Change in Bone Mineral Density among High Frequency Apheresis Blood Donors

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CHANGE IN BONE MINERAL DENSITY AMONG HIGH FREQUENCY APHERESIS BLOOD DONORS

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A Dissertation submitted to the Faculty of the Graduate School,
Marquette University,
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy

Milwaukee, Wisconsin
August 2018

ABSTRACT

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Exposure to citrate anticoagulant during apheresis blood donation induces significant decreases in serum ionized calcium with subsequent perturbations to parathyroid hormone, vitamin D, and markers of bone remodeling. Cross-sectional studies of bone mineral density (BMD) among apheresis donors exhibit conflicting results. Resolving the potential impact of the highest apheresis donation frequency represents a significant knowledge gap in ensuring adequate protections for volunteer apheresis blood donors. ALTRUYST (NCT02655055) was a randomized, longitudinal, controlled clinical trial designed to determine if repeated exposure to citrate through apheresis donation reduces BMD. Male donors, 18-65 years of age with no more than five previous apheresis donations and no diseases of bone or mineral metabolism, agreed to make ≥20 apheresis donations in the subsequent one year period. Dual-energy x-ray absorptiometry was performed at baseline and again after one year of participation. Paired t-test was used to assess change in mean BMD. Donors in the apheresis arm (n=26) made a median of 20 donations (range 4–22 donations) during the one-year study period with a mean donation interval of 17.8 days. Controls (n=15) made zero apheresis donations and a median of two whole-blood donations (range 0-6). Mean lumbar spine BMD at the end of the study period did not differ significantly from that at the beginning among donors in the control arm (mean change=-0.002 g/cm^2, 95% CI [-0.020, 0.016], p=0.78), nor did it change significantly among donors in the apheresis arm (mean change=0.007 g/cm^2, CI [-0.005, 0.018], p=0.24). Change in mean BMD at the total hip was not statistically significant for control donors (mean change=0.002 g/cm^2, CI [-0.006, 0.009], p=0.63) or apheresis donors (-0.004 g/cm^2, CI [-0.10, 0.002], p=0.16). Tests for differences in proportions of donors with change in BMD exceeding the least significant change (LSC) at the lumbar spine (0.00743 ±0.02058g/cm^2) between the apheresis and control arms in either a positive [apheresis 13 (50%), control 5 (33%), p=0.84] or negative direction [apheresis 8 (31%), control 6 (40%)] were statistically non-significant (p=0.87). Proportional increases [apheresis 6 (23%), control 6 (40%), p=0.25] and decreases [apheresis 11 (42%), control 3 (20%)] were not significantly different (p=0.15) at the total hip (LSC=0.00671±0.01859g/cm^2).

Table of Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

Apheresis blood collections produce life-saving therapies and represent an increasing fraction of all blood derived components in clinical use today. Approximately four apheresis derived platelet products were transfused in 2004 for every whole blood derived platelet product (1) (Table I). Six years later, the use of apheresis derived platelet products had increased to 10 apheresis derived platelet products transfused for every whole blood derived product (2). These data illustrate an increase in the demand for volunteer apheresis blood donors in the United States. This demand requires increasing the number of donors undergoing apheresis, increasing the frequency of apheresis procedures per individual donors, and/or increasing the number of units derived per donation.

YEAR	Hospitals		Blood Centers		Total		RATIO Apheresis : WBD
	Apheresis	WBD	Apheresis	WBD	Apheresis	WBD	
2004	681	184	44	10	725	194	3.7 : 1
2006	1,196	248	36	9	1,232	257	4.8 : 1
2008	1,399	195	34	38	1,433	233	6.2 : 1
2010	1,953	197	17	2	1,970	199	9.9 : 1

Table I. Summary data from *National Blood Collection and Utilization Survey Reports* showing the number of transfused apheresis-derived and whole blood-derived (WBD) platelet products for participating hospitals and blood centers from 2004 to 2010. Values are reported as thousands of doses (e.g. x 10^3).

Table II shows the relationship between the number of apheresis platelet and red blood cell procedures performed and the corresponding number of units derived over a six year period in the United States (1-4). While the number of apheresis red blood cell collections has increased, the proportion of products derived from each collection has remained unchanged (53% in 2004; 49% in 2010). In contrast, both the number of apheresis platelet procedures and the number of units yielded per procedure had increased. An additional 200,000 platelet apheresis procedures were performed in 2010 as compared to 2004; an increase of 17%. There was also an increase in the number of platelet product units derived from 1.3 per procedure in 2004 to 1.9 per procedure in 2010. Optimization in manufacturing practice might have played a modest role in this observed increase in blood product yield per procedure; however, blood collecting organizations were likely increasing the number of units derived from an individual donation. Operationally, apheresis donor recruitment strategies often focus efforts on retaining donors willing to donate often and who are capable of giving multi-product donations in part because the number of donations in the previous year has a positive association with donor return (5). These patterns of apheresis blood collection emphasize the importance of understanding the long-term health impacts of apheresis on blood donors.

YEAR	Apheresis Red Blood Cells		Plateletpheresis	
	Procedures Performed	Units Yielded	Procedures Performed	Units Yielded
2004	434	†824	1,164	1,527
2006	-	1,619	1,167	1,823
2008	1,022	1,926	1,352	2,130
2010	976	1,978	1,340	2,516

Table II. Summary data from *National Blood Collection and Utilization Survey Reports* from 2004 to 2010 showing the estimated number of apheresis procedures performed and number of corresponding component units yielded for participating hospitals and blood centers. Values are reported as thousands of units (e.g. x 10^3). †Estimate based on consultation with participating blood centers and average number of units produced per apheresis procedure.

Apheresis red blood cell collection guidelines are based on FDA criteria for allogeneic whole blood donation, which mandates a minimum of eight weeks between single- and 16 weeks between double-red blood cell donations [21 CFR 640.3(b) and 640.12]. Subsequent eligibility for donation is based on total red blood cell loss at the time of collection with a maximum loss of 1,540 mL per rolling 12 month period. Platelet apheresis donation guidelines are currently founded on studies showing that frequent apheresis platelet donors are able to maintain platelet counts within the normal reference range (6-8). These platelet apheresis studies supported an AABB comment and FDA policy increase in the number of apheresis donations an individual volunteer donor can make from 12 to 24 per rolling 12 month period with no lifetime maximum (9, 10). Federal regulations on paid source plasma donors allow 110 apheresis donations in a rolling 12 month period [21CFR640.65(8)]. Despite a wealth of information on the physiology of the apheresis donation experience, fundamental data are still needed to inform policy that maximizes donor health and maintains the national blood supply.

In contrast to whole blood collections, apheresis procedures require the use of intravenous (I.V.) citrate anticoagulation. Cross-sectional studies have reported that intermittent exposure to I.V. citrate is associated with significant declines in bone mineral density (BMD) (11, 12) or no changes at all (13). In contrast to I.V. citrate, oral potassium citrate formulations of much lower dose have been used to treat low BMD with well-documented efficacy (14). The impact of I.V. citrate received during apheresis, either positive or negative, is important given that BMD is a significant risk factor for low trauma fracture, a problem that affects more than two million people in the U.S. annually (15). The cross-sectional studies that have been performed on apheresis donors have important limitations that call into question the validity of the reported relationship between citrate exposure and BMD. Thus, it is ultimately unknown what effect repeated exposure to I.V. citrate has on skeletal health. As demand to preferentially transfuse apheresis blood products continues to increase, the importance of understanding the effects of repeated exposure to citrate on the skeletal health of volunteer donors is essential.

Hypotheses

Null Hypothesis: high frequency apheresis blood donation does not cause declines in BMD after a one year follow-up period.

Alternate Hypothesis: high frequency apheresis blood donation causes declines in BMD after a one year follow-up period.

Objectives

The objective of this project was to assess the impact of I.V. citrate exposure through apheresis blood donation on BMD using dual-energy x-ray absorptiometry. Our central hypothesis was that frequent, repeated exposure to I.V. citrate causes declines in BMD. Based on data from treatment of low BMD with oral citrate, we further hypothesized that infrequent exposure to I.V. citrate may be associated with improved BMD. The rationale for conducting this study was based on the need to obtain empirical data that will help ensure the health of volunteer donors, or, to develop protocols that protect members of this valuable community resource.

Specifically, we studied two groups: 1) donors who, for the first time, begin undergoing a series of apheresis blood donation procedures during the follow-up period, and, 2) healthy whole blood donor controls. This was a randomized, controlled study. Blinding was achieved through the use of a blinded external reviewer of all outcome data. The use of the same blood donor as their own control represented a simple and validated approach to account for variation in BMD in the general population, thereby avoiding fundamental design limitations of previous cross-sectional studies. We anticipated that findings from this study would serve as the foundation for larger, randomized trials addressing the role of repeated apheresis on BMD in blood donors, or, confirm that current blood collections standards are safe for donors.

CHAPTER TWO: BACKGROUND

Apheresis Blood Donation

In contrast to donating whole blood, making a blood donation by apheresis permits the collection of individual blood components such as platelets, plasma, or red blood cells. Despite longer average collection times, the blood collection community benefits immensely by allowing an individual donor to come back more frequently than every 56 days (in the case of platelets and plasma), decrease post-collection manufacturing costs, and optimizing transfusion therapy by matching donor-recipient attributes before a unit of blood is ever collected. Furthermore, transfusion of an apheresis-derived blood product minimizes recipient exposures to foreign antigens, thereby mitigating the risk of alloimmunization. During apheresis, whole blood exits the body through traditional venipuncture and is processed in an extracorporeal circuit that involves centrifugation, separation, and return of un-harvested blood components back to the donor. Normal blood coagulation would commence in this environment but is suppressed with anticoagulant (AC) treatment.

Apheresis blood collection guidelines are determined at the national level. This has resulted in some disparities by country in terms of the maximum number of allowable apheresis procedures per annum by an individual as well as the minimum inter-donation interval by component type. Of note, there is no maximum number of lifetime donations an individual donor can make as long as they continue meeting local donor eligibility criteria. Thus, regardless of your country of donorship, you may achieve the same number of lifetime donations as your expatriate counterpart.

The current inter-donation intervals for common blood donation procedures in the United States are provided in Table III. The maximum amount of time between apheresis blood donations is a matter of personal preference, so long as that interval is no shorter than that permitted by local regulations. It is possible that an individual volunteer blood donor can make as many as 26 apheresis blood donations in a one-year period. This is based on the assumption that the donor makes 24 single platelet apheresis donations and two double red blood cell donations. The primary limiting factor in terms of the number of allowable donations is based on blood donor plasma volume depletion. Platelets require some volume of plasma for suspension before transfusion. Similarly, a double red blood cell product does indeed contain a very small volume of plasma. Therefore, the small amount of plasma removed from the body is multiplied by the relatively high frequency of allowable apheresis procedures and leads to donor deferral for exceeding donated plasma volumes.

Donation Type	Inter-Donation Interval (days, based on average maximum by year)
Whole Blood	56
Double Red Blood Cells	112
Plasma	30
Platelets	14

Table III. Comparison of the average number of days between allowable blood donation by donation type in the United States. Platelet donations may occur more frequently than every 14 days, though the per year maximum number of allowable donations in the United States is 24.

The duration of an individual apheresis blood donation procedure is determined by a number of factors. A typical double red blood cell procedure can take approximately 40 minutes, but this depends on factors such as the donor's hematocrit (proportion of formed elements in the blood), their blood volume, as well as their ability to tolerate the procedure itself (16). Similarly for the collection of platelets, the amount of time that an individual spends donating is a function of their circulating platelet count, the number of component products they are donating (single, double, or triple platelet), and the ability to tolerate the procedure (17). A donor's ability to tolerate the procedure is heavily influenced by the effects of the AC solution (18, 19).

As whole blood leaves the site of venipuncture, citrate AC is administered before the extracorporeal blood enters the centrifuge housing. The amount of AC an individual donor receives during a given apheresis blood donation procedure depends, first, on the type of apheresis machine being used. There are continuous and intermittent flow devices used in apheresis blood collection with intermittent flow devices having slightly less total citrate burden to the donor (assuming all other determinants equal) (20). Because this disparity in citrate burden is relatively inconsequential, it is also one of the least important considerations when a blood collecting agency is determining which type of apheresis machine to use for collections. The result is that both types of machines are currently in use in the United States, as well as around the world.

In addition to the type of apheresis machine, the donor's total blood volume is an important determinant of the amount of AC administered during an apheresis procedure (21). Donors with smaller blood volumes, such as women, are not able to dilute the same amount of AC over as large a blood volume as their male counterpart. This corresponds

to an increased concentration of active AC in the blood at any given time, making women more likely to experience adverse side effects associated with citrate anticoagulation (22). This example illustrates the importance of concentration of citrate on the overall burden of citrate the donor experiences.

Another determinant in the concentration of citrate in the blood is the AC-to-whole blood ratio (AC:WB). At higher proportions of AC per unit of whole blood, apheresis blood collection times are decreased. The total blood collection time is also affected by the inlet pump rate, which in combination with AC:WB, determines the overall length of an apheresis procedure. Donors who can tolerate higher AC:WB ratios do not require as high of an inlet pump rate as a donor who is more sensitive to experiencing side effects associated with citrate anticoagulation. In contrast, sensitive donors often require a larger volume of whole blood to be processed which alters the inlet pump rate. The fact is that each apheresis blood collection is highly personalized and subject to values imputed in embedded algorithms, donor tolerance of citrate AC, and the overall goal of the apheresis procedure itself (i.e. the number of component products derived).

Finally, the type of blood component being collected modulates AC exposure. It has been shown that as much as 85% of the citrate introduced in the extracorporeal circuit is actually diverted to the actual apheresis plasma product (23). Thus, donors making apheresis plasma donations are experiencing a significantly reduced citrate burden as compared to those who are making plateletpheresis or double red blood cell donations. Apheresis plasma collections represent the most common form of apheresis in many European countries, whereas apheresis plasma products are less frequently obtained in

the United States and platelet collections by apheresis are common. Therefore, any long-term studies of the effect of AC on health outcomes in apheresis donors should carefully consider the type of apheresis procedure occurring, as well as the frequency of those collections.

Citrate Anticoagulation

Anticoagulant citrate has been used for decades to confer protection against extracorporeal thrombosis during apheresis. Citrate's mechanism of action involves binding ionized calcium and subsequently interrupting thrombus formation in the extracorporeal circuit. Many variables determine the amount of citrate an individual apheresis donor is exposed to, including the type and duration of an individual apheresis blood donation, as well as the number of lifetime donations made by an individual donor. Unlike many alternative AC solutions such as heparin, anticoagulation with citrate is superior in terms of pharmacokinetic half-life, risk of hemorrhage, and cost. Each molecule of citrate has three carboxylic acid subgroups that are negatively charged. As a result, the molecule itself confers anticoagulant properties by binding divalent metal cations, such as calcium, that are essential cofactors in the intrinsic clotting cascade. With the third carboxylic acid subgroup still ionized, complexed citrate remains solubilized in human blood and does not confer the risk of precipitation (24). The half-life of citrate is approximately 36 minutes (25). It undergoes rapid metabolism through the tricarboxylic acid cycle in organs rich with mitochondria such as the liver and skeletal muscle. What is not metabolized is simply excreted in complexed form in the distal convoluted tubule of the kidney nephron.

Exogenous citrate spends a variable amount of time in the extracorporeal circuit during apheresis. Nevertheless, the longest interval between administration and entry into the donor's vasculature is less than ten minutes and most of the citrate is still in its active form. This has been shown in several studies where the concentration of citrate was monitored in apheresis blood donors. There is an initial surge of citrate in the donor, many times greater than normal biological concentrations, in the minutes following apheresis initiation (22, 26). The concentration of citrate continues to increase throughout the procedure as more cycles of blood processing occur (22, 26). As expected, the concentration of citrate in peripheral blood plateaus at the termination of apheresis and begins declining rapidly thereafter. All residual citrate is metabolized or excreted within the 24 hour period after the termination of AC exposure (22).

As predicted by citrate's mechanism of action, the concentration of serum ionized calcium in the donor's blood decreases immediately upon the first return cycle of the apheresis machine (26). This indicates that there is active citrate that has not yet complexed with calcium in the extracorporeal circuit. This biologically active form of citrate begins to sequester ionized calcium in the peripheral circulation. Throughout the procedure donor peripheral blood continues to experience declines in serum ionized calcium concentrations to levels that are slightly below the normal healthy range (27). It is this decline in serum ionized calcium, as well as general blood cooling in the extracorporeal circuit, that is responsible for the adverse reactions experienced by apheresis blood donors (28).

Rates of citrate infusion can vary, though the industry standard range for ACD-A and ACD-B are between 1.0 and 1.8 mL/kg/min (29). It is well documented that doses of

citrate used in apheresis platelet collections positively correlate with serum citrate concentration during apheresis procedures (22, 26, 30). Routine apheresis collection procedures elicit a spike in serum citrate from 0.11mmol/L (±0.04) at baseline to 1.61mmol/L (±0.3). Because the concentration of citrate in peripheral blood is dose-dependent (26), the derivation of multiple apheresis blood products corresponds to a larger dose of IV citrate in the blood donor. Citrate's half-life in the circulation is approximately 36 minutes (25) and donors are able to fully metabolize exogenous citrate from apheresis collections within 24 hours of exposure (26, 31).

Modern apheresis machines report the amount of anticoagulant used after completion of an apheresis procedure. The common range of anticoagulant volume administered during an apheresis procedure is 200 – 800mL. Apheresis platelet collections can occur very frequently, up to 26 times per year, and are increasing in both number and duration. Thus, it is important to consider the effects of IV citrate exposure, both acutely and over time, in the blood donor population.

