in a centrifuge for 5 minutes at 12000rpm and 4˚C to spin out the insoluble matrix from the sample. 25µL of the soluble sample was removed from each microcentrifuge tube and transferred to the black 96-well plate. 75µL of 1x TE was added to each well to bring the sample volume 100µL. To prepare for the standard curve, the DNA standards were made with varying concentrations of DNA standard and 1xTE 100µL of each standard dilution were added to separate wells on the plate (See Figure 3). The Picogreen reagent (Molecular probes catalog #P-11496) was then diluted 1:200 in 1x TE and made the day of the assay. Due to its light and time sensitivity, it was wrapped in aluminum foil. 100µL of the diluted Picogreen reagents were added to each well, covered with foil, and placed on a shaker for 2-5 minutes. The plate was then read on a fluorescent plate reader at excitation/emission of 285/20, 530/25nm (See Figure 3). The optics position of the reader was top 50%, sensitivity 50, and height 7mm.

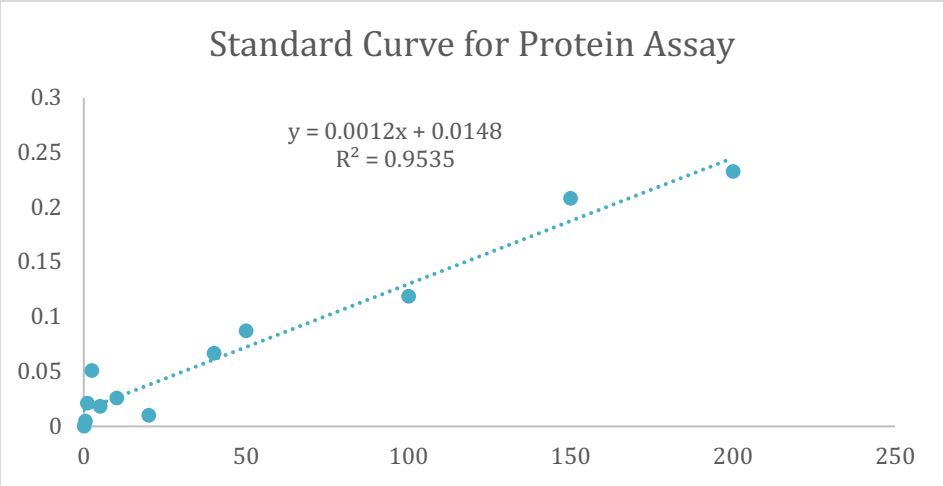
Figure 3- Representative image of DNA Plate assay and standard curve. Representative DNA assay standard curve (Patients 231, 237)



Protein Assay The protein assay was performed in a clear 96-well plate. 25µL of the soluble extract was first transferred to the 96-well plate from each microcentrifuge tube. As per the manufacturer's instructions, the working reagent (WR) for the protein assay was prepared using volume to volume ratios: 25:24:1 ratio of reagents MA:MB:MC from the Protein Assay Kit (Thermo Scientific Product No. 23225). For the total WR volume required for the assay, the formula (# of standards + # of unknowns + 2)(150µL WR) was used. The WR is not light sensitive but does need to be made on the day of assay. 125µL of 1X TE were added to each sample well and 100µL of 1X TE were added to each standard well. Then 25µL of extraction buffer was added to each standard well. 150µL of the WR was then added to each well and placed on the shaker for 30 seconds. The diluted Albumin (BSA) Standards are then prepared (See Figure 4). To prepare the set of protein standards, varying volumes of 1X TE and Vol/Source of BSA are used.

After the standards were made, 25µL of the standard were transferred to the wells in the 96-well plate that contained 1X TE and WR. The plate was then incubated at 37˚C for 2 hours and covered using a sealing tape. It was important to limit the incubations to less than or equal to 37˚C, otherwise there is a chance that high background and aberrant color development may occur. After 2 hours of incubation, plates were cooled to room temperature and read the absorbance at 562nm.

Figure 4- Representative image of Protein Plate assay and standard curve. Representative Protein assay standard curve (Patients 231, 237)

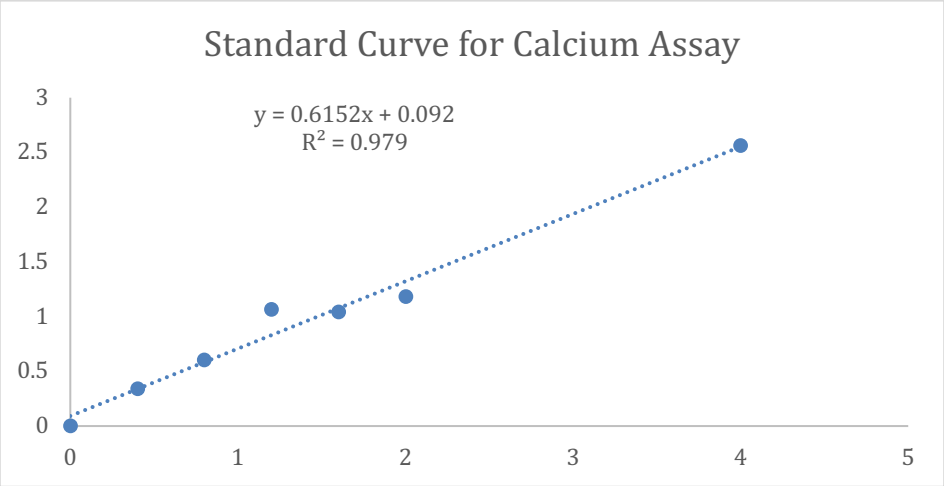


Hydrolysis of Insoluble Matrix The insoluble matrix in the microcentrifuge and the remainder of the soluble materials were then subjected to acid hydrolysis for the analysis of total calcium and collagen. 13.0µL of 12N HCl is added to each microcentrifuge tube and vortexed. This was left at room temperature for 30 minutes to allow for extraction. After 30 minutes, 55µL was removed for the calcium assay while the remaining sample was adjusted to final 6N HCL by adding 92µL of 12N HCl to the remainder of the samples in each microcentrifuge tube. Samples were placed in heating

blocks in the fume hood and hydrolyzed for 3 hours at 120˚C. After 3 hours, the microcentrifuge tubes were removed and cooled to room temperature. The samples were then spun down in a centrifuge to remove any particulate at 12000 RPM for 5 minutes at 18˚C. 200µL from each microcentrifuge tube were removed and transferred to a clear 96-well plate in preparation for the Hydroxyproline assay. This 96-well plate was first dried to remove the acid by incubating in a vacuum oven at 60˚C for 24 hours.