Citrate Physiology

Citrate's role as a metal chelating agent that binds divalent cations, such as calcium and magnesium, has been thoroughly characterized (32). Like endogenous citrate, sodium citrate dihydrate and citric acid in acid-citrate-dextrose (ACD) solutions chelate calcium ions in the blood by forming calcium-citrate complexes that disrupt coagulation (33, 34). Studies documenting changes in circulating divalent cation concentrations in apheresis blood donors have shown that there are similarities across donation type. In a study by Szymanski, 79 volunteer donors undergoing typical

apheresis procedures employing citrate anticoagulation demonstrated an average 22% decrease in serum ionized calcium (iCa; from 4.19 mg/dL to 3.27mg/dL) when comparing pre- and immediately post-procedure blood samples (30). Average decreases of 33% and 39% in circulating iCa and ionized magnesium (iMg), respectively, were observed in another study of volunteer blood donors undergoing plateletpheresis (26). Hester and colleagues showed that donors with a blood volume of four liters undergoing typical platelet apheresis procedures with citrate anticoagulation experienced decreases in iCa of 15% at 10 minutes and 31% at 90 minutes (27). Similar findings were reported for a study of healthy leukapheresis donors, where serum iCa and iMg decreased 35% and 56%, respectively (35). Ionized calcium is a tightly regulated molecule both intra- and extracellularly. Donors generally tolerate decreases in concentrations of iCa up to 20% before experiencing side effects (36) with women having a greater sensitivity to declining concentrations than men (22).

Figure I provides a simplistic diagrammatic representation of the movement of calcium throughout the body in a prototypical, healthy, adult individual over the course of one day. Much of the 1,000mg of ingested calcium from food is excreted in the feces. The 200mg net gain of calcium from the G.I. tract is lost in the urine. Calcium from metabolically active trabecular bone is in homeostasis.

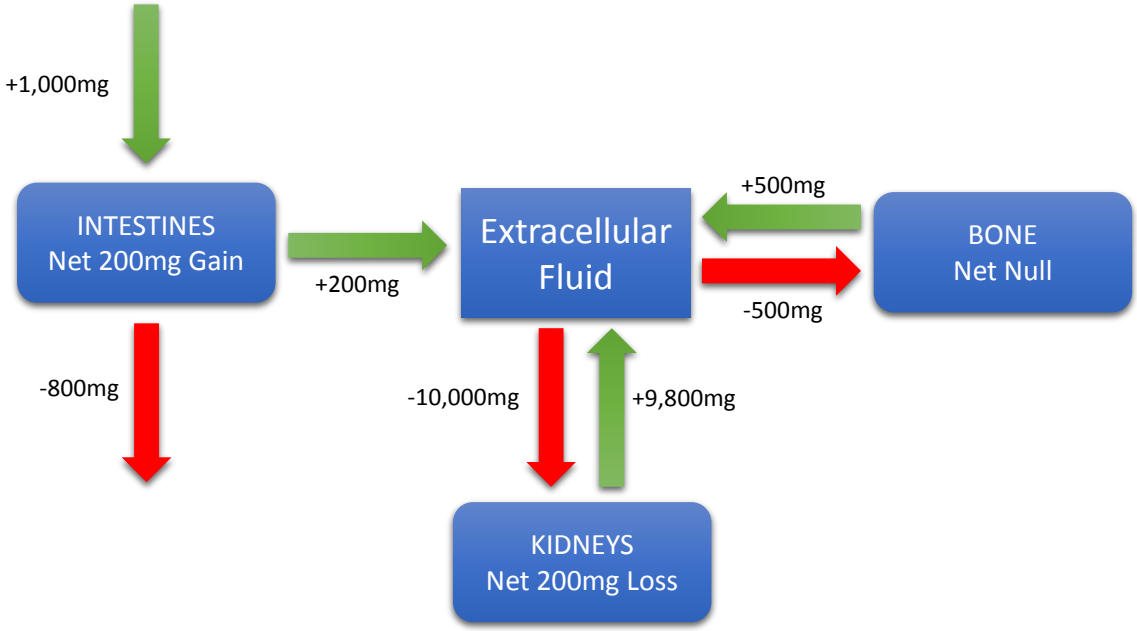


Figure I. The movement of total body calcium throughout the course of one day in a prototypical, healthy, adult individual whose calcium status is in balance.

G-protein coupled receptors on the surface of the parathyroid glands and kidneys directly sense declines in iCa concentration in the blood and stimulate secretory cells to release parathyroid hormone (PTH) (37). Alterations to blood ionized calcium concentrations are carefully monitored in the body due to calcium's central role in many biological functions. Monitoring occurs at the parathyroid glands; four pea-sized structures immediately adjacent to the thyroid gland at the base of the neck. G-protein coupled receptors, the calcium sensing receptors (or CaSR), located on the surface of chief cells are bathed in peripheral blood. In the absence of sufficient ionized calcium, these proteins change conformation and rapidly trigger the synthesis of PTH (37). The secretion of PTH into circulation is extremely rapid and can occur within minutes of a biological stimulus (38). Therefore, in the case of individuals with relatively low

concentrations of ionized calcium in the bloodstream, we anticipate high circulating concentrations of PTH as illustrated in Figure II.

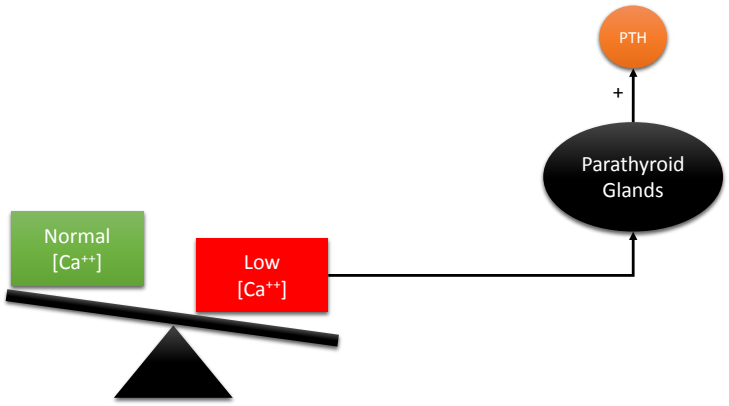


Figure II. Schematic representation of the stimulus, decreased ionized calcium in the bloodstream, affecting the secretion of PTH by the parathyroid glands.

PTH has three primary effector organs in the body and all are associated with replenishing the supply of ionized calcium in the bloodstream (Figure III). In the kidneys, PTH directly stimulates the reabsorption of filtered calcium in the nephron. PTH also stimulates 1-α hydroxylase that ultimately converts 25-hydroxy (OH) vitamin D to the active metabolite 1, 25 di-hydroxy ((OH)2) vitamin D. Increasing 1, 25 (OH)2 vitamin D levels upregulate calcium ATP-ase channels in the intestinal epithelium and promote calcium absorption. Finally, PTH stimulates osteoblasts to secrete the ligand for receptor-activator of nuclear factor kappa B (RANK-L) that, along with macrophage colony stimulating factor (MCS-F) lead to the terminal differentiation of hematopoetic precursors to active osteoclasts. Active osteoclasts liquefy the bone matrix through a process called resorption. Hydrochloric acid dissolves the mineral-rich hydroxyapatite that is subsequently associated with the release calcium into the bloodstream.

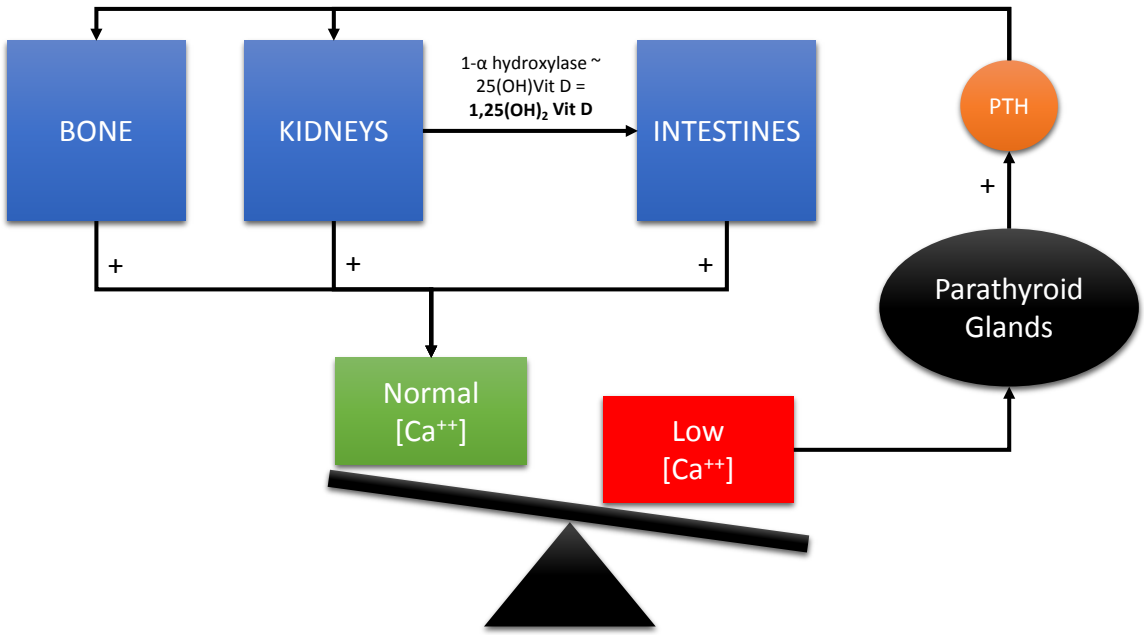


Figure III. The relationship between effector organs of secreted PTH and their role in replenishing low ionized calcium in peripheral blood.

PTH directly stimulates the reabsorption of filtered calcium in the kidney nephron. Renal control of reabsorption is under both osmotic and electrochemical control. Calcium is a divalent cation that can be specifically regulated in the kidney through the manipulation of the electrochemical gradient. Selective reabsorption of calcium requires that positively charged molecules be excreted in order to maintain balance. In the case of hypocalcemia, the nephron is stimulated by PTH to reabsorb the positively charged cation. In exchange, the body excretes positively charged phosphate ions into the filtrate leading to phosphaturia.

The influence of continuous exposure to citrate on the release of PTH was assessed in 12 healthy donors undergoing platelet apheresis donation in a study by Toffaletti, et al. They demonstrated that PTH reached maximum serum concentrations

within 5-15 minutes of the start of citrate infusion (38). This has been corroborated in a number of platelet apheresis donor studies (11, 26, 31, 39). Initial PTH surges in apheresis donors are short-lived and PTH concentration returns to near-baseline as early as 30 minutes after the infusion of citrate is terminated (26, 39). One study has shown that PTH may remain elevated up to one day after the procedure despite a termination in exposure (11).

The release of PTH into circulation simultaneously triggers all three of the body's main mechanisms to restore normal iCa: increased calcium reabsorption in the distal tubules of kidney nephrons, increased intestinal calcium absorption through a Vitamin D mediated pathway, and increased bone resorption (Figure III). Increases in serum PTH increase calcium reabsorption in the distal convoluted tubule. Citrate exposure through apheresis, however, increases urinary loss of calcium (40) despite concomitant increases in PTH. Loss of calcium in the urine has been shown to be citrate dose-dependent (26) and occurs during the 24 hour period after exposure to citrate (31, 35). Calcium reabsorption in the kidney is 98% efficient under normal conditions, suggesting that replenishment of iCa losses during apheresis will be minimal.

PTH also stimulates the activation of Vitamin D, which in turn increases intestinal absorption of calcium. For platelet apheresis donors, concentrations of activated Vitamin D have been shown to decrease 9% from baseline immediately after standard platelet apheresis. One day following the exposure to citrate, activated Vitamin D exceeds baseline concentrations by an average of 26% (31). Through a Vitamin D mediated pathway, some calcium can be replenished in apheresis donors through small intestine absorption (37). But despite providing large amounts of calcium as supplements, this

study showed that donors were not able to recover 100% of baseline iCa concentrations through this mechanism. Furthermore, calcium supplementation is, in practice, symptom dependent and not routinely employed outside of the setting of this particular study. Thus, the apheresis donor's body may rely on metabolically active trabecular bone to recoup lost calcium.

Bone Remodeling

Bone is a biological structure comprised of relatively few cell types and associated proteins. However, terminal differentiation of these cell types coupled with incessant metabolism make the human skeleton an incredibly dynamic human tissue. This constant evolution of organic matrix and mineral underpin the essential role of the human skeleton in human structure and function. Homeostasis of the human skeleton is determined by the cell types whose changing roles determine the various types of bone. Osteoblasts play the role of secreting organic proteins that form the structure upon which mineral apatite deposits. Alterations to osteoblast function affect the skeleton by decreasing its density and ability to resist fracture. Bone resorbing osteoclasts serve the crucial role of helping to maintain systemic mineral homeostasis, but can cause significant problems with skeletal integrity if disproportionately activated. Few studies have explored the impact of apheresis on skeletal remodeling.

Bone is in a constant state of remodeling that is a coupled process linking the resorption of bone by osteoclasts and the deposition of new bone by osteoblasts. The deposition of bone is performed by osteoblasts: mesenchymal stem cell-derived cells under the master regulation of RUNX-2 (41). Bone deposition involves the secretion of the organic matrix elements of bone, namely Type 1 collagen, by osteoblasts on the

surface of bone. Collagen assembles into helical fibrils that form cross-links leading to an overlapping structure. The alignment of collagen fibrils is offset, leaving a small proportion of the fibril exposed and creating a potential nucleation site for mineral deposition (42). Mineral deposition is spontaneous and involves substituting subgroups of the hydroxyapatite molecule with various minerals which lead to the creation of apatite. Osteoblasts either undergo apoptosis, or, are embedded within the newly formed matrix and undergo terminal differentiation to osteocytes.

The interplay between bone resorption and deposition is so tight that indeed, the very cells that deposit bone are essential to the stimulation of the cells that resorb it. Upon stimulation by various cytokines, osteoblasts secrete the ligand for receptor activator of nuclear factor kappa B (RANK-L). RANK-L, along with macrophage colony stimulating factor (MCS-F), bind receptors on hematopoetic osteoclast precursors stimulating fusion, multinucleation, and differentiation into osteoclasts. These very large cells form a tight bond on the surface of bone called the sealing zone. Hydrochloric acid is subsequently secreted to dissolve the mineral element of bone, apatite (43). Apatite is rich in important minerals such as calcium and magnesium. Cathepsin K is also secreted (44), leading to the breakdown of type 1 collagen, the primary organic component of bone. Osteoclast plasma membranes express a unique morphology during this process by forming highly convoluted festoons called the ruffled border that increase the surface area of the cell maximizing the absorption of the newly liquefied components of bone (45). Migration of these components through the cell and into peripheral circulation illustrates the incredibly important role that bone serves in maintaining systemic mineral homeostasis.

In addition to secreting osteoclast stimulating factors, osteoblasts secrete a decoy receptor protein called osteoprotegerin. This decoy receptor can sequester otherwise potent activators of osteoclast differentiation (46). In effect, osteoblasts have complete control over osteoclast activation by manipulating the relative concentrations of these substances in the immediate proximity of active bone metabolism. This known phenomenon may represent an optimal therapeutic target for diseases of osteoclast over expression, however, RANK-L is non-specific and such therapies could have significant off-target effects.

There are several markers of bone resorption, however only a few have been measured in apheresis donors. C-terminal telopeptides, such as β-CTX, are both sensitive and specific measures that quantify the breakdown of type 1 collagen (47). In a randomized, placebo-controlled study of blood donors, citrate infusion increased serum concentrations of β-CTX in apheresis donors whereas controls not receiving citrate had no change in their serum β-CTX ($p < 0.0001$ for between-exposure comparisons) (48). This finding held true for donors in another study where both serum and urine concentrations of β-CTX were elevated by as much as 26% and 17%, respectively, and remained elevated for up to 24 hours post-donation (11). The greatest measured increases in β-CTX have been observed at the completion of an apheresis procedure (48) suggesting that bone resorption begins during exposure to citrate. When concentrations of β-CTX are compared to concentrations of osteocalcin (OC), a protein secreted by bone-forming osteoblasts, the proportion of these two markers throughout the procedure increases suggesting that bone metabolism may shift toward resorption during apheresis.

Additional data in the weeks following apheresis are needed because formation occurs at the end of the remodeling cycle.

Phosphate is also an essential component of hydroxyapatite in bone. As elevations in PTH stimulate the dissolution of hydroxyapatite by osteoclasts, release of phosphate from bone increases. PTH simultaneously reduces reabsorption of phosphate ions in the proximal convoluted tubule of kidney nephrons, allowing excess phosphate to be excreted and ensuring that concentrations of serum phosphate do not exceed normal physiologic concentrations. Serum phosphate concentrations decrease modestly during apheresis and return to pre-apheresis concentrations within the 24 hour period following apheresis (11). However, the concentration of fibroblast growth factor (FGF) 23, the protein that stimulates the expression of sodium-phosphate co-transporters in the nephron, has not been measured in apheresis blood donors.

There is some evidence that exposure to citrate from apheresis actually favors bone deposition, not resorption. OC has been shown to remain slightly elevated at 24 hours post-apheresis donation (31). Furthermore, concentrations of osteoprotegerin (OPG), an inhibitor of the maturation of bone degrading osteoclasts, were observed to decrease following 120 minutes of citrate exposure and recover to baseline at 24 hours post-exposure (11). Tartrate-resistant acid phosphatase (TRAP), an enzyme expressed by osteoclasts, has been shown to be a useful marker of bone resorption because of its limited variability in vivo (49, 50). In apheresis platelet donors TRAP was observed to be lower than baseline at both 120 minutes and 24 hours post-exposure suggesting that apheresis acutely suppresses bone resorption. The authors do not address the paradoxical nature of this finding, especially considering their claim that a finding of lower bone

density in apheresis donors relative to controls is a “true finding”. It should be mentioned that a limitation of using TRAP to assess bone resorption in healthy people may be the inability to make meaningful interpretations when threshold concentrations below that of pathological conditions are not met (51).

The process of remodeling bone is a process that occurs over a 4-6 month period. The availability of physiological data in apheresis blood donors spanning an inter-donation interval of two weeks is relatively sparse. Rather than creating a complete profile of prolonged effects of IV citrate exposure in apheresis donors, researchers have begun looking into bone-related health outcomes. Consequently, the opportunity exists to fully catalog apheresis blood donor physiology in the weeks following IV citrate exposure. With some studies showing evidence of bone remodeling 14 days after exposure to citrate (52), it is difficult to use the available data to predict long term effects on bone health in this donor population. More careful characterization of the effects of exposure duration, and especially frequency, is needed as the body’s recovery following exposure has not been sufficiently studied.