Calcium Assay The assay is performed using a clear 96-well microplate, using the Calcium Colorimetric Assay Kit (Sigma-Aldrich Catalog No. MAK022). Prior to starting, the calcium assay buffer (Sigma-Aldrich Catalog No. MAK022A) and chromogenic reagent (Sigma-Aldrich Catalog No. MAK022B) were allowed to come to room temperature before use. 55µL of calcium extract from hydrolysis of the insoluble matrix was transferred into the microplate. To prepare the calcium standard curve, 10µL of 400nM calcium standard stock (Sigma-Aldrich Catalog No. MAK022C) in 990µL of 1N HCl was made to a final concentration of 5nM of stock for the curve (0.2µg/µL) (see Figure 5). Once the standard was prepared, add 50µL of each standard to a separate well of the 96-well plate. After, add 60µL of calcium assay buffer followed by 90µL of chromogen to each well of the plate. Cover the plate with foil and incubated the plate at room temperature for 10 minutes. Then, read the absorbance at 575nm.

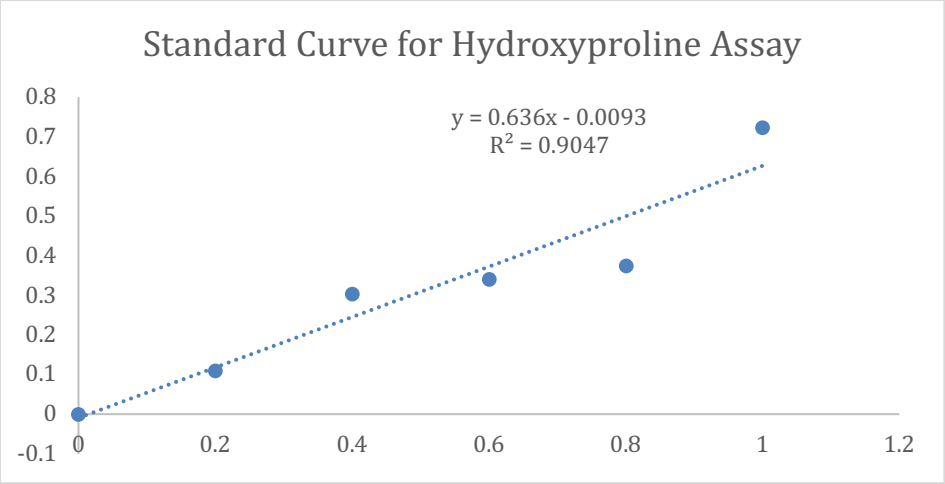
Figure 5- Representative image of Calcium Plate assay and standard curve
Representative Calcium assay standard curve (Patients 231, 237)



Hydroxyproline Assay This assay was performed in a clear 96-well microplate using a Hydroxyproline Assay Kit (Sigma-Aldrich Catalog No. MAK008). The oxidation buffer (Sigma-Aldrich Catalog No. MAK008A) and 4-(Dimethylamino) benzaldehyde (DMAB) (Sigma-Aldrich Catalog No. MAK008D) concentrate were used at room temperature. These two assay reagents are stable for 2-3 hours after preparation, so they should be prepared after sample preparation and just prior to the start of the assay. It should also be considered to make as much reagent that is needed for the number of samples and standards to be assayed. To prepare the Chloramine T/Oxidation Buffer Mixture, a total of 100µL per well was needed; therefore, 6µL of Chloramine T Concentrate (Sigma-Aldrich Catalog No. MAK008B) and 94µL of Oxidation Buffer were multiplied by the number of samples and standards to determine the amount needed. To prepare the DMAB/Perchloric Acid/Isopropanol Solution, a total of 100µL per well was needed; therefore, 50µL of DMAB concentrate and 50µL of Perchloric Acid/isopropanol (Sigma-Aldrich Catalog No. MAK008C) were multiplied by the number of samples and

standards. To make the hydroxyproline standards for colorimetric detection, 10µL of the 1 mg/mL hydroxyproline Standard Solution (Sigma-Aldrich Catalog No. MAK008E) was diluted with 90µL of ultrapure water to prepare a 0.1mg/mL standard solution. Then 0, 2, 4, 6, 8, and 10µL of the 0.1 mg/mL hydroxyproline standard solution was placed into the 96-well plate generating the 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1.0µg concentration per well standards. After, 100µL of the Chloramine T/Oxidation Buffer Mixture was added to each sample and standard well. This solution was then incubated at room temperature for 5 minutes. Then 100µL of the diluted DMAB Reagent was added to each sample and standard well (See Figure 6). This was allowed to be incubated for 24-48 hours at 60˚C. After incubation, the absorbance was read at 560nm.

Figure 6- Representative image of Hydroxyproline Plate assay and standard curve. Representative Hydroxyproline assay standard curve (Patients 231, 237)



Patient Demographics

Patients (N=10) in this study underwent total hip arthroplasty surgery at Boston Medical Center between 2019 and 2020, where the MSCs were directly harvested from

marrow harvested from remaining aspirates from the placement of the acetabular cup. The patients' ages ranged from 36 to 58 years old and had an average of 47 years old. For the purpose of this study, 10 patient samples were chosen based on those, which had previously been assessed for osteogenic function in primary cultures. Prior data for the primary MSC growth and osteogenic assay were derived from a prior study (Margaret Dunlap, Demographic Variation in Bone-Marrow Derived Mesenchymal Stem Cell Analytes, 2020 Masters of Medical Sciences Boston University Graduate Medical Sciences) and used to compare to the current study. Patients identified themselves as White (N=5), Black (N=3), Hispanic (N=1), Asian (N=0), and not available (N=1). The BMI of these patients ranged from 23.29 to 41.39. For the purpose of this study, those who had a BMI greater than or equal to 30 were categorized as overweight, while the remaining were categorized as healthy. The patients' vitamin D levels were also taken. Those with levels less than 19.9 (N=3) were considered deficient and those above 20 were considered within the normal range. In addition, it was noted whether they smoked or not, one patient identified as a smoker, three identified as non-smokers, and the remaining six identified as former smokers (See Table 1).

Table 1- Patient Demographics

Patient #	Sex	Age	Ethnicity	BMI (kg/m^2)	Diabetes	History of Steroid Use	Smoker	Vitamin D
226	M	50	N/A	27.31	No	No	No	NA
231	F	37	White	34.76	No	No	Former	13.4
232	F	52	African American	35.79	Pre-diabetes	No	Former	39.7
236	M	41	Hispanic or Latino	41.39	No	No	Former	14.1

237	M	54	White	31	Pre-diabetes	No	Former	26
238	F	56	White	38.03	No	No	no	24.4
240	M	36	White	23.29	No	Yes*	Former	40.8
242	M	58	African American	24.03	No	No	Yes	29.6
246	M	39	African American	28.87	No	No	No	10
247	M	47	White	30.63	No	No	Former	24.6

*Patient was on prednisone on and off

Biochemical Assay Data and Statistical Analysis

Any wells that resulted in values of 0 for a DNA measurement were excluded when taking the well averages across a patient plate. Prior statistical correlations to comorbidities for the primary cultures were based on the analysis presented in Dunlap 2020 while these findings involve a smaller data set, so our conclusions are preliminary in nature until the additional matched samples are assessed. For the analysis for each biochemical assay, the optical density of the blank was subtracted from the optical density as well as standard curve values that were measured. Once the subtracted values were found, they were graphed with the line of best fit to find the concentration of each well of the biochemical marker that was being measured. The concentrations found were then converted to nanograms or micrograms per mL, depending on the biochemical marker. Then the average of the 11 wells were taken to determine the patient's mean concentration values of DNA, ALP, calcium, hydroxyproline, and protein. To determine a significant difference in ALP, calcium, hydroxyproline, and protein normalized values between the primary and secondary cultures, a one-way ANOVA was conducted. The p-value was less than 0.05 to determine the significance in difference between the two cultures.

RESULTS

The first question that is addressed in this study is whether there is a greater environmental impact on osteogenic and mineralization potential in primary versus sub cultivated cultures. Therefore, a comparison between the patient's primary and secondary cultures was compared for significant difference in biochemical markers based on normalized values. Graphical assays for each of the assays are presented first while a summary of the statistical analysis is presented at the end of this section in Table 2.

The next question investigated in this study was to determine the positive correlation between DNA, ALP, calcium, and hydroxyproline. With an increase in MSC growth and mineralization, we hypothesized that there would also be an increase in the overall levels of the expressed biochemical markers. A comparison was made within each biochemical marker.

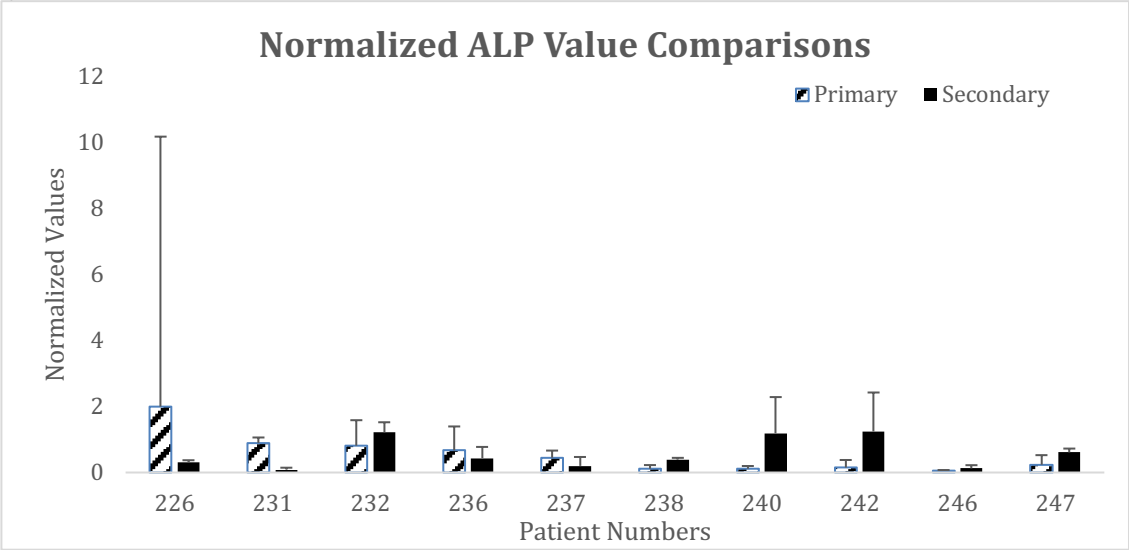
Biochemical Assay Variables Comparison

ALP There was no significant difference between the DNA normalized values of primary and secondary ALP values. The mean ALP normalized values for the primary and secondary cultures were 0.5501 and 0.5800 respectively (See Table 2). Because the F-ratio=0.0156 and p-value=0.9019, the results do not show significance at $p<0.05$. However, the overall concentration values were significantly different between the ALP concentrations of the two cultures. The mean ALP concentration values for the primary and secondary were 118.7607 nM/ml and 831.1413 nM/ml respectively (See Table 2). Because the F-ratio=9.2226 and p-value=0.0071, the results show significance at $p<0.05$. Therefore, with a large f-ratio value, we can reject the null hypothesis and conclude that

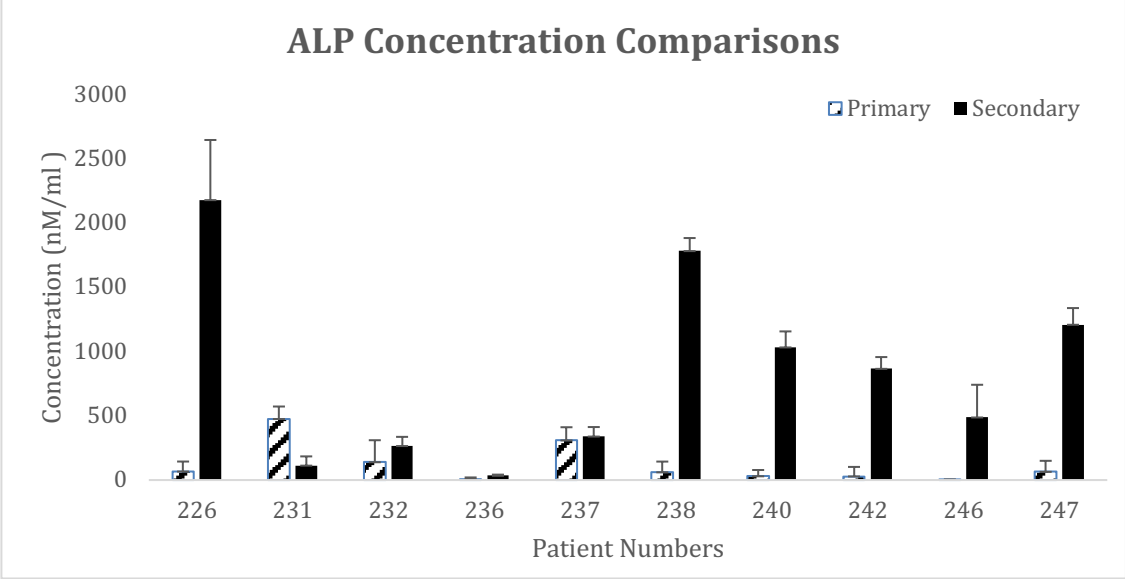
there is a significant difference in ALP concentration between the primary and secondary cultures (See Figure 7).