Steddon and Cunningham (53) noted in their review of calcium receptor manipulation therapies that short periods of elevated PTH favor bone formation by means of expediting the maturation of osteoblasts. Further, it has been conceptualized that large and rapid increases in PTH followed by normalization, such as that stimulated by calcilytic drug therapies, may translate in bone-anabolic effects (54). Finally, we should not ignore that oral potassium citrate is a common treatment for low bone density with documented efficacy (14). Thus, intermittent exposure to citrate through apheresis blood donation could theoretically have beneficial effects on bone. The conclusions of many of

these studies have been derived from clinical trials of postmenopausal women only, all of whom have declining estrogen. Because of estrogen's central role in bone metabolism, the generalization of these findings to apheresis blood donors warrants very careful attention and additional research.

Citrate anticoagulation affects calcium homeostasis which underlies the concern about potential declines in bone density. Possible long-term health effects of IV citrate exposure during apheresis may have important implications given that decreased bone density is a significant risk factor for low-trauma fractures. If repeated citrate infusion during apheresis procedures adversely affects bone density, it is likely that the frequency and/or amount of citrate exposure correlate with the severity of bone catabolic effects. Results from a bone density study of 102 apheresis platelet donors with a lifetime average of 85 apheresis procedures (range 16 – 633) as compared to non-blood donor controls demonstrated significantly lower bone density at the lumbar spine (Z-score P=0.038) for apheresis donors as compared to controls (11). No significant differences in bone density were seen at the hip and femoral neck (Z-score P=0.36 and P=0.72, respectively). The authors conclude that donor-specific differences in bone metabolism are unlikely, but that these donors differ in the way they regulate mineral homeostasis when challenged metabolically by exposure to citrate. It is not clear if the authors intend this to be a donor-specific predisposition or some type of response-mediated adaptation to citrate exposure. Given that the available data suggest that disturbances to the body's homeostatic maintenance of bone may span the inter-donation interval of frequent apheresis donors, effects on bone density in high intensity apheresis donors could be sizable. However, the most important limitation of this study was in the cross-sectional evaluation of BMD

which the authors acknowledge. Because individuals achieve different peak bone densities, longitudinal comparisons using donors as their own controls are essential to drawing accurate conclusions about citrate exposure through apheresis and BMD.

Two complimentary studies have examined the effect of repeated apheresis blood donation and bone-related health outcomes. The first is a prospective National Institutes of Health study that incorporates a longitudinal assessment of bone density (NCT00073060). No data are as yet available. The other study was conducted on the Scandinavian Donations and Transfusions (SCANDAT) database (55). This retrospective cohort study (56) provided the first large-scale data on the effects of frequent and long-term apheresis donation on the risk of fractures. In this study, all available data on Swedish blood donors who experienced one or more apheresis blood donations during the period 1990 through 2012 were analyzed until death, emigration, or the end of the follow-up period. Donor fractures were sourced from the Swedish patient register and classified according to the International Classification of Diseases, Revisions 9 and 10. Fractures, including all and osteoporosis-related fractures, were analyzed and correlated with apheresis blood donation using Poisson regression. There was no association between the number of lifetime apheresis donations, the recentness of apheresis donation, nor gender or age on a blood donor's risk of fracture in any analyses. The Swedish blood donor population is different from the U.S. blood donor population in terms of race, ethnicity, body mass index, and other determinants of bone density. Furthermore, the vast majority of apheresis donations made during the analysis period were plasma donations where citrate burden to the donor is as much as 85% reduced compared to platelet apheresis donations (23). These large-scale epidemiology studies have previously shown utility in

examining potential associations between the blood donation activity and long term health outcomes (57-60), however, these studies are observational in nature only. Thus, a longitudinal, randomized study is needed in blood donors to assess whether or not repeated apheresis is associated with declines in bone mineral density.

Conclusions

The collection of blood products using apheresis technologies has been increasing over the previous decade. Citrate anticoagulation confers protection to the donor by sequestering ionized calcium in the donor's blood stream. Parathyroid glands sense the decline in ionized calcium in donor serum and secrete parathyroid hormone. The physiologic response to parathyroid hormone has been measured in blood donors undergoing apheresis and there are indications that bone homeostasis is perturbed. There are very limited longitudinal data on whether or not apheresis-induced modifications to normal calcium homeostasis impact bone density.

CHAPTER THREE: ALTRUYST Methodology

In this prospective, longitudinal, randomized, controlled clinical trial (NCT02655055) we tested the hypothesis that high frequency apheresis donation is associated with declines in BMD after a one year follow-up period. Eligible donors who had made no more than five apheresis blood donations in the past were enrolled using informed consent (Appendix 1). All donors also agreed to make close to the maximum number of allowable volunteer apheresis blood donations during the follow-up period (n=26) to help limit attrition and control for potential selection biases. Dual-energy x-ray absorptiometry (DXA) was performed at baseline for all enrolled men aged 18 to 65 years who had no known skeletal medical conditions. Following DXA, donors were randomly assigned to continue with their apheresis donation program ('treatment'), or, control (no apheresis blood donation during the follow-up period). Individual subjects also served as their own controls through repeated measures analysis (baseline and approximately twelve months later). The primary outcome measure was the change in total lumbar spine bone mineral density as assessed by DXA. A secondary outcome measure included change in total hip bone mineral density. Two additional outcome measures included femoral neck BMD and the trabecular bone score, a gray-level textural metric that can be extracted from the two-dimensional lumbar spine DXA image (61).

Study Population

This was a prospective, randomized, single-center study that assessed the effect of high frequency intravenous citrate exposure on bone density in volunteer apheresis blood

donors. Eligible participants were male, 18-65 years of age, with no more than five lifetime apheresis blood donations. There are known side effects of apheresis including paresthesia (due to hypocalcemia) and chills that deter future donation. Thus, this study recruited male donors who had made at least one previous donation to help mitigate attrition. All eligible participants were recruited from a population of donors beginning an apheresis blood donation program. Donors were encouraged to donate nearly the annual maximum of 24 donations per year (i.e. 20 – 26 donations during the study). Apheresis donation procedures included platelets, red blood cells, plasma, and multi-product donation types. All donation types were allowable in the present study because citrate anticoagulation is universally administered, procedure times are comparable, and even modest amounts of citrate exposure are associated with supraphysiologic spikes in parathyroid hormone.

Inclusion and Exclusion Criteria

Inclusion criteria

- Male
- eligible volunteer blood donor
- ≥ 18, and, ≤ 65 years of age at enrollment
- ≥ 1, and, ≤ 5 prior apheresis blood donation procedures

Exclusion criteria

- Female
- Age < 18 or > 65 years at enrollment
- ineligible for whole blood donation

- BMD Z-score <(-2.0) or >(2.0) at any measurement site upon baseline assessment
- metal prosthesis at measurement site
- weight > 300 lbs (136 kg)
- previous fracture of the lumbar spine or hip (femoral neck, intertrochanteric hip)
- any fragility fracture, defined as a fracture resulting from a fall of standing height or less, during adulthood (specifically ≥18 years of age at the time of fracture)
- previous lumbar spinal fusion surgery
- cystic fibrosis
- emphysema
- celiac disease
- Crohn's disease
- Current or past (>1month duration) use of medications known to affect BMD including, not limited to:
- (phenytoin, phenobarbital, corticosteroids)
- Current Osteoporosis Medication use including, but not limited to:
- (Forteo, oral biphosphonates, Reclast, Prolia, calcitonin)
- Unable or unwilling to donate high frequency apheresis

Control Population

The primary outcome measure was change in BMD and therefore, each participant served as their own control. Additionally, this was a randomized study. Eligible participants were randomly allocated to exposure (high frequency apheresis) or control (no apheresis allowed; whole blood donation allowed). Randomization occurred

after informed consent and baseline assessment but before the first ‘on-study’ blood donation. The control group was included in this single-center study design to ensure that any regional changes in BMD among the larger population could be detected and accounted for in the analysis.

Recruitment and Enrollment Methods

This study used a four-tiered recruitment program that aims to capture donors at points of intake.

Tier 1: In-Person Strategies:

Recruitment strategies used in prior studies involving face-to-face encounters have proven to be one of the most highly productive methodologies employed, especially for targeted donor groups (e.g. PR00022441, PRO00023331, PRO00022435). We therefore employed similar strategies in this protocol using face-to-face recruitment methods and an informational flyer (Appendix 2). Subjects were recruited in person by research staff at fixed or mobile collection sites. Research coordinators primarily approached donors at the time of registration following routine procedures for research with volunteer blood donors. Donors expressing a lack of interest in participation were thanked. Donors expressing interest in participating participated in further discussions about the study with a trained research coordinator using the informed consent to guide talking points. The researchers collaborated with local site managers to ensure that recruitment activities did not interfere with BloodCenter of Wisconsin operations and

donor intake. In collaboration with BCW Donor Group Recruiting, in-person recruitment also occurred at mobile blood drives. The overall success of this study was based on the team's ability to recruit new apheresis donors who were beginning to undergo these procedures. To improve efficiency and accuracy of recruitment efforts, as well as avoiding recall bias on the part of the donor, the research team requested an appointment list from IT/IS for eligible donors scheduled to donate at sites where in-person recruitment is planned. The appointment list included donor name, date of appointment, location of appointment, time of appointment, donor gender, number and type of donations in the previous 24 months, and donor ID. This list was distributed only to those research staff that are trained and certified in managing human subjects research related identifiers. Donors on the list were approached using the same strategy as for any other donor approached in person. These lists were maintained on the BloodCenter of Wisconsin secure server and any printed copies were kept in a locked file cabinet at the Blood Research Institute.

To enhance the functionality of the informational flyer, the research coordinator collaborated with leadership in Donor Services and Volunteer Services Departments to identify ‘study champions’ at each fixed blood center location. Through didactic and interactive workshops led by the study team, endemic blood center staff and volunteers learned which donors were potentially eligible for the study and offered to put donors in touch with members of the research team. Study champions served the dual purpose of enhancing recruitment and maximizing data integrity by serving as a trained liaison. Donor Services personnel placed an ALTRUYST sticker on the donor’s summary sheet

to help Volunteers identify which individuals in café were potentially eligible for the study. The word “ALTRUYST” was all that appeared on this sticker.
Ultimately, the research coordinator performed study screening before enrollment to ensure the highest degree of protocol compliance.

Tier 2: Targeted, Active Strategies

BloodCenter of Wisconsin fixed donation centers track apheresis donations made by individuals using a hanging file folder identified by the donor’s name. Within the folder is a record of the number of apheresis donations and procedure run sheets. A donor’s initial apheresis donations are captured in a similar way but are arranged alphabetically in a shared hanging file folder. Both of these resources provided an opportunity to tailor recruitment strategies to eligible male donors. The Tier 2 recruitment strategy allowed the research team to review these records for eligible subjects on site. Each time an eligible donor was identified the research team member placed a study card (business card designed specifically for the study) on the donor’s record. This practice cued front line staff members to provide the donor with the informational flyer (Appendix 2) upon presentation. Donor staff then put the donor in contact with the research team or the donor could have chosen to self-identify at a later time. This approach ensured anonymity and consistent recruitment messaging.

Tier 3: Targeted, Passive Strategies

This strategy was implemented using an ongoing apheresis conversion initiative led by BloodCenter of Wisconsin to expand the apheresis blood donor base. Beginning in the spring of 2015, all whole blood donors were being evaluated for their capacity to donate apheresis platelets. Automated reports were be generated from endemic BloodCenter of Wisconsin data systems to identify donors who initiated apheresis donation in response to this operational recruitment strategy. Targeted strategies for recruitment included mailings and emails at both fixed and mobile blood drives. The text for mailings and emails were provided as attachments to the protocol (Appendix 3). Additionally, BCW Call Center scripts were developed and integrated with endemic direct and automated appointment tools. Messaging content was derived from text provided in Appendices 2 and 3 because of the dynamic nature of donor recruitment strategies.

These population-based approaches to sampling are subject to non-participation bias (i.e. eligible donors who choose not to enroll). Due to the relatively low proportion of donors who would have converted to an apheresis donation regimen and the relatively short enrollment period, all interested and eligible donors were enrolled in the study (i.e. no random sampling).

Tier 4: Non-targeted, Passive Strategies

The research team provided ALTRUYST informational flyers (Appendix 2) to BCW Donor Services Managers who posted them in blood center common areas. Interested subjects could then self-identify their interest in participating by either calling the research hotline or emailing clinicalresearch@bcw.edu. Front line blood collections

staff received training from Donor Services Managers (who will receive training from the research team) to answer any questions about the study from donors by providing contact information for the research team to ensure that consistent messaging occurs. Study champions augmented the supervisor's need to provide information by serving as a trained liaison.

Finally, the BCW website (www.bcw.edu) has modalities that directly support clinical trial recruitment. Using text blended from across other IRB-approved media (informational flyer, email script, letter) the research team collaborated with corporate web developers to create electronic capture mechanisms (hyperlinks) that were displayed broadly across research oriented web content (BCW domain only). These links were intended to funnel self-identifying individuals to direct contact with the research team (email or telephone).

Measurement Methodology

Self-Report Survey

Subjects who provided written authorization for participation first completed a survey to confirm eligibility and to evaluate increased risk of fracture including questions on race, ethnicity, family history of osteopenia/osteoporosis, family history of fracture, personal history of fracture, daily calcium intake, activity level, medication use, and other parameters that affect baseline BMD prior to DXA [Appendix 4]. These are considered standard of care intake questions for patients being evaluated for osteopenia/osteoporosis contributing to the validation of risk factors, with and without bone density

measurements, to improve accuracy of fracture prognostication tools. The survey was administered twice: once prior to the baseline assessment and once prior to the final assessment. The paper-based survey was self-administered by the study subjects and was reviewed by research staff to ensure complete capture of the required information. The survey was designed to capture responses affecting a donor's eligibility in the study and therefore, donors who were ineligible based on responses did not have baseline DXA performed.

Bone Mineral Density

After informed consent and before the first on-study blood donation, participants had dual energy x-ray absorptiometry (DXA) performed [see Appendix 5]. DXA assesses BMD by way of emitting two fan beam x-ray energies that quantify bone density in mg/cm^2. Baseline BMD measurements were collected before the first on-study donation using DXA at the lumbar spine (L1 – L4), left and right hip (femoral neck, greater trochanter, and intertrochanteric region), and full body using standardized equipment. Subjects with metal prostheses and/or prior fracture at any measurement site were not eligible for participation because of interference with BMD measurement. Likewise, subjects weighing more than 300 pounds were not eligible due to DXA scan table mass limits. Subjects with a fragility fracture, defined as any fracture from a fall of standing height or less, during adulthood (i.e. ≥18 years of age at time of fracture) were also excluded.

DXA systems, as with all measurements, introduce a form of variation implicitly. This study used the GE iDXA™ with Encore version 11.40.004 software for all DXA

measurements. This machine was maintained according to manufacturer specifications which included periodic phantom scans, visual inspection of phantom scans, phantom scans after any service, and service logs. A recent study reported the greatest numerical difference in BMD at the total hip for same-subject assessments at 0.007g/cm^2 (62). Calibration data were collected for the measurement instrument and normalized prior to data analysis. This instrument is located in the Department of Physical Therapy, Cramer Hall, Marquette University, Milwaukee, WI.

Individual bone density results can be standardized to population values for subjects of the same age, sex, and race and will be reported as Z-scores. Due to ethical considerations, any subject with a BMD Z-score <(-2.0) or >(2.0) at any measurement site during the study was notified of the finding [Appendix 6] and advised to seek medical attention. It is likely that these subjects, upon medical consultation, would begin an efficacious treatment regimen that would have an impact on follow-up BMD values. While excluding BMD outliers limits the generalizability of this study's findings, the strategy employed has the advantage of addressing the question of how the vast majority (~95%) of eligible donors respond while eliminating possible confounding introduced by therapy prompted by abnormal BMD findings. Ongoing monitoring of the true prevalence of this finding in this study's sample during the enrollment period was performed. Because the prevalence of baseline BMD out of this range was higher than expected, the final enrollment sample size was adjusted to achieve the desired analytic sample size.

All BMD test results outside of this range, or within 0.1 standard deviations, were reported to this study's Medical Monitor, Dr. Robert D. Blank, who is a clinical expert in

metabolic bone disease (see Section PROTECTION AGAINST STUDY RISKS). To preserve blinding, all BMD measurements (baseline and follow-up) were sent to an external adjudicator, Dr. Joseph Shaker, a clinical expert in metabolic bone disease, at the end of the study but before analysis. All BMD reports were blinded in terms of subject randomization and date of scan. The adjudicator thus served the role of interrogating all BMD scans to ensure quality and validity of study values. Finally, cross-calibration of multiple DXA systems was not required in this study because only one instrument was used and all scans were performed by a single technologist.

Biospecimens

Enrolled participants had peripheral tubes of blood collected both pre- and post-apheresis. Collection of these samples did not require an additional venipuncture because it was diverted from normal blood collection kits. All specimen processing was conducted in designated laboratories at BCW by trained personnel. Laboratory testing was performed at ARUP clinical research laboratories in Salt Lake City, UT. Results from testing were used only for the purpose of post hoc analysis and hypothesis generation. Analytes included elements of a comprehensive metabolic panel, testosterone, parathyroid hormones, complete blood counts including reticulocytes, and markers of bone and mineral metabolism. Laboratory testing occurred in batches well after specimen acquisition, and therefore results were not shared with participants at any time. Frozen aliquots were stored at BCW for up to three years after the study is closed to allow time for testing.

Citrate Anticoagulation

BloodCenter of Wisconsin utilizes anticoagulant citrate dextrose solution (ACD-A) for the collection of apheresis products. Sodium citrate dihydrate is derived from anhydrous citric acid ($C_6H_8O_7$) and together these constitute 2.2g and 0.73g per 100mL of the ACD-A formulation used during apheresis, respectively. Data on ACD-A exposure is captured on apheresis run sheets the blood center. Table IV shows the citrate exposure endpoints (time of exposure, dose) that were collected for this study.