Figure 7- Representative Images of Primary and Secondary Culture Comparisons for ALP (primary culture data acquired from Margaret Dunlap's thesis (Demographic Variation in Bone-Marrow Derived Mesenchymal Stem Cell Analytes, 2020) A) Normalized ALP values comparison B) Concentration ALP values comparison

A)



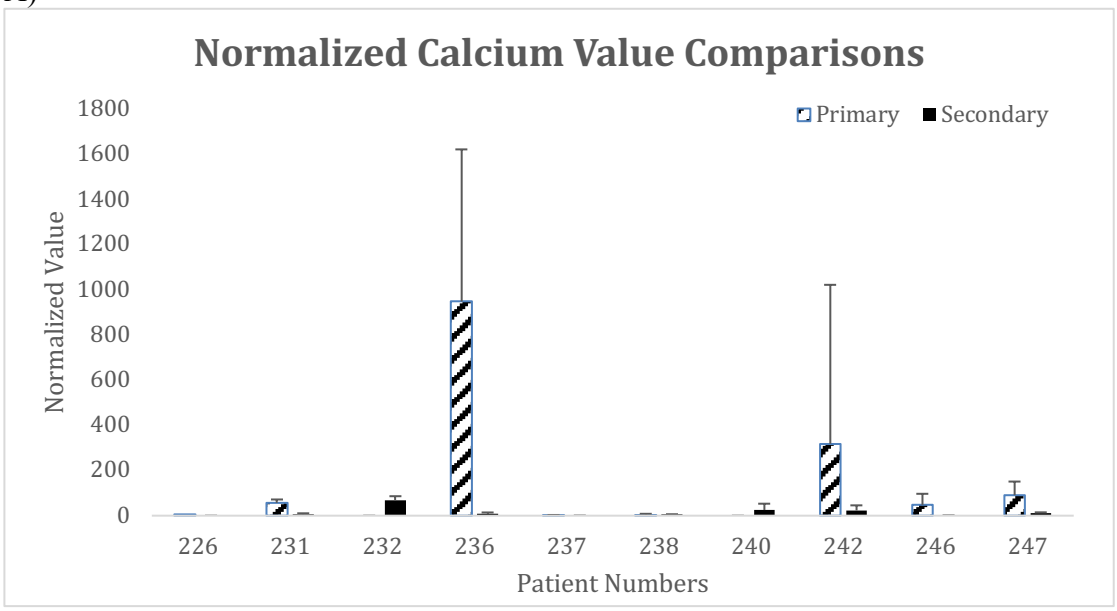
B)



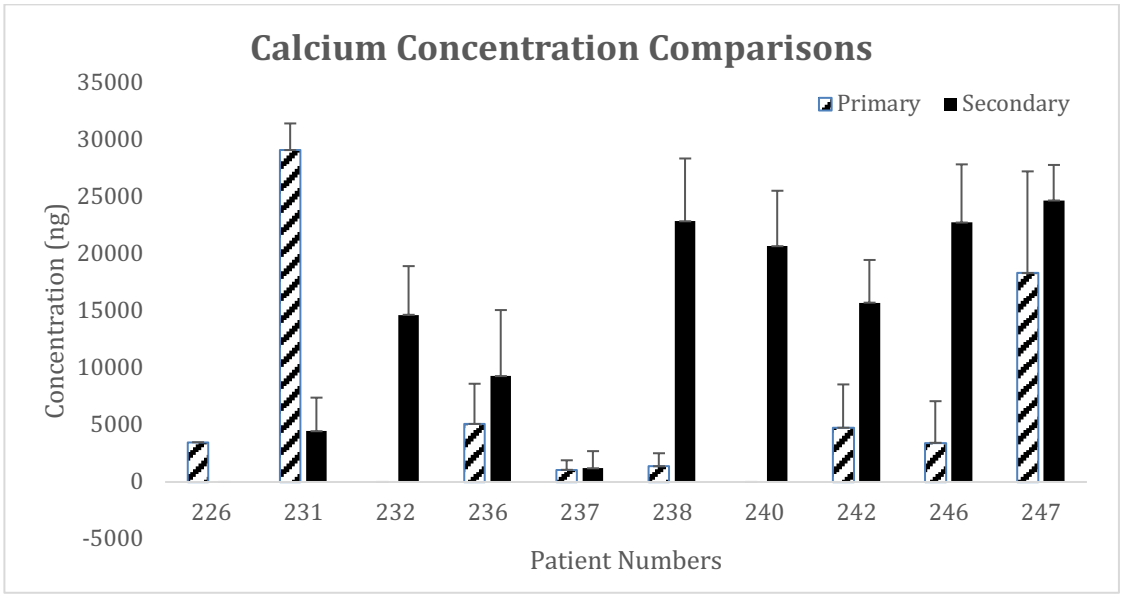
Calcium There was not a significant difference between normalized calcium concentrations. The mean calcium normalized values for the primary and secondary cultures were 147.1798 and 14.8552 respectively (See Table 2). Because the F-ratio=1.9668 and p-value=0.1778, the results do not show significance at $p<0.05$. Therefore, we cannot reject the null hypothesis and conclude that there is not a significant difference in calcium normalized values between the primary and secondary cultures. Furthermore, looking at the concentration values of the calcium, a significant difference between calcium concentrations was not evident after running an ANOVA. The mean calcium concentrations for the primary and secondary cultures were 6663.2604ng and 13628.5772ng respectively. Because the F-ratio=2.7241 and p-value=0.1162, the results do not show significance at $p<0.05$. Therefore, we cannot reject the null hypothesis and conclude that there is not a significant difference in calcium concentration between the primary and secondary cultures. Despite not showing significant difference, it can be noted that it is trending towards significance in difference (See Figure 8).

Figure 8- Representative Images of Primary and Secondary Culture Comparisons for Calcium (primary culture data acquired from Margaret Dunlap's thesis (Demographic Variation in Bone-Marrow Derived Mesenchymal Stem Cell Analytes, 2020) A) Normalized Calcium values comparison B) Concentration Calcium values comparison

A)



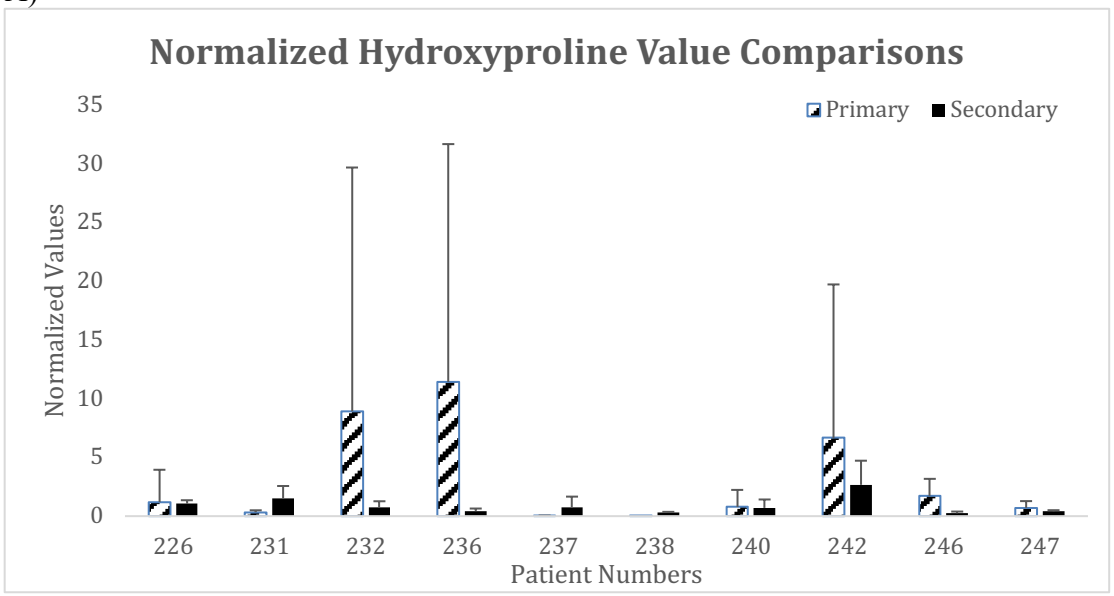
B)



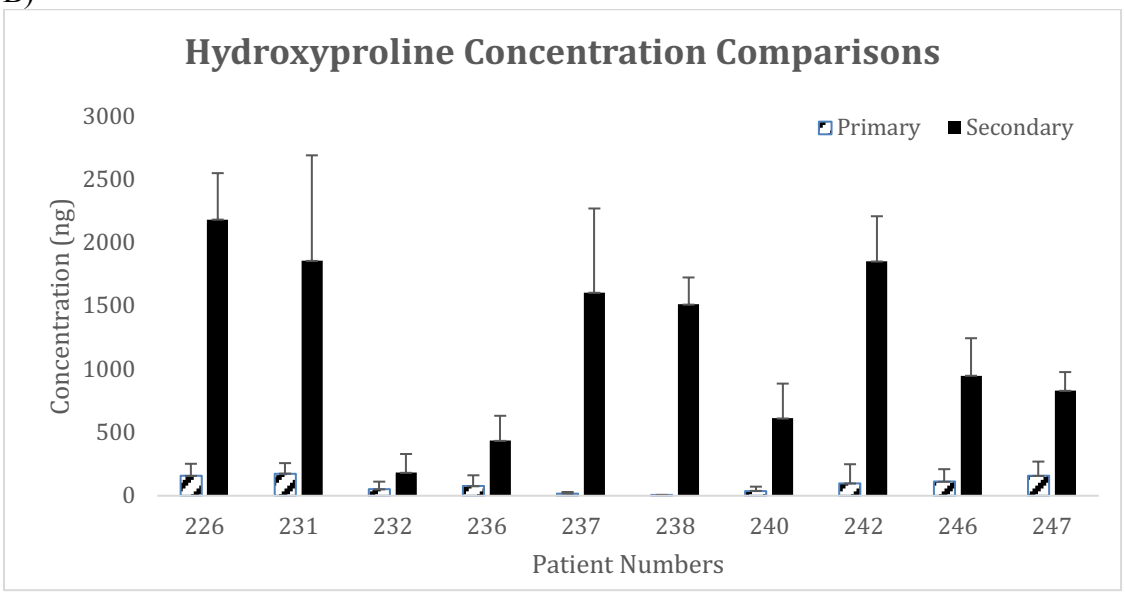
Hydroxyproline There was not a significant difference between the primary and secondary hydroxyproline normalized values. The mean hydroxyproline concentration for the primary and secondary cultures were 3.1753 and 0.8871 respectively (See Table 2). Because the F-ratio=2.88 and p-value=0.1069, the results do not show significance at $p<0.05$. Therefore, with a low f-ratio value, we cannot reject the null hypothesis and conclude that there is no significant difference in hydroxyproline normalized values between the primary and secondary cultures. However, looking at the concentration values, a significant difference was found between the primary and secondary cultures. There was a significant difference between hydroxyproline concentrations. The mean of hydroxyproline concentrations for the primary and secondary cultures were 89.4594ng and 1204.2877ng respectively. Because the F-ratio=25.9880 and p-value=0.0001, the results show significance at $p<0.05$. Therefore, with a large f-ratio value, we can reject the null hypothesis and conclude that there is a significant difference in hydroxyproline concentration between the primary and secondary cultures (See Figure 9).

Figure 9- Representative Images of Primary and Secondary Culture Comparisons for Hydroxyproline (primary culture data acquired from Margaret Dunlap's thesis (Demographic Variation in Bone-Marrow Derived Mesenchymal Stem Cell Analytes, 2020) A) Normalized Hydroxyproline values comparison B) Concentration Hydroxyproline values comparison

A)



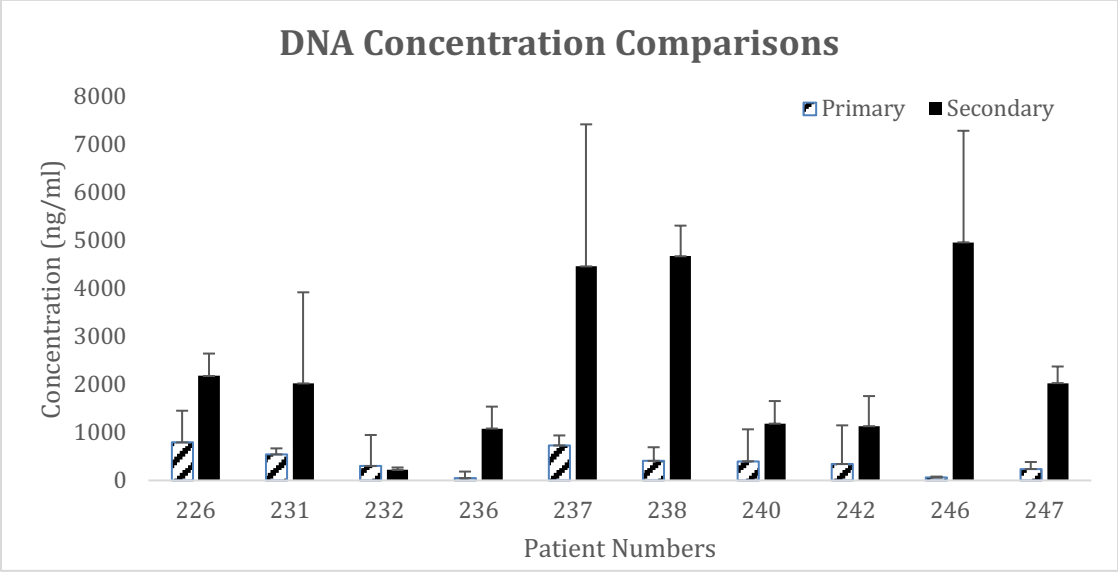
B)



DNA To investigate the difference in mineralization of the MSC cultures between primary and secondary culture, the concentration in DNA was measured. Although a

direct comparison cannot be made with concentration values, it is worth noting that there was a significant difference in DNA concentration between the two cultures. An ANOVA was run to determine significance differences. The mean DNA concentrations for primary and secondary cultures were 386.8162 ng/ml and 2397.4118 ng/ml respectively. As the f-ratio=13.7252 and had a p-value=0.0016, the results showed significance at $p<0.05$ (See Figure 10). Therefore, with a large f-ratio value, we can reject the null hypothesis and conclude that there is a significant difference in DNA concentration between the primary and secondary cultures.