The type of apheresis donation being made (platelet, red cell, plasma, or multi-product), volume of ACD-A (mL) infused, and duration of apheresis procedure (minutes) was abstracted from blood center flow sheets for each visit over the study period. Platelet product yield was classified as single ($3.1 – 6.6 \times 10^{11}$), double ($6.7 – 9.9 \times 10^{11}$), and triple ($\geq 10.0 \times 10^{11}$).

CITRATE EXPOSURE ENDPOINTS		
Collections Information	Machine	Trima, Amicus
	Run Sheet	Yes
	Concurrent Products[1]	Yes
Exposure Time	Start Time	Yes
	End Time	Yes
	Total Time	Yes
Citrate (AC) Parameters	Type of AC[2]	ACD-A
	[AC][3]	Yes
	Lot #	Yes
	Expiry	Yes
	Volume AC Used	Yes
	Volume AC in PLT	Yes
	Actual Volume Infused[4]	Yes

Yes = collected regularly on blood center apheresis flow sheets
1 Concurrent plasma collection is allowed.
2 AC = anticoagulant
3 concentration of AC
4 = $V_{AC\ used} - V_{PLT\ AC}$

Table IV. Availability of citrate exposure endpoints collected from BloodCenter of Wisconsin apheresis run sheets.

Subject Remuneration

Participation in this study was time and travel intensive. At two time points (baseline and final visits) donors traveled to Marquette University's campus to have bone density scans performed. Because of the large geographic catchment area of BCW, study participants received $50 per visit (no more than $100 total) to cover travel costs (Figure IV). Additionally, participants were offered full body composition analysis reports (at the end of the study only) upon request. Throughout the study donors were asked to comply

with many guidelines including blood donation types, blood donation frequency, fracture reporting, questionnaires, and study visits. Study participants were evaluated for protocol compliance at the time of the final visit. Compliance with all of the study's guidelines and completion of all study visits resulted in payment of $100 and $250 by check for no apheresis and apheresis groups, respectively. Total remuneration did not exceed $200 for donors in the control group and $350 for donors in the apheresis group. Compensation was not tied to blood donation, but solely for the clinical assessment and completing research study protocol(s). Please refer to the Final Appointment Letter (Appendix 7).

To proactively address any issues that may arise between the voluntary attribute of the derived blood component products and study participation, the research team contacted the FDA's Center for Biologics Evaluation and Research (CBER) Office in February 2016. The remuneration plan outlined above was presented alongside the open question as to whether or not component products could be labeled as "voluntary units" (21 CFR 606.121(c)(8)(v) and Compliance Policy Guide 230.150, Blood Donor Classification). The response received from FDA indicates that this remuneration plan is acceptable as captured in the attached communication (Appendix 8).

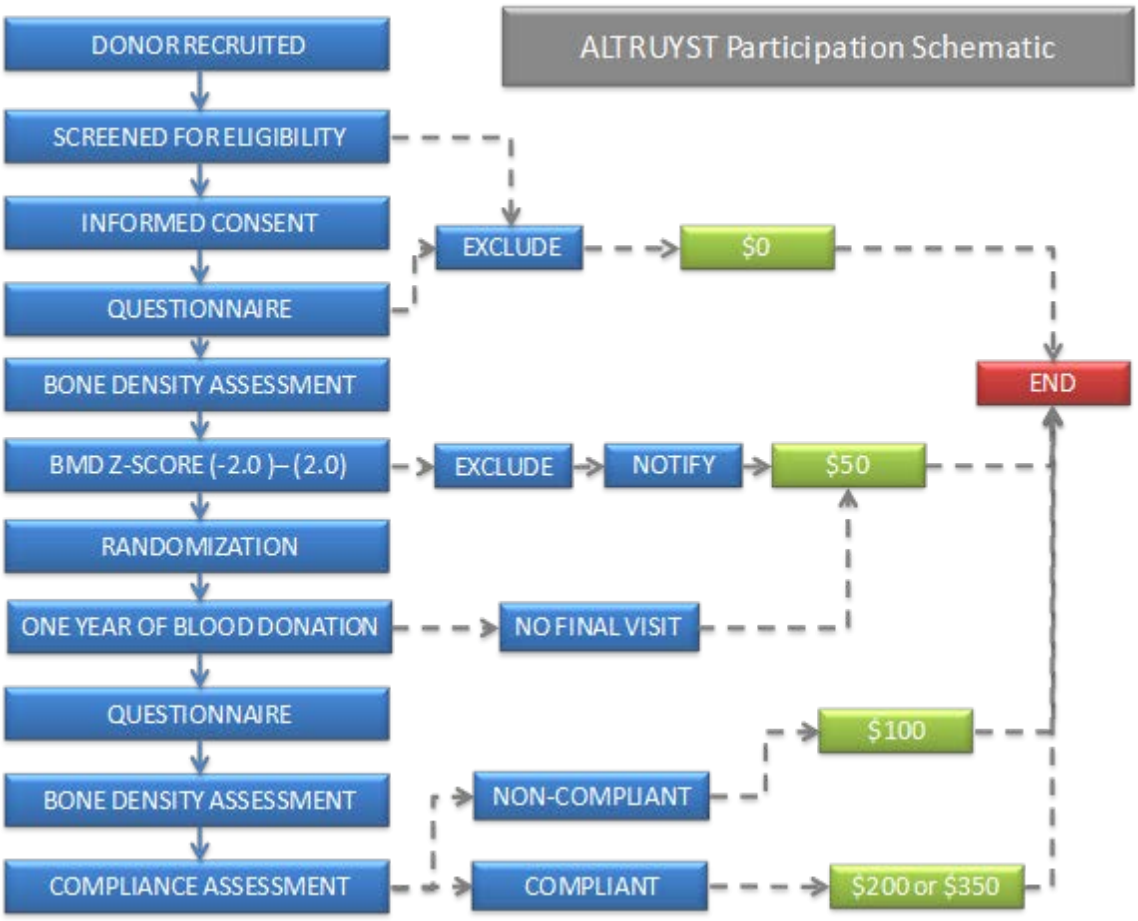


Figure IV. Participation schematic for ALTRUYST participants including amounts of subject remuneration based on study completeness.

Statistical Methodology

The hypothesis tested was that high frequency apheresis would cause a decline in BMD from baseline assessment to follow-up. Specifically, the null hypothesis was that for men aged 18 - 65 years and otherwise satisfying eligibility criteria, there would be no difference in the magnitude of change in bone mineral density between a baseline and follow-up bone mineral density assessment for high frequency apheresis donors (H_o: $\mu X = 0$, where X is change in BMD). The alternate hypothesis was that men aged 18 - 65 years and otherwise satisfying eligibility criteria would experience a decline in BMD between a baseline and follow-up assessment following one year of high frequency

apheresis (H_a: $\mu X < 0$). The T-test statistic was used to compare change over the one year period in comparison to zero, or no change. This was a randomized study and the control group did not donate apheresis blood products during the follow-up period. The null hypothesis for comparing the treatment group (high frequency apheresis group) to this control group was that change in BMD was no different between the treatment and control groups (H_o: $\mu X = \mu Y$, where X was change in BMD in the treatment group and Y was change in the control group). The alternate hypothesis tested was that change in the treatment group was greater in magnitude than change in the control group (H_a: $\mu X < \mu Y$).

There is natural variation in an individual's peak BMD (63). This source of variation plays a role in the derivation of power. NHANES 2009 – 2010 examination data were used in estimating the mean and standard deviation of total lumbar spine BMD for men aged 25-50. NHANES is a stratified, multistage probability sample of the civilian non-institutionalized population of the United States. Data are freely available on the Centers for Disease Control and Prevention website: http://www.cdc.gov/nchs/nhanes/nh3data.htm.

The demographic and examination data sets were downloaded from NHANES in April 2015. Using R, the two data sets were merged using sequence number (seqn) as the linking variable. Using R studio, females were excluded using the gender variable (riagendr); men aged <25 or >50 were excluded using the age variable (ridageyr); Caucasian, non-Hispanic men were selected using the race/ethnicity variable (ridreth1). Mean (1.054925 gm/cm^2) and standard deviation (0.1345117 gm/cm^2) of total lumbar

spine BMD (dxxosbmd) was then computed from the restricted data set. The complete programming syntax for this procedure is included as [Appendix 9].

PRECISION ASSESSMENT

An *in vivo* precision assessment of the individual technologist performing all bone mineral density scans in this project was performed in August of 2015 (lumbar spine) and again in July 2017 (total hip). Precision assessment was performed per ISCD guidelines (64). In summary, 15 patients were scanned on the same DXA machine thrice with repositioning in between assessments. Eligible participants were male, between 18 and 65 years of age, generally healthy and otherwise eligible for the research study. Precision assessment was performed on the lumbar spine (L1 – L4, Table V) and at the total hip (Table VI). Volunteers were offered full body composition analysis (optional). If a full body scan was performed, it was conducted before the serial L1-L4 and total hip measurements. Repositioning entailed removal of the leg support block, sitting followed by standing, several paces of normal walking, and then complete repositioning of the volunteer. All scans contributing to the precision assessment were performed within two weeks of each other.

Subject	1st Scan	2nd Scan	3rd Scan	SD	SD^2
LSC 1	1.221	1.225	1.221	0.00231	0.00001
LSC 2	1.532	1.522	1.524	0.00529	0.00003
LSC 3	1.153	1.153	1.171	0.01039	0.00011
LSC 4	1.118	1.122	1.103	0.01002	0.00010
LSC 5	0.908	0.918	0.902	0.00808	0.00007
LSC 6	1.217	1.217	1.219	0.00115	0.00000
LSC 7	1.106	1.101	1.100	0.00321	0.00001
LSC 8	0.979	0.970	0.971	0.00493	0.00002
LSC 9	1.226	1.227	1.225	0.00100	0.00000
LSC 10	1.182	1.187	1.166	0.01097	0.00012
LSC 11	1.253	1.238	1.239	0.00839	0.00007
LSC 12	1.433	1.435	1.436	0.00153	0.00000
LSC 13	1.104	1.123	1.109	0.00985	0.00010
LSC 14	1.104	1.098	1.08	0.01249	0.00016
LSC 15	1.002	0.994	0.990	0.00611	0.00004
				Sum	**0.00083**
				Sum/n	**0.00006**
				RMSD	**0.00743**

Table V. Serial mean lumbar spine bone density values (g/cm^2) for L1 – L4 in 15 male subjects scanned in August 2015 with repositioning between scans. SD = standard deviation; n = 15; RMSD = root mean square deviation, or, precision error.

Subject	1st Scan	2nd Scan	3rd Scan	SD	SD^2
LSC 16	1.160	1.158	1.149	0.005859	0.000034
LSC 17	1.195	1.204	1.192	0.006245	0.000039
LSC 18	1.002	1.008	0.998	0.005033	0.000025
LSC 19	0.955	0.956	0.940	0.008963	0.000080
LSC 20	1.093	1.102	1.090	0.006245	0.000039
LSC 21	1.023	1.015	1.022	0.004359	0.000019
LSC 22	1.236	1.201	1.234	0.019655	0.000386
LSC 23	0.987	0.990	1.001	0.007371	0.000054
LSC 24	1.136	1.152	1.144	0.008	0.000064
LSC 25	1.118	1.121	1.110	0.005686	0.000032
LSC 26	1.268	1.267	1.271	0.002082	0.000004
LSC 27	0.886	0.890	0.898	0.00611	0.000037
LSC 28	0.998	1.001	0.995	0.003	0.000009
LSC 29	1.100	1.097	1.107	0.005132	0.000026
LSC 30	1.034	1.030	1.028	0.003055	0.000009
				Sum	**0.000860**
				Sum/n	**0.000045**
				RMSD	**0.006708**

Table VI. Serial mean femoral neck bone density values (g/cm^2) for the total hip in 15 male subjects scanned July – September 2017 with repositioning between scans. SD = standard deviation; n = 15; RMSD = root mean square deviation, or, precision error.

POWER COMPUTATIONS

Step 1 involved simulating the distribution of T under the null hypothesis (H0). First, population parameters for μ (population mean), σ (population standard deviation), and ρ (correlation) were set. Next, a covariance matrix for each group (exposure and

control) was established. We are not able to make the assumption that the two variables being measured, Xbefore and Xafter, or Ybefore and Yafter, are independent. Therefore, simulation occurred for various sample sizes with 10,000 iterations of a multivariate, normal variable random sampling method. Average change and standard deviation in bone mineral density for exposure and control were calculated. α was set to 0.05 and distributions of test statistics were visually inspected through histogram. The programming code was developed and executed in R: a language and environment for statistical computing (65) and is provided as [Appendix 10].

Step 2 involved simulating the distribution T under Ha. μ was set to the population mean for all time points except for a 3% reduction in the exposure group. σ was set to be the same for both groups at all time points. ρ was set to be equal to ρ as for H0. The same covariance matrix was used for each group. Simulation also occurred with 10,000 iterations of a multivariate, normal variable random sampling method. Average change and standard deviation were calculated. α was set to 0.05 and data were visually inspected through histogram. The programming code was developed and executed in R (65) and is provided as Appendix 10.

Step 3 involved determining the critical value. In R, the quantile function was used to derive the critical value where the probability of the test statistic is greater than the critical value under H0. This value is equal to $1 - \alpha$.

Step 4 involved computing power. In R, the power was computed by adding the values of the test statistic under Ha where this value was greater than the critical value. This sum was then divided by the number of simulated iterations.

Power simulations were conducted for a two measurement design: a baseline assessment of BMD and an assessment after one year of treatment (apheresis) or control (no apheresis). Mean and standard deviation of lumbar spine BMD were calculated from NHANES III as above and a 3% decline in BMD in the exposure group was selected as the primary outcome. Using this approach, power was calculated for various sample sizes and $\rho = 0.1, 0.5, 0.9, 0.925$, and 0.95. Because healthy men comprising this cohort have no change in BMD over a one year period on average (Looker, Melton et al. 2010), it was assumed that there would be a high degree of correlation between baseline and follow-up assessments (e.g. $\rho = 0.99$). The primary sources of variation in this model would be the technologist's LSC and the variance of change in BMD for the exposure group.

Attrition and Randomization

Studies in blood donor populations with very similar interventions demonstrate highly varied degrees of attrition (e.g. (66, 67)). In the present study, all enrolled donors were recruited from a pool of donors willing to undergo high frequency apheresis. Nevertheless, it was expected that donors randomized to continue with apheresis as planned would demonstrate a higher rate of attrition relative to those randomized to forgoing apheresis for the one year follow-up period. To preserve this study's analytic sample size, unequal attrition estimates were used to compute required enrollment sample sizes.

In conclusion, an estimated 80% power was achieved with approximately 20 subjects analyzed as treatment and 15 analyzed as controls. It was expected that donors randomized to apheresis would be more likely to undergo attrition. If one in five donors

randomized to treatment dropped out of the study (i.e. 20% attrition), then the probability of preserving the desired analytic sample size in this group was approximately 91% when 28 donors are enrolled. The probability of preserving the desired analytic sample size in the control group, estimated to experience 5% attrition, was approximately 95% when 17 donors were enrolled. Total enrollment cohort size in this scenario was n = 45 (28 + 17 = 45). With approximately two thirds of the randomized cohort being assigned to treatment (62%), a 2:1 randomization was used. A projected subject workflow with numbers is provided in Figure V.

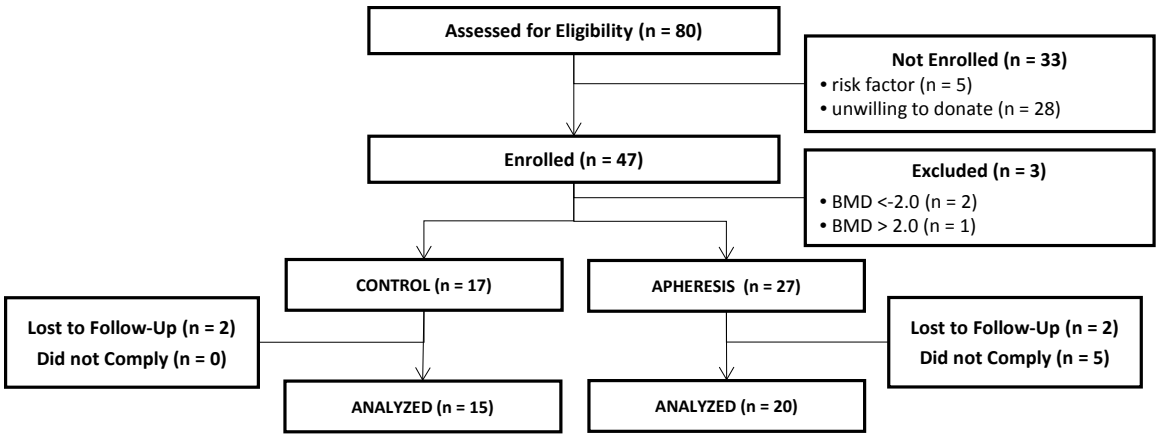


Figure V. Projected subject workflow for ALTRUYST study showing effects of anticipated subject attrition rates and preservation of analytic sample size.

Human Subjects Considerations

This study was submitted to local Institutional Review Board (IRB) review prior to any human subjects participation. Informed consent was required due to radiation

exposure associated with DXA and the randomized study design. There was no direct benefit to subjects who participated in this study other than remuneration for each DXA visit and study compliance, and, full body composition reports from DXA after study completion. If a participant was eligible based on the screening questionnaire, then there was a minimum of one suite of DXA scans performed at baseline. For participants who completed the study, a total of two suites of DXA scans were performed (one at baseline and one at follow-up). Each completed DXA assessment resulted in payment of $50 to the participant to remunerate costs associated with time and travel. Total remuneration for two suites of DXA scans was $100. In addition, participants who complied with the protocol intervention into which they were randomized (20 – 26 apheresis procedures, or no apheresis procedures during the follow-up) received additional compensation at the end of the study (equal to $100 or $250). The most an individual participant received as part of their participation in this study was $200 or $350, based on randomization and compliance. There was no remuneration granted to participants for any blood donation activities – these were all voluntary donations per FDA guidance.

Women were not included in this study due to a high degree of variability in bone density both across age strata and over the life course (Looker, Melton et al. 2010). Because all participants were male, there was no need to assess child-bearing status before DXA in this cohort. The inclusion of racial minorities was not precluded; however, powering the study to detect between-race interactions was untenable based on the low representativeness of various racial groups in the blood donor population.

Potential Risks

Potential risks for participation in this study were associated with risks of venipuncture, exposure to radiation from DXA, and loss of confidentiality. Risks of venipuncture were the same as those for routine apheresis and whole blood donation which eligible subjects regularly accept. These risks were presented to volunteer blood donors at the time of every donation. This risk was considered minimal and was discussed in the informed consent document signed by all participants.

Pencil beam BMD scanners employ a narrow (2-3 mm) x-ray beam with a single detector, while fan beam scanners use broader, fan shaped beams. The original version utilizes an array of multiple detectors in a single pass while the newer, narrow angle fan beam scanners have a smaller detector array and perform multiple, overlapping passes (68). Overall, fan beam scanners offer shorter scan times, better resolution, and slightly higher radiation doses than pencil beam scanners. DXA measurement was performed using fan beam scanners exclusively in this study.

Although DXA conveys a relatively low radiation dose to the patient, the areas irradiated include sensitive organs such as the bone marrow, and in some instances, reproductive organs. Radiation dose is contingent upon the method and mode of delivery and the significance of the exposure depends upon the body part irradiated. The Radiological Society of North America (69) provides a comparison of radiation exposure from various procedures to natural environmental exposure. A typical whole body DXA scan is equivalent to three hours of natural background radiation. Overall, results show

that DXA is a low dose examination compared to other ionizing radiation procedures such as standard radiographs (70). Radiation exposure data are summarized in Table VII.

TYPE OF EXPOSURE	EFFECTIVE RADIATION DOSE (µSv)
Mammogram (70)	450
Air Flight JFK to ORD (71)	390
Chest X-Ray (70, 72)	100
Daily Natural Exposure (70)	8
Lumbar Spine DXA (73, 74)	0.7
Femoral Neck DXA (73, 74)	0.7
Whole Body DXA (72, 74)	3
All 5 DXA Scans	**5.8**

Table VII. Effective radiation dose for select natural exposures and exposures associated with dual energy x-ray absorptiometry (DXA) bone scans.

Protection Against Study Risks

While the radiation dose is low, there still may be concern with reproductive organ exposure. Any attempt at shielding has the potential to compromise scan results. Reproductive organ exposure to scattered radiation during the lumbar spine scan does occur, and exposure during hip scans is dependent on the size of the scan field (75). Although DXA scans deliver low radiation doses to patients compared to general-purpose

radiographic systems, technical competency is necessary to minimize unnecessary exposure and to produce accurate results. Improper patient positioning may result in unintended and unnecessary exposure to the patient as well as staff. The scan operator was therefore appropriately trained to perform scans safely, accurately, and reliably. The DXA facility performed quality control and precision testing to ensure accuracy and reproducibility of results (76).

Staff persons involved in this project had extensive experience implementing clinical studies where confidential information is collected, stored, and utilized for research purposes. All original documentation were assembled in donor records maintained in secure locations at BloodCenter of Wisconsin and Marquette University facilities. Information on enrolled donors were identified by a unique, anonymized subject ID for the purposes of anonymity and laboratory testing. DXA measurement data remained in a secure-access room, on the access-restricted hard drive, at Marquette University or in the paper-based chart assembled at BloodCenter of Wisconsin. The only people with access to identifiable information in this study were CITI certified and listed as personnel on the IRB-approved protocol.

Due to the limited interventional nature of this study (randomization and DXA) reporting requirements were limited to abnormal BMD discovered through DXA, losses of confidentiality, and adverse outcomes associated with venipuncture. All abnormal DXA results from this study [BMD Z-score <(-2.0) or >(2.0)] were reviewed by this study's Medical Monitor. The recommendation to seek personal medical attention in the form of an IRB-approved letter was provided to subjects. These reports were generated on behalf of the Medical Monitor, shared with all study personnel, and reported to the

IRB as required. Adverse events associated with venipuncture were addressed per local protocol and reported to the IRB as required.

This study's Medical Monitor was an endocrinology-trained physician experienced in the diagnosis and treatment of patients with osteoporosis. Dr. Robert D. Blank, Chief of Endocrinology, Metabolism, and Clinical Nutrition at the Medical College of Wisconsin served as the Medical Monitor. This physician reviewed all abnormal DXA reports and adjudicated adverse findings as needed. Specifically, any BMD Z-score ≤(-1.9) or ≥(1.9), as determined by the DXA technologist were forwarded for review by the Medical Monitor. It is likely that upon notification of abnormal BMD, subjects would be evaluated, and possibly treated, for a bone or metabolism-related disease by their physician, thus affecting follow-up BMD measurements and confounding study results. Therefore, all subjects were asked as part of the risk assessment survey whether or not they are currently or have ever taken medications that are used in the treatment of low BMD. These data were used first to screen for eligibility and later upon data analysis. All subjects were given the results of their DXA results upon request, but only after completing their participation in the study to help mitigate potential modifications to behavior during the study.

Statistical Analysis

The distributions of continuous and ordinal variables at baseline were compared using the t-test statistic and Fisher's exact test, respectively. The primary outcome was defined *a priori* as change in lumbar spine BMD and the secondary outcome was change

in total hip BMD. The intention to treat analysis included all subjects who completed follow-up. A compliance analysis was also performed that included only those subjects who complied with the criteria of their randomization. Multivariable logistic regression was performed as an exploratory analysis using the questionnaire, laboratory, baseline BMD data, and, treatment arm as predictors of change in bone density exceeding the LSC. Bilirubin, alkaline phosphatase, and anion gap were log transformed to achieve normal distributions. Automated stepwise backwards elimination was subsequently performed to identify significant predictors of both positive and negative response. The programming code was developed and executed in R: a language and environment for statistical computing (65) and is provided as [Appendix 11].

TIMELINE

The projected timeline for the ALTRUYST trial is presented in Table VIII.

Activity	**Period**
Power Computation	Spring 2015
DXA Precision Assessment	Summer 2015
Finalize Proposal	Fall 2015
Clinical Trials.gov Registration	Winter 2015
IRB Submission	Spring 2016
Enroll Subjects	Summer 2016
Enroll Subjects & Follow-Up	Fall 2016
Follow-Up	Spring 2017
Final Visits	Summer 2017
Final Visits & Analysis & Publication	Fall 2017

Table VIII. ALTRUYST project timeline.

CHAPTER FOUR: ALTRUYST Results

Human Subjects Approvals and Enrollment

BloodCenter of Wisconsin Quality Support Services supported the application for a determination that the proposed study remuneration plan was compliant with 21 CFR 606.121(c)(8)(v) and local Compliance Guide 230.150 in February, 2016. The Blood and Tissue Compliance Branch (BTCB) at the US Food and Drug Administration (FDA) acknowledged that these requirements had been met and that the proposed donor incentive did not constitute monetary payment (Appendix 8) allowing labeling and transfusion of derived component blood products as “Volunteer Blood Donor”. Thereafter, the study team sought ceded IRB approval from the Marquette University Institutional Review Board (IRB). The Inter-Institutional Liaison for Human Research Protections approved the request in February, 2016 (Appendix 12). The study was then submitted to the Medical College of Wisconsin (MCW) IRB for approval. The Department of Physiology at MCW provided an attestation that the project had scientific merit, used procedures consistent with sound research design, and was likely to yield the expected knowledge; and that the proposal was a complete and coherent one on April 5, 2016 (Appendix 12). Radiation verification and approval from the Radiation Safety Office at MCW was received on April 28, 2016 (Appendix 13). The study was reviewed by Institutional Review Board #3 on April 26, 2016. The Committee determined that the study met all criteria under 21 CFR 56.111 and provided an approval notice (Appendix 14). IRB-approved screening methods commenced and the first subject was enrolled per protocol on May 18, 2016 and the final subject was enrolled on January 28, 2017. Figure

VI shows 100%, 75%, and 50% of enrollment goals with actual enrollment numbers (black line) and the number of eligible enrollees (dashed line) superimposed over the projected 100% (green), 75% (yellow) and 50% (red) cumulative accrual numbers.

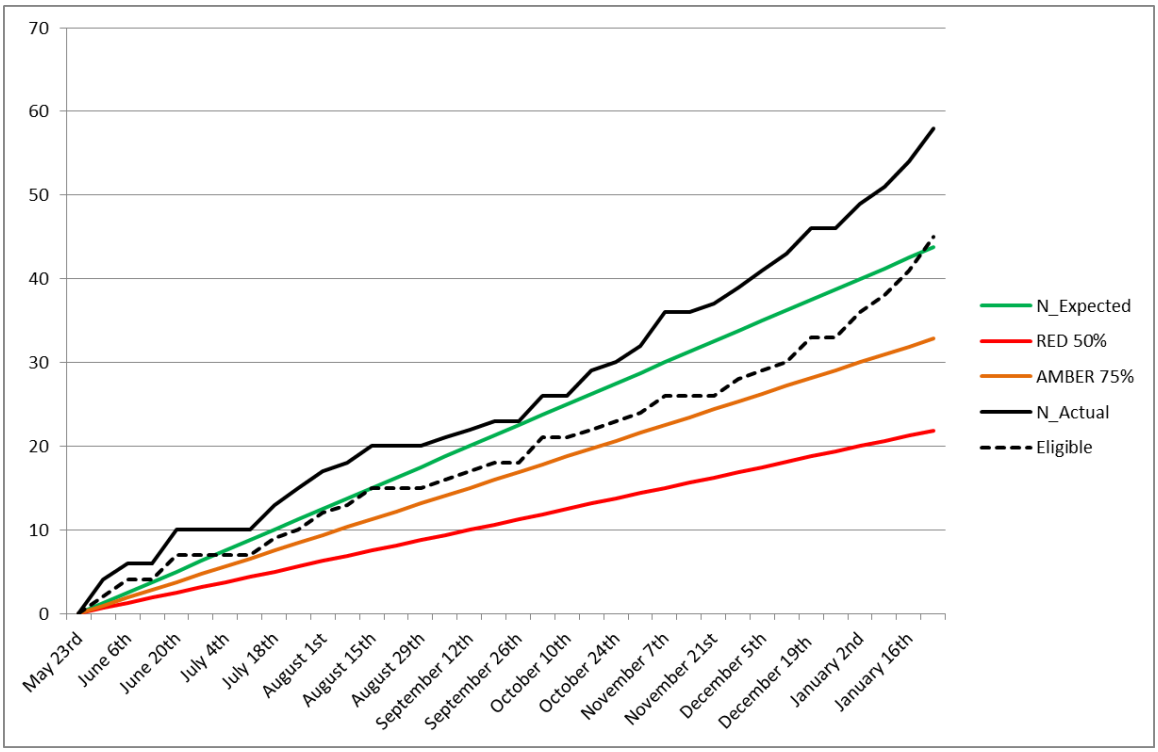


Figure VI. Cumulative enrollment chart showing 100%, 75%, and 50% enrollment goals with actual and eligible numbers superimposed.

Enrollment and Analytic Samples

Among 120 volunteer blood donors assessed for eligibility, 58 enrolled in the study (Figure VII). Seven enrollees were subsequently excluded for having a bone density Z score <-2.0 or > 2.0 at enrollment. Three additional subjects were excluded for having previously undergone apheresis more than five times and one subject was excluded for weighing more than 300 pounds. Ultimately, 32 subjects were randomized

to apheresis and 15 to the control arm. Approximately 20% of subjects from the apheresis arm were not available at follow-up: two subjects voluntarily withdrew from the study (6%) and four subjects were lost to follow-up (11%). All subjects in the control arm were available at follow-up.

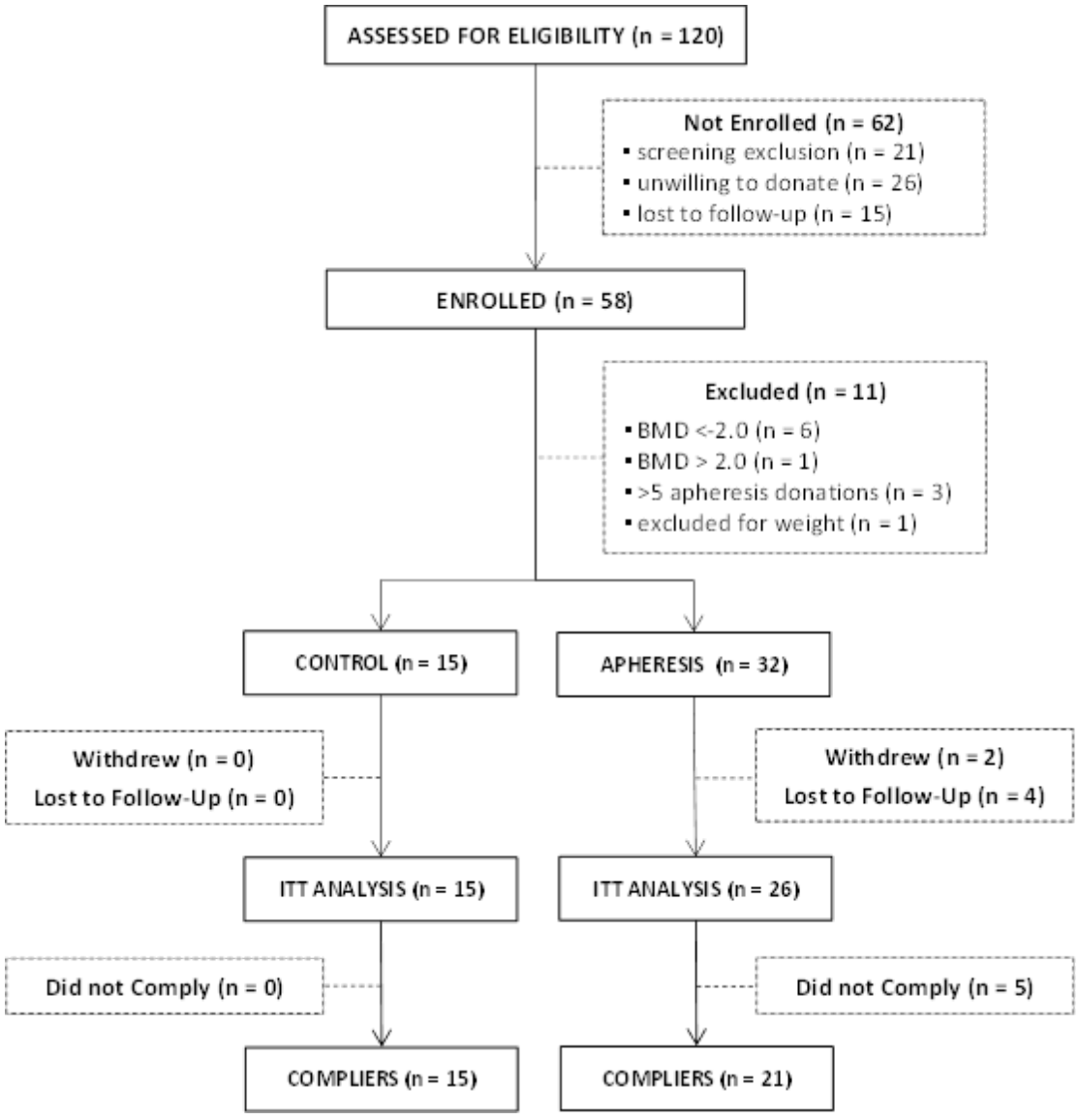
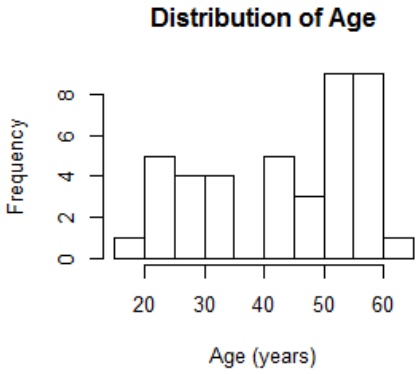
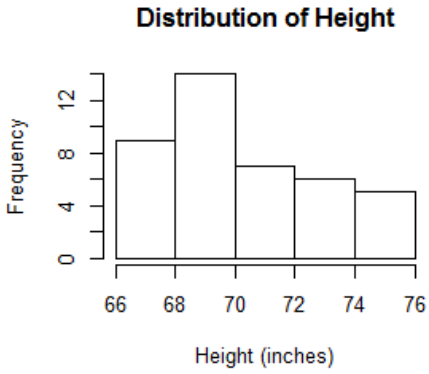


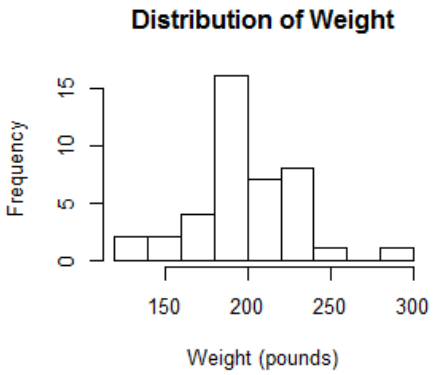
Figure VII. Enrollment schematic showing recruitment, enrollment, ineligibility, and losses to follow-up for the ALTRUYST trial. ITT = Intention to Treat

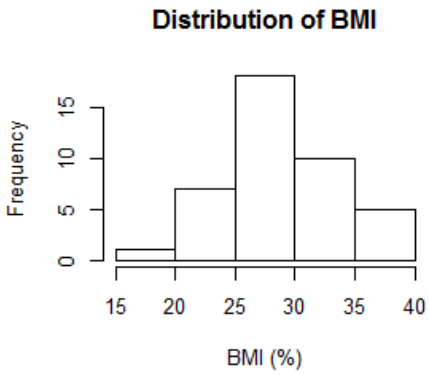
Baseline Demographics, Laboratory Test Results, and Donation History

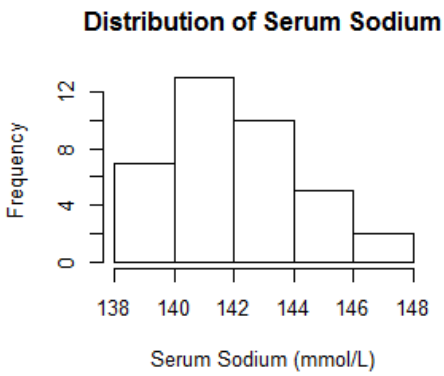
The distributions of baseline characteristics are provided in Figure VIII. A peripheral blood sample was not available for four participants. Bilirubin, alkaline phosphatase, and anion gap were log transformed for all donors to achieve normal distributions (Figure IX). Male, adult testosterone concentrations were below the lower normal range cutoff in three donors: two in the treatment arm and one in the control arm. Mean age at enrollment was 43.8 years (SD = 13.5) with one donor (2%) reporting African American race and another (2%) reporting Hispanic ethnicity (Table IX). Mean height (p=0.89), weight (p=0.25), and body mass index (BMI) (p=0.38) were no different between study arms. Baseline laboratory parameters were similar between groups (p=0.14-0.97), as were the number of previous whole blood (median = 5, p=0.73) and apheresis (median = 3, p=0.49) donations. Though lumbar spine bone density was not different between study arms (p=0.26), bone density at the total hip was, on average, 0.107 g/cm^2 higher among those donors randomized to the apheresis arm (p=0.03) (Table IX). The lower limit of the reference range for testosterone is 300ng/dL and three individuals were below that value at enrollment. The only individual among these three who experienced clinically meaningful change in BMD was a 25 year old male in the control group who experienced a 4.3% decline in total hip BMD and 2.2% decline at the L-spine. The only other laboratory value out of the normal reference range was anion gap: value 18 mmol/L (ref range 7 - 15).