Figure 10- Representative Image of Primary and Secondary Culture Comparisons for DNA (primary culture data acquired from Margaret Dunlap's thesis (Demographic Variation in Bone-Marrow Derived Mesenchymal Stem Cell Analytes, 2020) Concentration Hydroxyproline values comparison

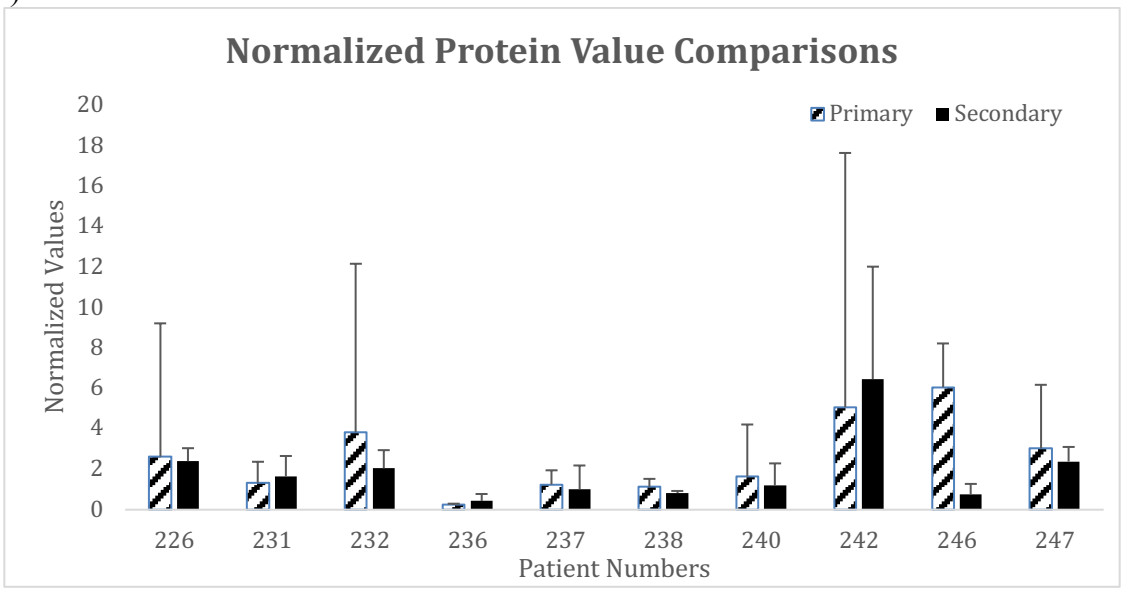


Protein There was not a significant difference between the primary and secondary protein normalized values. The mean of normalized protein values for the primary and secondary cultures were 2.6171 and 19.1382 respectively (See Table 2). Because the F-ratio=0.7552 and p-value=0.3963 the results do not show significance at

p<0.05. Therefore, with a low f-ratio value, we cannot reject the null hypothesis and conclude that there is no significant difference in protein normalized values between the primary and secondary cultures. However, looking at the concentration values, a significant difference was found between the primary and secondary cultures. There was a significant difference between protein concentrations. The mean protein concentrations for the primary and secondary cultures were 4629.7150 µg/ml and 27791.1010 µg/ml respectively. Because the F-ratio=16.3859 and p-value=0.0008, the results show significance at p<0.05. Therefore, with a large f-ratio value, we can reject the null hypothesis and conclude that there is a significant difference in protein concentration between the primary and secondary cultures (See Figure 11).

Figure 11- Representative Images of Primary and Secondary Culture Comparisons for Protein (primary culture data acquired from Margaret Dunlap's thesis (Demographic Variation in Bone-Marrow Derived Mesenchymal Stem Cell Analytes, 2020) A) Normalized Protein values comparison B) Concentration Protein values comparison

A)



B)

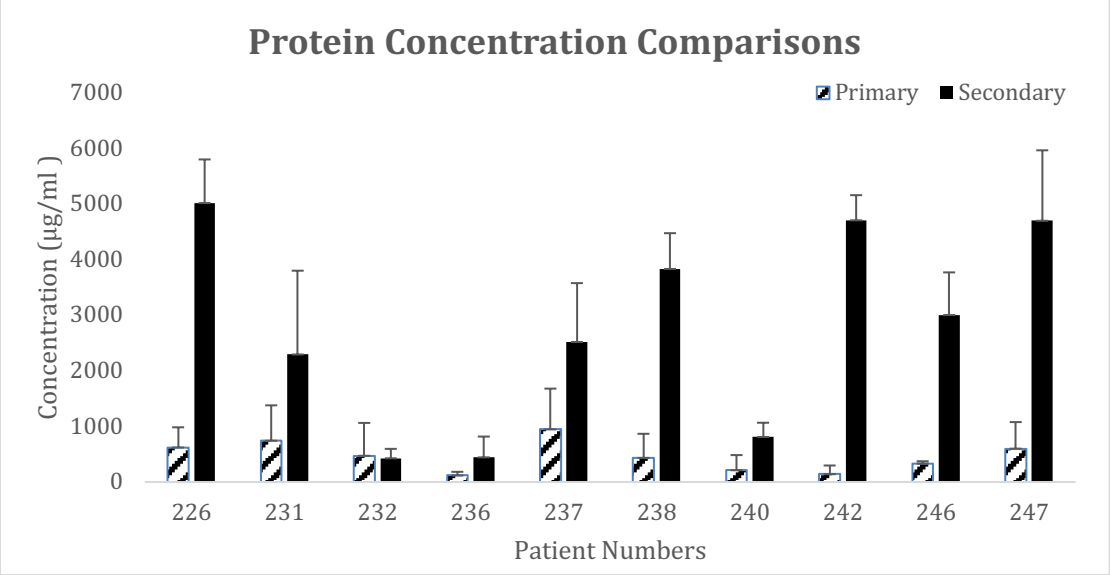


Table 2- ANOVA Statistical Analysis for Normalized and Concentration Values of Each Biochemical Assay

		Primary	Secondary
ALP	**N**	10	10
	Total Sum	5.5014 (1187.6070 nM/ml)	5.8001 (8314.1301 nM/ml)
	Mean	0.5501 (118.7607 nM/ml)	0.5800 (831.1413 nM/ml)
	Standard Deviation	0.5962 (154.6165 nM/ml)	0.4652 (725.7949 nM/ml)
	Standard Error	0.1885 (48.8940 nM/ml)	0.1471 (229.5165 nM/ml)
	f-ratio value	0.0156 (9.2226)	
	p-value	0.9019 (0.0071)	
Calcium	**N**	10	10
	Total Sum	1471.7980 (66632.0640 ng)	148.5516 (136285.7700 ng)
	Mean	147.1798 (6663.2604 ng)	14.8552 (13628.5772 ng)
	Standard Deviation	297.661 (9510.5993 ng)	20.5798 (9361.9914 ng)
	Standard Error	94.1287 (3007.5156 ng)	6.5079 (2960.5216 ng)
	f-ratio value	1.9668 (2.7241)	

	p-value	0.1778 (0.1162)	
Hydroxyproline	**N**	10	10
	Total Sum	31.7527 (894.5944 ng)	8.8714 (12042.8770 ng)
	Mean	3.1753 (89.4594 ng)	0.8871 (1204.2877 ng)
	Standard Deviation	4.2012 (60.8329 ng)	0.7279 (688.8660 ng)
	Standard Error	1.3285 (19.2371 ng)	0.2303 (217.8386 ng)
	f-ratio value	2.88 (25.9880)	
	p-value	0.1069 (0.0001)	
DNA (Concentration values only)	**N**	10	10
	Total Sum	3868.1620 ng/ml	23974.1180 ng/ml
	Mean	386.8162 ng/ml	2397.4118 ng/ml
	Standard Deviation	249.1153 ng/ml	1698.0119 ng/ml
	Standard Error	78.7772 ng/ml	536.9585 ng/ml
	f-ratio value	13.7252	
	p-value	0.0016	
Protein	**N**	10	10
	Total Sum	26.1710 (4629.7150 µg/ml)	19.1382 (27791.1010 µg/ml)
	Mean	2.6171 (462.9715 µg/ml)	1.9138 (2779.1101 µg/ml)
	Standard Deviation	1.8771 (271.2575 µg/ml)	1.7396 (1788.9309 µg/ml)
	Standard Error	0.5936 (85.7792 µg/ml)	0.5501 (565.7096 µg/ml)
	f-ratio value	0.7552 (16.3859)	
	p-value	0.3963 (0.0008)	

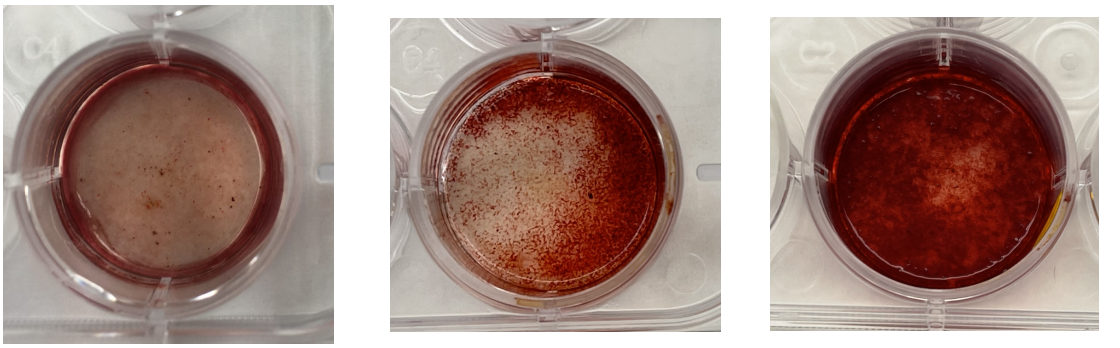
*Values in parenthesis are concentration values for that biochemical assay

Alizarin Stain Nodules Though there were no primary cultures to use as a comparison, the secondary cultures showed Alizarin staining and nodule formation suggesting significant evidence of mineralization for all patients. As described in the

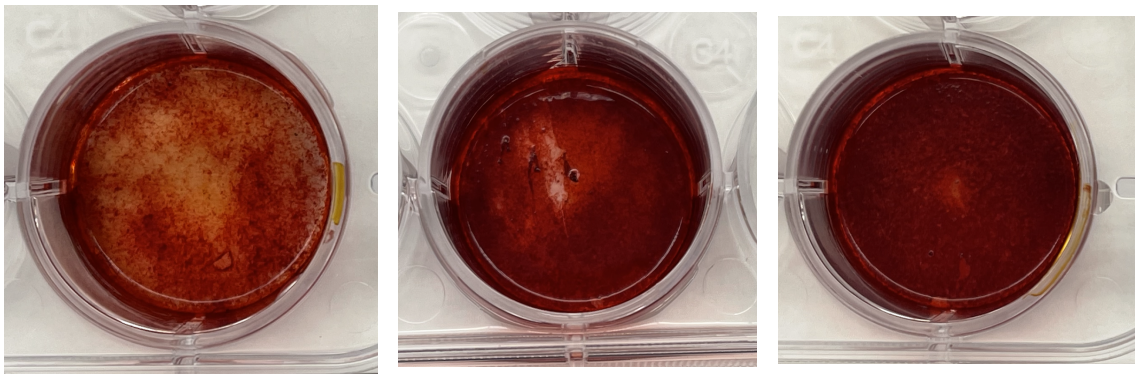
methods section, Alizarin staining was scored from 0 (no staining) to two (well completely stained) (See Figure 12).

Figure 12- Alizarin Stain Nodules Scored Each stain was scored between 0 and 2. 0= no mineralization, 1= little mineralization, 2= complete mineralization (a) Patient 226, Score: 1 (b) Patient 232, Score: 1 (c) Patient 240, Score: 2 (d) Patient 242, Score 2 (e) Patient 246, Score 2 (f) Patient 247, Score 2

(a) (b) (c)



(d) (e) (f)



Biochemical Assay Variables

The second question proposed in this study was to determine the positive correlation within the biochemical markers. Therefore, a Pearson correlation coefficient test was run comparing DNA, ALP, calcium, and hydroxyproline for correlation within the group.

When comparing DNA to ALP, a weak positive correlation was found. The value of R=0.189 and since it is closer to the value of zero, it has a weak relationship. DNA compared to calcium had a value of R=0.0837. This also has a R value closer to zero, meaning they have a weak, but positive correlation. DNA and hydroxyproline also had a weak positive correlation with the R=0.3513. When ALP was compared to calcium, it was found that they also have a weak positive correlation with a R value = 0.1396. ALP was also compared to hydroxyproline and it was found that they also have a moderate positive correlation with the R value = 0.4426. Lastly, calcium was compared to hydroxyproline and it was found that they have a negative moderate correlation, with a R value = -0.4915 (See Table 3).