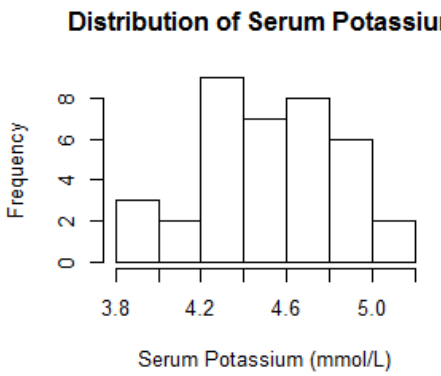

Distribution of Age
Frequency
0
2
4
6
8
20
30
40
50
60
Age (years)

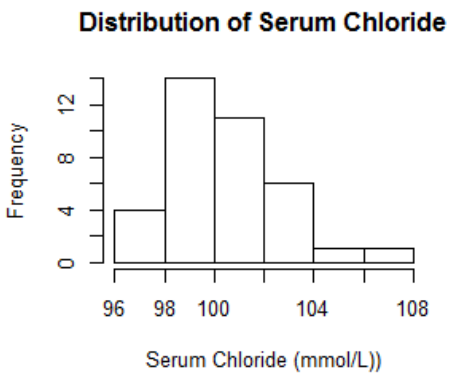

Distribution of Height
Frequency
0
4
8
12
66
68
70
72
74
76
Height (inches)

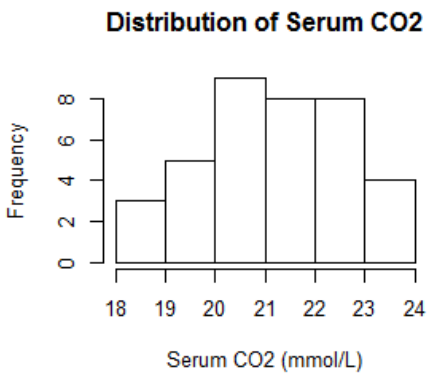

Distribution of Weight
Frequency
0
5
10
15
150
200
250
300
Weight (pounds)

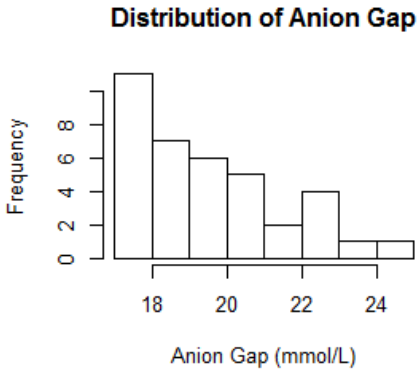

Distribution of BMI
Frequency
0
5
10
15
15
20
25
30
35
40
BMI (%)

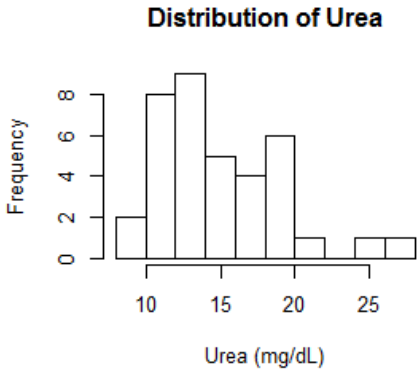

Distribution of Serum Sodium
Frequency
0
4
8
12
138
140
142
144
146
148
Serum Sodium (mmol/L)

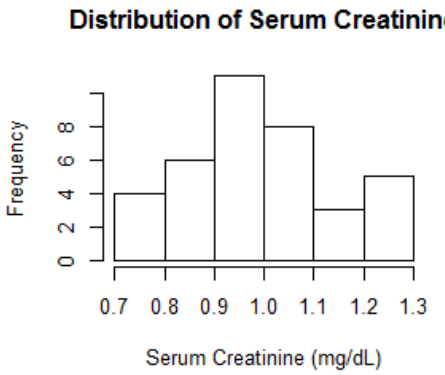

Distribution of Serum Potassium
Frequency
0
2
4
6
8
3.8
4.2
4.6
5.0
Serum Potassium (mmol/L)

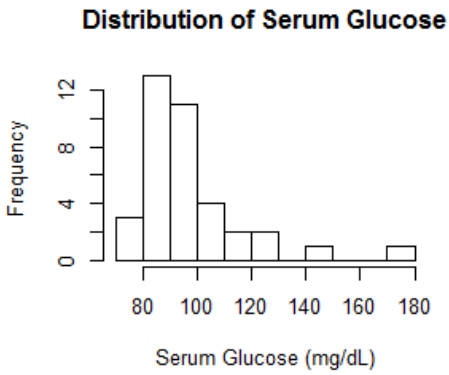

Distribution of Serum Chloride
Frequency
0
4
8
12
96
98
100
104
108
Serum Chloride (mmol/L))

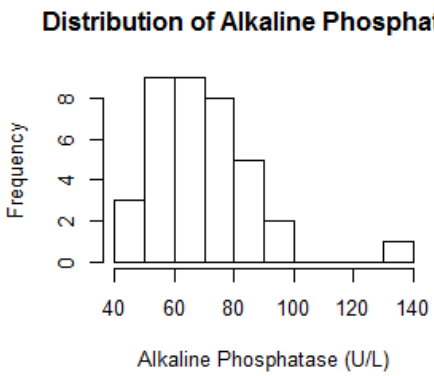

Distribution of Serum CO2
Frequency
0
2
4
6
8
18
19
20
21
22
23
24
Serum CO2 (mmol/L)

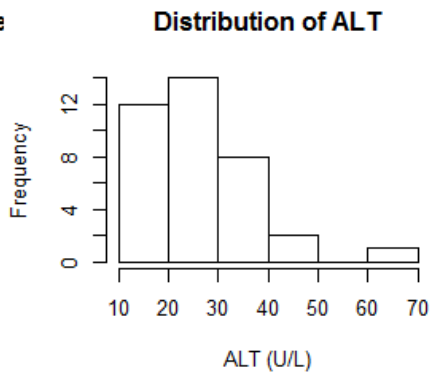

Distribution of Anion Gap
Frequency
0 2 4 6 8
18 20 22 24
Anion Gap (mmol/L)

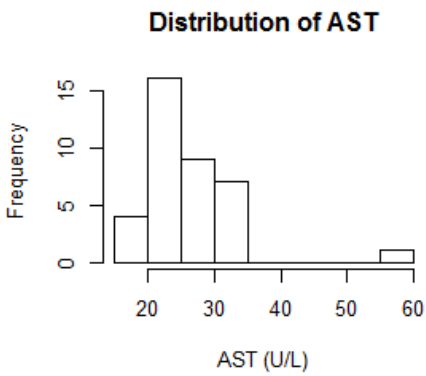

Distribution of Urea
Frequency
0 2 4 6 8
10 15 20 25
Urea (mg/dL)

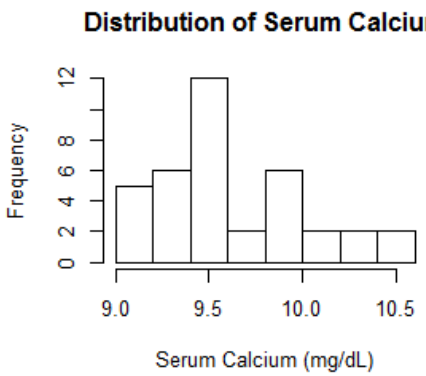

Distribution of Serum Creatinine
Frequency
0 2 4 6 8
0.7 0.8 0.9 1.0 1.1 1.2 1.3
Serum Creatinine (mg/dL)


Distribution of Serum Glucose
Frequency
0 4 8 12
80 100 120 140 160 180
Serum Glucose (mg/dL)


Distribution of Alkaline Phosphatase
Frequency
0 2 4 6 8
40 60 80 100 120 140
Alkaline Phosphatase (U/L)


Distribution of ALT
Frequency
0 4 8 12
10 20 30 40 50 60 70
ALT (U/L)


Distribution of AST
Frequency
0 5 10 15
20 30 40 50 60
AST (U/L)


Distribution of Serum Calcium
Frequency
0 2 4 6 8 12
9.0 9.5 10.0 10.5
Serum Calcium (mg/dL)

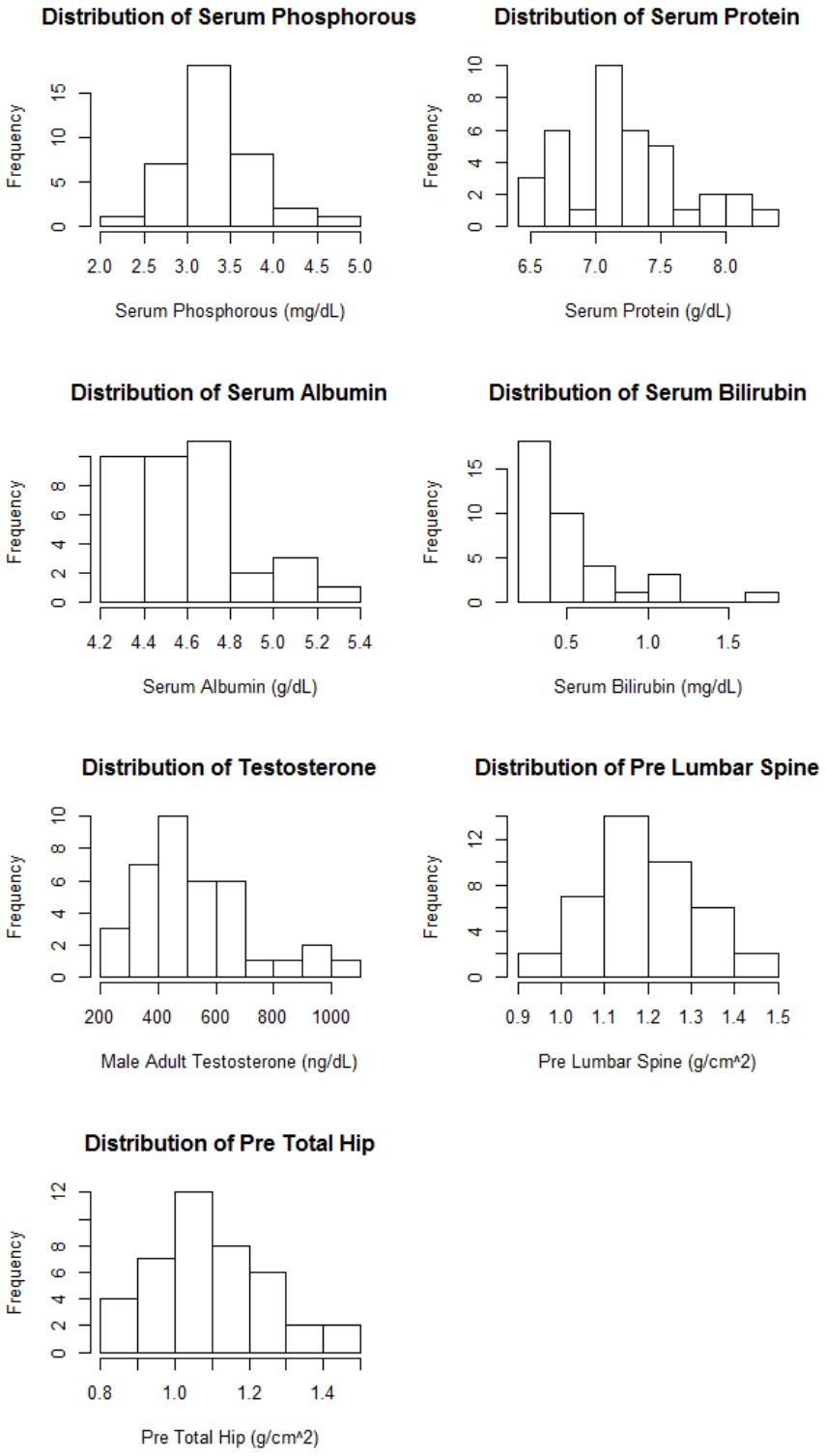


Figure VIII. Distributions of variables at baseline (N=37).

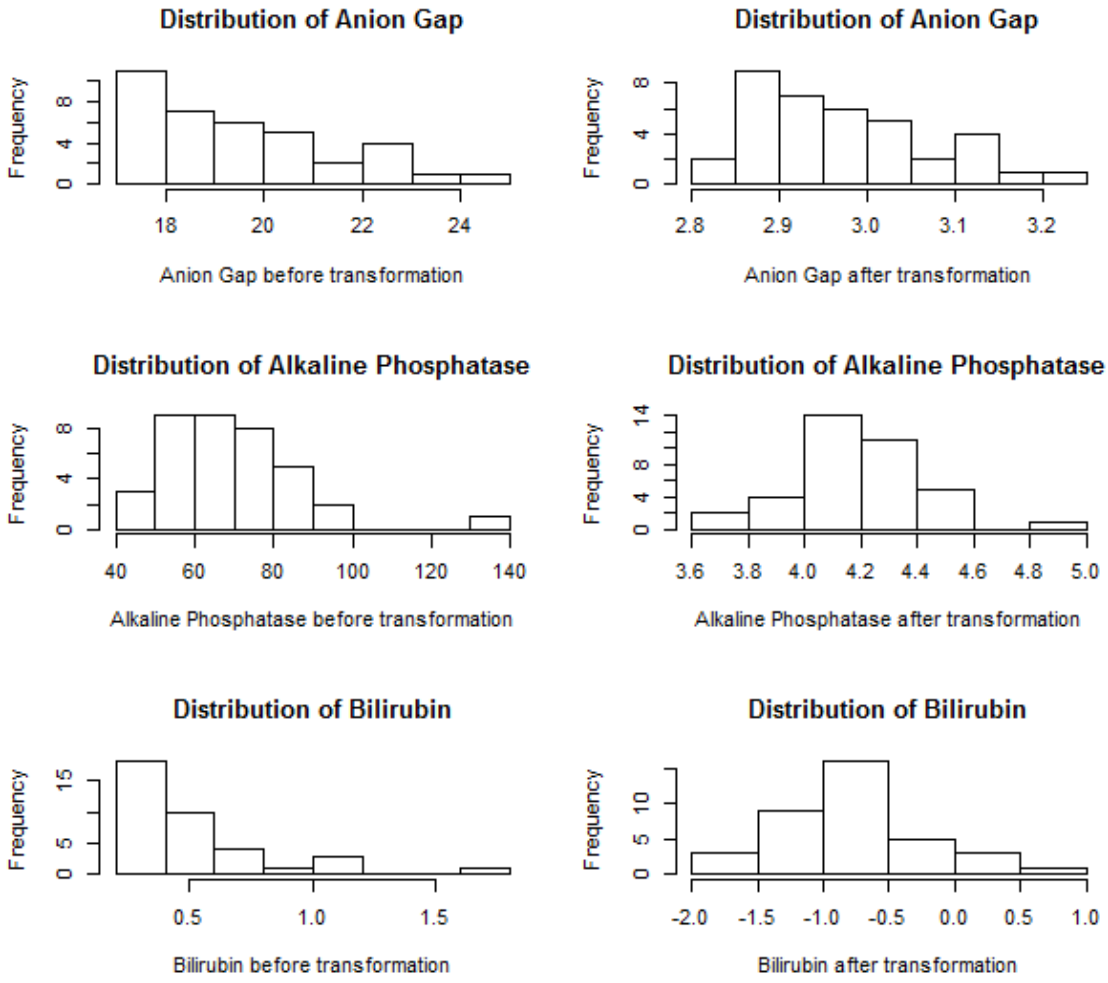


Figure IX. Effect of log-transforming anion gap, alkaline phosphatase, and bilirubin on their distributions (N = 37).

Table IX. Descriptive characteristics of ALTRUYST blood donors at enrollment.

	Apheresis	No Apheresis	Total	p
n	26	15	41	-
DEMOGRAPHICS				
Age (mean, SD)	42.6 (13.1)	45.9 (14.3)	43.8 (13.5)	0.46
Caucasian (n, %)	25 (96%)	15 (100%)	44 (98%)	-
Latino/Hispanic (n, %)	1 (4%)	0 (0%)	1 (2%)	-
ANTHROPOMETRICS[1]				
Height (inches)	70.4 (2.4)	70.2 (3.2)	70.3 (2.7)	0.89
Weight (pounds)	203.2 (32.2)	191.7 (29.0)	199.0 (31.2)	0.25
Body Composition (% body fat)	28.9 (4.7)	27.5 (5.0)	28.4 (4.8)	0.38
LABORATORY DATA[1]				
Serum Sodium (mmol/L)	143 (2.3)	142 (2.1)	143 (2.3)	0.24
Serum Potassium (mmol/L)	4.5 (0.3)	4.7 (0.3)	4.6 (0.3)	0.38
Serum Chloride (mmol/L)	101 (1.9)	100 (3.0)	101 (2.3)	0.37
Serum Carbon Dioxide (mmol/L)	22 (1.5)	21 (1.5)	22 (1.5)	0.19
Anion Gap (mmol/L)	20 (2.2)	20 (1.8)	20 (2.0)	0.34
Serum Urea Nitrogen (mg/dL)	16 (3.7)	15 (5.3)	15 (4.2)	0.80
Serum Creatinine (mg/dL)	1.00 (0.14)	0.99 (0.15)	1.00 (0.14)	0.83
Serum Glucose (mg/dL)	97 (16)	101 (25)	98 (19)	0.66
Alkaline Phosphatase (U/L)	67 (15)	75 (22)	70 (18)	0.33
Aspartate Aminotransferase (U/L)	26 (8)	27 (4)	26 (7)	0.53
Alanine Aminotransferase (U/L)	25 (12)	25 (7)	25 (10)	0.97
Serum Calcium (mg/dL)	9.7 (0.4)	9.7 (0.5)	9.7 (0.4)	0.93
Serum Inorganic Phosphorous (mg/dL)	3.3 (0.5)	3.5 (0.4)	3.4 (0.5)	0.14
Serum Total Protein (g/dL)	7.2 (0.4)	7.4 (0.5)	7.3 (0.5)	0.39
Serum Albumin (g/dL)	4.6 (0.3)	4.7 (0.3)	4.6 (0.3)	0.49
Serum Total Bilirubin (mg/dL)	0.6 (0.4)	0.6 (0.3)	0.6 (0.3)	0.95
Adult Male Testosterone (ng/dL)	520 (198)	515 (202)	518 (196)	0.94
PREVIOUS BLOOD DONATIONS[2]				
Whole Blood (n)	6	4	5	0.73
Apheresis (n)	3	3	3	0.49
BONE DENSITY[1]				
Lumbar Spine (g/cm^2)	1.214 (0.130)	1.168 (0.120)	1.197 (0.127)	0.26
Total Hip (g/cm^2)	1.133 (0.149)	1.026 (0.140)	1.094 (0.153)	0.03

[1]*mean (SD)*
[2]*median*

Blood Donations During ALTRUYST

ALTRUYST donors made a total of 534 combined blood donations during the one year study period (Figure X). All 15 (100%) donors randomized to the control arm complied with the protocol (three made zero donations, Figure X), whereas five (19%) apheresis donors did not achieve a minimum of 20 apheresis donations (Figure X). The most common apheresis donation type was a double platelet donation with mean interval between donations of 17.8 days (Table X). Donors in the apheresis arm experienced a median of 20 apheresis blood donations during the one year study period with the amount of citrate exposure by donation type ranging from 164mL – 657mL (Table X). The duration of each donation ranged from just under 30 minutes to more than two hours in length.

Table X. Apheresis collection and anticoagulant exposure characteristics for donors randomized to the treatment arm (high frequency apheresis) during the ALTRUYST trial.

		Single	**Double**	**Triple**
Collection Information	Number of Platelet Apheresis Donations	110	320	62
	Concurrent Plasma Collection *n* (%)	0 (0%)	4 (1%)	0 (0%)
	Mean (SD) Collection Time (*minutes*)	57.6 (21.8)	89.4 (23.3)	97.1 (24.9)
	Mean (SD) Inter-Donation Interval (*days*)		17.8 (14.7)	
Anticoagulant Exposure	Type of Anticoagulant		ACD-A[1]	
	Mean (SD) Volume (*mL*) AC Infused per Procedure	299 (104)	469 (111)	498 (117)

[1]*ACD-A = anticoagulant citrate dextrose solution, solution A (2.13% free citrate ion)*

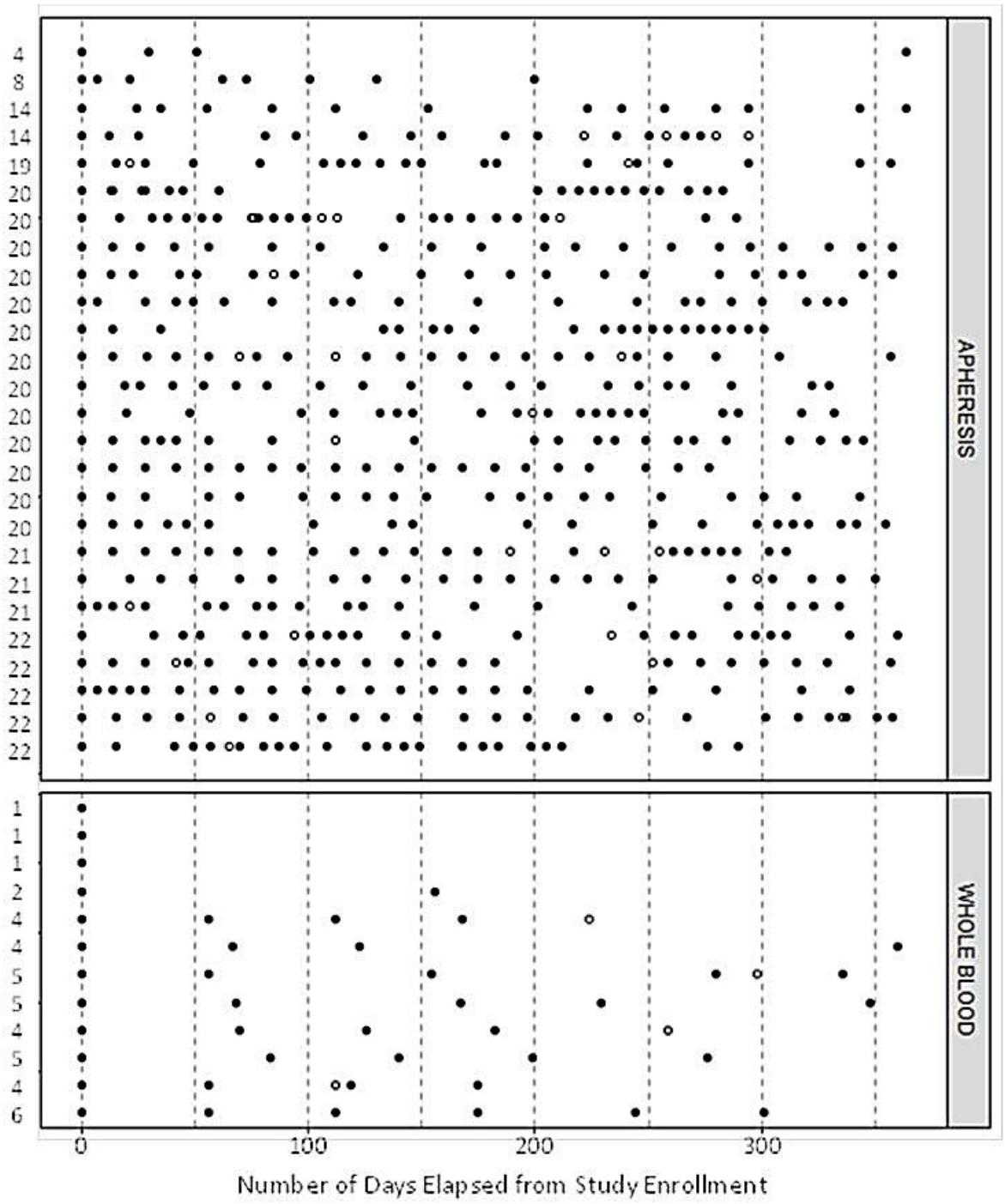


Figure X. Cleveland dot plot of donations (solid dots, n = 543) and deferrals (open dots, n = 38) from donors during the ALTRUYST study. The top panel shows donors randomized to apheresis and the bottom panel shows donors randomized to no apheresis (i.e. whole blood only). Each donor is represented as a row with the number of successful donations made during the study shown to the left.

Primary Outcome

Lumbar spine bone mineral density did not change among donors in the control arm after one year of participation (1.168 g/cm^2 at enrollment, 1.170 g/cm^2 at follow-up, mean change = -0.002 g/cm^2, 95%CI [-0.020, 0.016], p=0.16), nor did it change among donors in the apheresis arm (1.214 g/ cm^2 at enrollment, 1.213 g/cm^2 at follow-up, mean change = 0.007 g/cm^2, 95%CI [-0.005, 0.018], p=0.24) (Figure XI). Tests for differences in proportions of donors with change in BMD exceeding the least significant change (LSC) at the lumbar spine (0.00743 ±0.02058g/cm^2) between the apheresis and control arms in either a positive [apheresis 13 (50%), control 5 (33%), p=0.84] or negative direction [apheresis 8 (31%), control 6 (40%)] were statistically non-significant (p=0.87) (Figure XII). Performing the analysis with only those donors who complied with the protocol (i.e. apheresis donors making ≥20 apheresis donations) did not meaningfully alter these results.

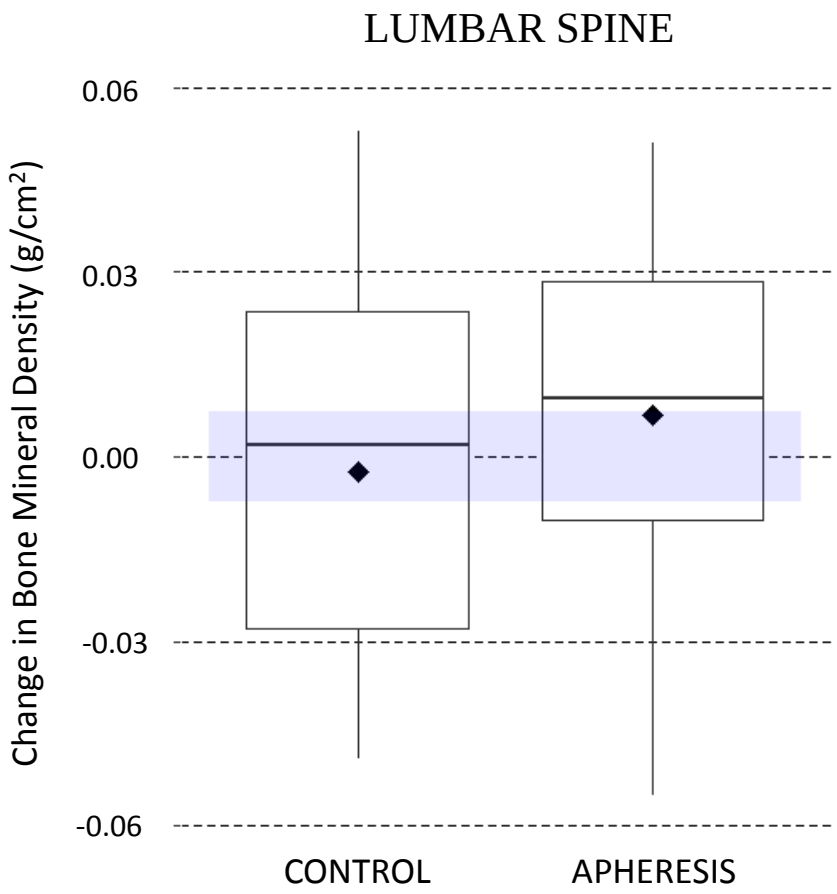


Figure XI. Boxplot showing change in bone mineral density at the lumbar spine for control (left, n = 15, mean change=-0.002 g/cm2, 95% CI [-0.020, 0.016], p=0.78) and apheresis (right, n = 26, mean change=0.007 g/cm2, CI [-0.005, 0.018], p=0.24) donors in the ALTRUYST trial. Diamonds indicate mean values; median change is represented as the central horizontal bar within the interquartile range box. The blue shaded region indicates the least significant change for the technologist.

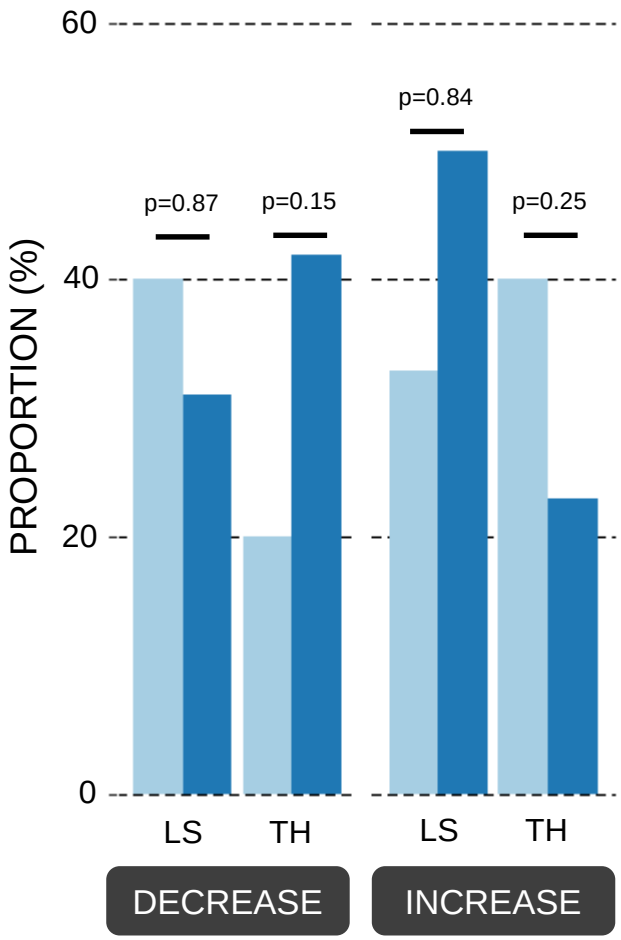


Figure XII. Proportion of subjects experiencing a decrease (left) (LS = lumbar spine, apheresis 8 (31%), control 6 (40%)) or increase (LS right, apheresis 13 (50%), control 5 (33%)) in BMD exceeding the least significant change. Proportion of subjects experiencing a decrease (left) (TH = total hip, apheresis 11 (42%), control 3 (20%) or increase (LS right, apheresis 6 (23%), control 6 (40%)) in BMD exceeding the least significant change for LS and TH. Donors in the apheresis arm are in light blue and donors in the control arm are in dark blue.

Secondary Outcomes

Change in mean BMD at the total hip was not statistically significant for control donors (1.026 g/cm^2 at enrollment, 1.028 g/cm^2 at follow-up, mean change = 0.002 g/cm^2, CI [-0.006, 0.009], p=0.63) or apheresis donors (1.133 g/cm^2 at enrollment, 1.129

g/cm^2 at follow-up, mean change = -0.004 g/cm^2, CI [-0.10, 0.002], p=0.16) (Figure XIII). Proportional increases [apheresis 6 (23%), control 6 (40%), p=0.25] and decreases [apheresis 11 (42%), control 3 (20%)] were also not significantly different (p=0.15) at the total hip (LSC=0.00671±0.01859g/cm^2) (Figure XII). Performing the analysis with only those donors who complied with the protocol (i.e. apheresis donors making ≥20 apheresis donations) did not meaningfully alter these results.

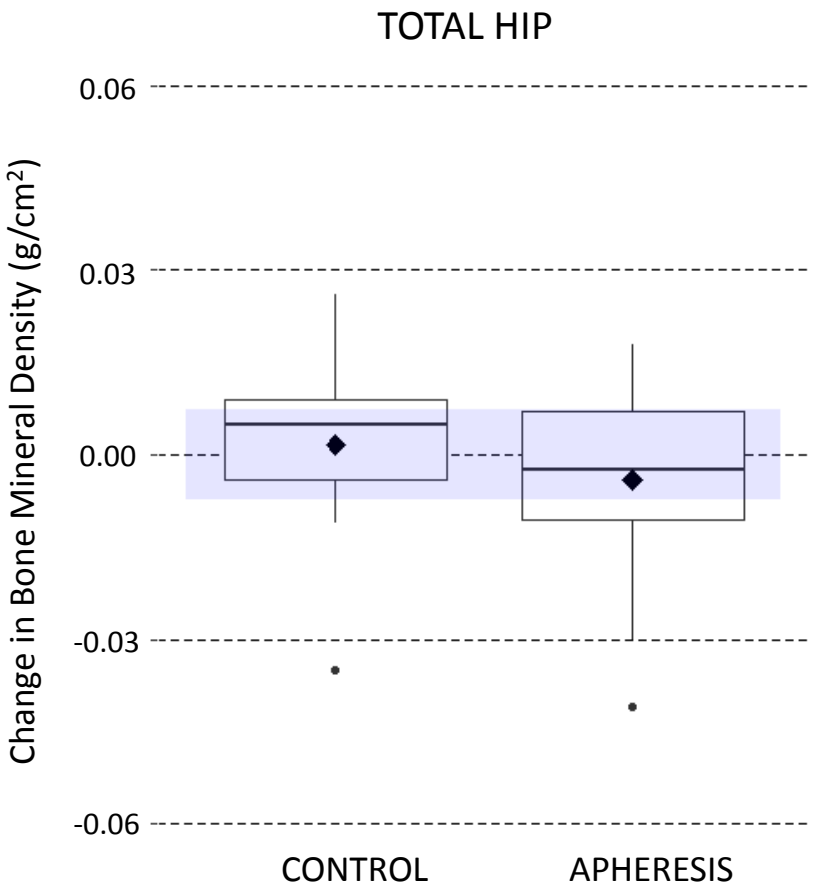


Figure XIII. Boxplot showing change in bone mineral density at the total hip for control (left, n = 15, mean change=0.002 g/cm^2, 95% CI [-0.006, 0.009], p=0.63) and apheresis (right, n = 26, mean change-=0.004 g/cm2, CI [-0.010, 0.002], p=0.16) donors in the ALTRUYST trial. Diamonds indicate mean values; median change is represented as the central horizontal bar within the box representing the interquartile range. The blue shaded region illustrates the least significant change for the technologist.