Table 3- Pearson Correlation Coefficient Results Testing for Correlation Within Biochemical Assays

	DNA (ng/ml)	**ALP (nM/ml)**	**Calcium (ng)**	**Hydroxyproline (ng)**
Total Sum	23974.1180	8314.1301	136285.7700	12042.8770
Mean	2397.4118	831.1413	13628.5772	1204.2877
R value	**DNA + ALP:** 0.189 **DNA + Calcium:** 0.0837 **DNA+ Hydroxyproline:** 0.3513 **ALP + Calcium:** 0.1396 **ALP + Hydroxyproline:** 0.4426 **Calcium + Hydroxyproline:** -0.4915			
R^2 value	**DNA + ALP:** 0.0357 **DNA + Calcium:** 0.0088 **DNA+ Hydroxyproline:** 0.1234 **ALP + Calcium:** 0.00195 **ALP + Hydroxyproline:** 0.1959 **Calcium + Hydroxyproline:** 0.2416			

DISCUSSION

Comparison Between Primary and Secondary Cultures

To determine the extent to which environmental comorbidities affect the expression of osteogenic biochemical markers and mineralization of marrow stromal cultures primary and secondary cultures were compared. It was hypothesized that there would be a significant difference between the primary and secondary cultures in terms of normalized values within each biochemical marker of the primary and secondary cultures. However, it was found that when comparing the normalized assayed values there was no significance. However, when comparing the overall concentration values of DNA, ALP, calcium, hydroxyproline, and protein, it can be seen that there were much greater concentration values in the secondary than primary cultures.

Biochemical Markers

Total DNA and protein content were measured to evaluate the overall growth and metabolic activities of the primary and secondary cultures. The assessment of DNA content between the primary and secondary cultures showed that there was an overall greater growth of the secondary cultures compared to the primary cultures. . Interestingly when examining the normalized protein values the normalized values showed no significant differences between the primary and secondary cultures suggesting that the overall metabolic activity of the cultures were the same. These data suggest that there was an impact on the overall cell number and/or initial growth of the primary MSC cultures, possibly due to the patient comorbidities. However, on a per cell basis the overall metabolic activity was similar between the primary and secondary MSC cultures.

The examination of parameters that assessed osteogenic activity included alkaline phosphatase enzyme activity, overall accumulation of collagen via measurements of hydroxyproline content, and total accumulation of minerals via the measurements of calcium. When looking at the means of the normalized values of ALP, there was no significant difference between the primary and secondary cultures while the overall mean values of the secondary culture was slightly increased. However, the difference in concentrations obtained from the biochemical assays showed significant differences. The secondary culture had a mean of 621.98 nM/ml, while the primary only had 11.8105 nM/ml. The inconsistency between the normalized value and the evidence of increased mineralization and growth could be due to the presence of outliers within the individual plate assays. As an example the DNA values obtained for patient 226 ranged from 0.831 to 204.374 thus producing extreme normalized ALP values ranging from high to low concentration values over varying DNA values skewing the overall values for the data. Furthermore, having a small sample size would further exacerbate outliers within the data.

The calcium data obtained from the secondary culture had a greater normalized mean value compared to the primary culture with 162.2523and 34.7161 respectively. However, this difference was not significant. The calcium concentration prior to normalization was also not significant. However, it is worth noting that the mean calcium concentration for the secondary culture was increased compared to the primary culture with a mean of 3344.994 and 1521.4807 respectively. The lack of significant difference in the values could be due to a lack of data for a few of the patients. Although

the primary cultures were not grown and analyzed in this project the larger number of wells showing a zero-concentration value is clearly the result of the broader variability in expansion of stem cells or progenitors within individual wells that is lost when these cells are expanded and replated. When conducting this experiment again, it would be helpful to have a greater sample size so that the data can be compared over a much larger sample size.

Although it was hypothesized that the secondary cultures would have greater normalized values than secondary values, this was not evident in the hydroxyproline normalized values. In fact, the primary cultures had a greater normalized mean value than the secondary. Upon further inspection however, it can be seen that the secondary hydroxyproline concentrations had a greater mean than the first. One possible difference for the overall differences was that the secondary cultures were grown for one week less than the primary cultures under mineralizing conditions.

Further mineralization analysis was conducted with the Alizarin Nodule Stain. It was evident that layers of mineralization occurred within the wells of the plate, as they stained completely. For those wells with lack of stains, it was consistent with decreased values in DNA, ALP, calcium, hydroxyproline, and protein acquired for the patient. For future analyses, alizarin stains could be run for both primary and secondary cultures and compared side-by-side to reduce variabilities that occur at running the experiments at separate times.

Correlation Within Biochemical Markers

The second hypothesis within this study was determining if there were positive correlations between the biochemical markers, DNA, ALP, calcium, and hydroxyproline. A Spearman rho test was run to determine correlation and it was found that within all the biochemical markers, they had positive, but weak correlations with the exception of calcium and hydroxyproline. However, for calcium and hydroxyproline a negative correlation was observed. This correlation is significant though and consistent with what is observed as mineralization proceeds both in vivo and in vitro as the organic fraction of bone material decreases as mineral is deposited in the matrix (Gerstenfeld, 1987).

Issues, Limitations, and Future Direction

The recurrent issue within this study is the small sample size and lack of consistency between the data in the primary and secondary cultures. Having a small sample size allowed for a much greater range in concentration values, including outliers that skewed the data, especially for normalized values. In addition, although comorbidities were noted such as diabetes, BMI, and smoking status, there could be other factors that may have contributed to the variability of bone mineralization. Furthermore, as this was the first time growing out cells from frozen stock, there were issues with growing out cells at the start of this study. When repeating this experiment, protocols in growing out frozen cells will be more solidified, allowing for better growth and mineralization for all patients. Furthermore, conducting these two experiments at two separate times could have caused additional mishaps, resulting in varying data for the biochemical assays. In addition, some variability in the data could have risen from the assay kits. Problems with the calcium and protein assays could have led to

uninterpretable data. For example, issues with the protein standards could have resulted in errors with calculating the concentration values. Therefore, given the limitations, results should be interpreted with caution.

This study examined the impact of comorbidities on patients by comparing mineralization of primary and secondary subcultures of MSCs. Initially the hypothesis was that there would be a significant difference between the primary and secondary cultures; however, data showed that in fact there was no significant difference between the two subcultures. On the other hand, when observing just the concentration values, the data showed drastic increases in concentration values from secondary to primary cultures. Furthermore, the second hypothesis predicted that there would be positive correlations among the biochemical markers like DNA, ALP, calcium, hydroxyproline, and protein, and showed that although they were weak correlations, there were in fact positive correlations, with the exception of calcium and hydroxyproline. As osteoporosis continues to be a major and increasing health problem for the ageing populations, it is important to understand the mechanisms and pathogenesis of osteoporosis, so that interventions for both individuals and communities can utilize effective prevention methods and treatment options (Wark, 1993).

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