Mean femoral neck bone mineral density did not change among donors in the control arm after one year of participation (0.969 g/cm^2 at enrollment, 0.969 g/cm^2 at follow-up, mean change = 0.000 g/cm^2, 95%CI [-0.009, 0.009], p=0.63), nor did it change among donors in the apheresis arm (1.094 g/cm^2 at enrollment, 1.093 g/cm^2 at follow-up, mean change = -0.001 g/cm^2, 95%CI [-0.010, 0.007], p=0.74) (Figure XII).

Mean trabecular bone score was 1.388 (SD = 0.098) in the control group at enrollment and did not significantly change over the one year study period (1.406 (SD = 0.112) at follow-up, mean change = -0.003, 95%CI [-0.024-0.019], p=0.79). Donors in the apheresis arm had a mean trabecular bone score at enrollment of 1.474 (SD = 0.105) and it did not change over the one year study period (1.475 (SD = 0.133) at follow-up, mean change = 0.001, 95%CI [-0.022, 0.024], p=0.92).

Multivariable Logistic Regression

Multivariable logistic regression with change exceeding the LSC in both positive (gain in BMD) and negative (loss of BMD) directions using automated stepwise backwards elimination did not identify baseline covariates that were significantly associated with either outcome (Table XI). This was an exploratory analysis as the study was not powered to identify significant predictors. The regression took the general form:

$$logit[pr(Y=1)] = \beta 0 + \beta 1\,(apheresis) + \beta 2\,(age) + \beta 3\,(risk\ factors=1) + \beta 4\,(risk\ factors=2) + \beta 5\,(risk\ factors=3) + \beta 6\,(risk\ factors=1) + \beta 7\,(family\ history=1) + \beta 8\,(family\ history=2) + \beta 9\,(family\ history=3) + \beta 10\,(health\ conditions=1) + \beta 11\,(health\ conditions=2) + \beta 12\,(medication\ use=1) + \beta 13\,(diet=3) + \beta 14\,(diet=4) + \beta 15\,(diet=5) + \beta 16\,(diet=6) + \beta 17\,(diet=7) + \beta 18\,(medication\ use=2) + \beta 19\,(BMI) + \beta 20\,(pre\_(site)) + \beta 21\,(Na) + \beta 22\,(K) + \beta 23\,(Cl) + \beta 24\,(CO_2) + \beta 25\,(anion\ gap) + \beta 26\,(urea) + \beta 27\,(creatinine) + \beta 28\,(glucose) + \beta 29\,(alkaline\ phosphatase) + \beta 30\,(AST) + \beta 31\,(ALT) + \beta 32\,(Ca) + \beta 33\,(P) + \beta 34\,(protein) + \beta 35\,(albumin) + \beta 36\,(bilirubin) + \beta 37\,(testosterone)$$

Table XI. Results of exploratory multivariable logistic regression analysis with change in bone mineral density as the outcome [coefficient (p value)].

	Negative Change		Positive Change	
	Lumbar Spine	**Total Hip**	**Lumbar Spine**	**Total Hip**
Apheresis versus No Apheresis	0.49 (0.04)	1.64 (0.36)	0.48 (0.19)	-0.80 (0.53)
Age	-0.03 (0.03)	0.00 (0.92)	0.04 (0.09)	0.02 (0.53)
Risk Factors = 1	-0.54 (0.06)	-0.25 (0.79)	-0.25 (0.38)	0.06 (0.94)
Risk Factors = 2	-0.15 (0.22)	1.28 (0.54)	-0.23 (0.44)	-1.17 (0.52)
Risk Factors = 3	-0.65 (0.09)	0.33 (0.87)	-0.83 (0.24)	-0.17 (0.92)
Risk Factors = 4	-2.70 (0.03)	2.57 (0.54)	2.79 (0.11)	-2.59 (0.49)
Family Risk Factors = 1	-0.27 (0.17)	0.70 (0.69)	-0.95 (0.17)	-1.78 (0.36)
Family Risk Factors = 2	-0.96 (0.04)	0.33 (0.81)	-0.11 (0.71)	-1.51 (0.34)
Family Risk Factors = 3	-1.09 (0.07)	1.56 (0.64)	0.18 (0.76)	-4.15 (0.30)
Health Conditions = 1	1.13 (0.03)	0.86 (0.58)	0.06 (0.83)	-0.12 (0.92)
Health Conditions = 2	2.99 (0.05)	-0.52 (0.92)	1.78 (0.26)	3.30 (0.51)
Medication Use = 1	-0.53 (0.09)	-0.76 (0.64)	-0.95 (0.16)	0.02 (0.99)
Diet = 3	0.31 (0.11)	0.71 (0.65)	-0.04 (0.87)	-0.05 (0.97)
Diet = 4	-0.11 (0.36)	0.57 (0.75)	0.55 (0.27)	-0.09 (0.95)
Diet = 5	0.91 (0.04)	-0.29 (0.81)	-1.69 (0.08)	-0.47 (0.67)
Diet = 6	1.40 (0.03)	-0.85 (0.60)	-1.16 (0.14)	1.45 (0.39)
Diet = 7	5.05 (0.02)	-0.97 (0.77)	-1.05 (0.31)	2.58 (0.45)
Body Mass Index	0.04 (0.07)	-0.06 (0.61)	0.01 (0.75)	0.11 (0.38)
Baseline BMD	4.54 (0.03)	-4.69 (0.50)	-5.65 (0.08)	4.21 (0.48)
Serum Sodium	-0.11 (0.11)	-0.62 (0.41)	-0.30 (0.14)	0.44 (0.47)
Serum Potassium	0.60 (0.08)	-0.59 (0.75)	-2.16 (0.08)	-0.35 (0.82)
Serum Chloride	-0.15 (0.10)	0.54 (0.46)	0.11 (0.43)	-0.63 (0.37)
Serum Carbon Dioxide	-0.12 (0.07)	0.06 (0.87)	0.29 (0.11)	0.10 (0.77)
Serum Urea Nitrogen	0.15 (0.02)	-0.05 (0.59)	-0.12 (0.09)	0.05 (0.55)
Serum Creatinine	-4.15 (0.03)	4.25 (0.60)	2.44 (0.17)	-3.84 (0.58)
Serum Glucose	0.00 (0.40)	0.02 (0.50)	-0.02 (0.14)	-0.05 (0.25)
Alkaline Phosphatase	-0.01 (0.13)	0.02 (0.50)	0.02 (0.18)	0.00 (0.98)
Aspartate Aminotransferase	0.01 (0.18)	0.08 (0.53)	0.03 (0.23)	-0.03 (0.74)
Alanine Aminotransferase	-0.06 (0.03)	0.04 (0.65)	0.08 (0.09)	-0.04 (0.60)
Serum Calcium	-0.51 (0.10)	0.56 (0.83)	0.37 (0.40)	-2.51 (0.39)
Serum Inorganic Phosphorous	-0.02 (0.67)	0.56 (0.63)	-0.40 (0.19)	-0.39 (0.69)
Serum Total Protein	-0.24 (0.28)	-0.58 (0.75)	-2.24 (0.11)	0.23 (0.88)
Serum Albumin	-0.51 (0.18)	0.68 (0.83)	4.42 (0.07)	1.79 (0.55)
Serum Total Bilirubin	-0.15 (0.22)	1.06 (0.62)	-1.55 (0.08)	-2.09 (0.36)
Adult Male Testosterone	0.00 (0.04)	0.00 (0.65)	0.00 (0.85)	0.00 (0.81)

Dual-Energy X-ray Absorptiometry Calibration

Appendix 15 shows the quality assurance data for the period of image acquisition (May 1, 2016 – February 1, 2018). During this period, 597 phantom scans were performed per protocol. A phantom scan was performed within the 48 hour period preceding every ALTRUYST scan. Density of the phantom is 1.497, 0.995, and 0.497 g/cm^2 at the high, medium, and low bands, respectively. The percent coefficient of variation was 0.3%, 0.6%, and 1.3% for high, medium, and low BMD regions during the period of scan acquisition, respectively. Overall BMD precision was 0.24% (Appendix 15). The machine was not moved during the project period, and, all routine service and maintenance were performed per manufacture specifications.

Conclusion

ALTRUYST was a prospective, longitudinal, randomized controlled trial evaluating the role of high frequency apheresis blood donation on change in BMD. There were no significant alterations to BMD at the lumbar spine or total hip. Therefore, the null hypothesis that high frequency apheresis blood donation does not cause significant declines in BMD after a one year follow-up period cannot be rejected. Analyses of secondary outcome measures indicate that there were no significant changes in mean femoral neck BMD, nor trabecular bone scores. Multivariable logistic regression was not able to identify significant predictors of either positive or negative change at any site measured due to potential lack of statistical power. An analysis of those apheresis donors who complied with the protocol did not alter these conclusions.

CHAPTER FIVE: ALTRUYST Discussion

ALTRUYST was a prospective, longitudinal, randomized controlled trial evaluating the role of high frequency platelet apheresis blood donation on change in BMD in males and found no significant alterations to BMD at the lumbar spine or total hip. Previous studies demonstrated a higher prevalence of low BMD among apheresis donors when compared to non-blood donors (11) or whole blood donor controls, (12) independent of donor gender or age. One study reported no difference among apheresis and whole blood donors (13). These conflicting results likely stem from the cross-sectional designs employed in these previous investigations. Important determinants of BMD include genetic (77) and behavioral factors (78) that have been integrated into previous study designs to varying degrees. Using an individual blood donor as their own control represents a key feature of ALTRUYST supporting the conclusion that very high frequency platelet apheresis, and concomitant exposure to large doses of citrate AC, over a one year period do not induce changes in BMD. Furthermore, BMD of a control group randomly assigned to no apheresis remained unchanged during the study period, consistent with large studies of the male US population 18-65 years of age (79).

In the absence of fracture, low BMD is the single best predictor of fracture risk (64) and the corresponding association is exponential (80). Analysis of more than 140,000 Swedish blood donors over a 23 year period demonstrated no association between the risk of fracture and, mostly (94-98%), plasma apheresis (56). Plasmapheresis collections expose donors to a fraction of the AC that platelet apheresis donors receive (23) and high frequency platelet apheresis donors were not present in the SCANDAT2

analysis. Donors in the ALTRUYST trial almost exclusively donated platelets by apheresis (<1% of donations involved concurrent plasma collections) and received an average of between 300mL (single platelet apheresis) and 500mL (triple collections) of AC per procedure with an average of 17 days between exposures. Most ALTRUYST donors in the apheresis arm (21/26, 81%) achieved 20 or more apheresis donations during the one year study period. No donors in the ALTRUYST study experienced fracture during the follow-up period. Risk for low trauma fractures among otherwise healthy males 18-65 years of age is essentially zero,(81, 82) and, ALTRUYST was not powered using significant change in fracture risk as an outcome. Nevertheless, the finding that the upper limit of citrate AC exposure, both in terms of dose and frequency, failed to produce significant alterations to BMD among these donors indicates that current apheresis collection guidelines are adequate in protecting the bone health of the volunteer blood donor population and do not likely alter fracture risk in this donor population.

Dual-energy x-ray absorptiometry (DXA) is the industry standard for assessing bone mineral density and has many strengths relative to other BMD technologies. Access to large reference databases, including the National Health and Nutrition Examination Survey, permitted estimation of population mean and standard deviation bone mineral density values, specifically for the age- and gender-eligible group, before enrolling subjects in ALTRUYST. This feature of DXA allowed for robust modeling of power and sample size to occur in advance of the study itself. DXA is also the most accurate way of measuring BMD. The fact that not all insurance providers cover BMD assessment using DXA does not impact the decision to employ this as the primary outcome as all scans were provided to subjects free of charge. However, the acquisition of dual photon

absorptiometry (DPA) images requires significantly more time, which may have made recruitment for ALTRUYST challenging. Quantitative computed tomography (CT) provides three-dimensional images at the lumbar spine and has superior ability to measure changes in trabecular bone earlier than DXA (83). A lack of large reference databases for quantitative CT, inferior precision, and effective radiation doses several orders of magnitude higher than DXA are fundamental drawbacks to using this technology. Ultimately, the ability for DXA to accurately measure areal BMD at the lumbar spine and total hip, with minimal costs to subjects in terms of time and comfort, enriched the overall feasibility of performing this clinical trial.

ALTRUYST was 80% powered to detect a 3% change in bone mineral density over the one year study period. Though mean bone mineral density was higher in the ALTRUYST cohort relative to a gender- and age-matched sample of the US population, this is expected due to a well known “healthy donor effect” where volunteer blood donors consistently present with health indices superior to population norms (84). Furthermore, the variability about the measure of central tendency among ALTRUYST donors indicates that individuals within the cohort were not contributing any outlier effects that could bias our assessment of the study’s outcomes. This is, in part, due to the fact that ALTRUYST deliberately excluded individuals with BMD that fell outside of a 95% population-based estimate of mean BMD (i.e. ±2 standard deviations of Z-score). Seven individuals enrolled in ALTRUYST met this criterion and required exclusion per protocol. The higher than anticipated prevalence of abnormal BMD in the enrolled cohort cannot be explained, other than the possibility that such an observation was spurious. The exceedingly low dose of radiation these individuals experienced represents another aspect

of DXA that illustrates its superiority in assessing BMD in clinical studies. Other limitations of DXA are primarily related to potential sources of error, including differences observed among the three commercial manufacturers, differences in edge detection softwares, the need for rigorous quality control across systems when multiple sites are involved due to differences in x-ray tube or detector functionality, technologist-related sources of error, and various sources of shift and drift in these finely tuned and calibrated systems. ALTRUYST was a single-center study that used one GE DXA instrument, one technologist performed all study scans, and shift and drift were quantified throughout the period of measurement. These design features represent a strength in the ALTRUYST study and illustrate the much larger sample sizes that would be required to perform such a study at multiple centers.

BMD over the life course is largely influenced by heritability. Individual aberrancies in BMD were accounted for using the exclusion criterion for BMD exceeding two standard deviations of an age- and gender-matched mean value at enrollment. Family history of fractures was solicited from participants at enrollment through the use of the questionnaire. Any familial predisposition to disease of bone and mineral metabolism were coded as an ordinal variable, where the mean value was *one* and the median value was also *one*, were added into the multivariable logistic regression. This predictor did not achieve significance for changes either in the positive or negative direction. Further, the randomized nature of this study ensured that the likelihood of being assigned to either group was equivalent. Nevertheless, confounding could occur if subjects experienced changes to other important determinants of BMD including physical activity, diet, and medication use. In addition to excluding subjects with known diseases of bone and

mineral metabolism, subjects were deliberately excluded if they were taking medications known to impact BMD. Upon follow-up assessment, no changes in medication were noted meaning that any confounding from medication use was absent. Physical activity did not differ between the two randomized groups, and, changes in physical activity sufficient to invoke changes in BMD over the one year interval did not occur. The self-report questionnaire was administered by the investigative team to avoid any non-response bias. These design features and observations indicate that the findings of ALTRUYST are extremely unlikely to have known confounding that could have impacted the results of the study and though the potential for residual, unmeasured confounding can never be completely eliminated, the complete lack of any significant change in any outcome measured suggests that any such effect was minimal if present.

In addition to progressive declines in bone density, microarchitectural deterioration of bone is a fundamental part of the definition of diseases of reduced BMD (85, 86). Among these microarchitectural features are the number of trabeculae, separation between trabeculae, and the density of connections between trabeculae. Changes in these three dimensional features are associated with increasing bone fragility and susceptibility to fracture, (14, 87) even when bone density is the same between two samples. The trabecular bone score (TBS) is an analytic tool that quantifies the extent of between-pixel differences in x-ray attenuation from dual-energy x-ray absorptiometry images approximating microarchitectural features of bone. In contrast to alternative techniques for examining bone microarchitecture, obtaining TBS is non-invasive and uses standard dual-energy x-ray absorptiometry images of the lumbar spine, making it ideally suited to quantify microarchitectural changes in healthy volunteers. TBS did not

change among ALTRUYST donors in the apheresis arm, or among donors in the control arm. This finding is consistent with previous research showing that the magnitude of change in TBS is less than that of areal BMD in the spine.(88-90) Findings from ALTRUYST are also consistent with the observation that TBS remains relatively unchanged before the age of 45 years (91). Future research examining the potential anabolic effect of repeated apheresis blood donation among aging women would be an appropriate setting to explore changes in TBS in addition to BMD.

Apheresis induces the secretion of parathyroid hormone (26, 38, 39) and three cascading physiologic processes ensue: increased calcium reabsorption in the distal convoluted tubule, increased intestinal calcium absorption, and increased bone resorption. Data from ALTRUYST indicate that any increase in bone resorption among apheresis donors did not alter bone density more than what was defined as clinically meaningful *a priori*. Surprisingly, apheresis-induced surges in parathyroid hormone produce renal calcium loss (35, 92). Absorptive hypercalciuria is associated with increased urinary calcium loss resulting from increased absorption of calcium in the intestine. The elevated absorption of calcium can be controlled with ingestion of cellulose phosphate that binds free calcium in the gut (93). Hypercalciuria is also one of the clinical manifestations of Bartter's syndrome where genetic mutations in renal sodium-potassium-chloride co-transporters serve as the etiological foundation for this disease (94) and suggest genetic modifiers of mineral homeostasis could also contribute to the likelihood of an individual donor's response to repeated apheresis. In all cases of hypercalciuria, the formation of calcium stones (nephrolithiasis) is an associated untoward health outcome. Therefore, the observation that apheresis donors experience hypercalciuria subsequent to donation

invokes the possibility that apheresis could meaningfully increase the risk of nephrolithiasis among donors. Because the formation of calcium stones is a health outcome with a relatively long latency, case-control studies could theoretically examine this possibility if medical records and blood donation records were linked.

Disrupting the production of parathyroid hormone to circumvent the renal excretion of calcium in apheresis donors could theoretically be achieved by providing oral or intravenous calcium to supplant the biological stimulus for parathyroid hormone production (namely, reduced serum ionized calcium). Because citrate administration occurs at the point of venipuncture, any co-administration of calcium avoids interference with citrate's role in providing anticoagulation in the extracorporeal circuit. However, urinary loss of calcium was not suppressed in a placebo-controlled study where oral calcium was provided to apheresis donors (31). Furthermore, prophylactic intravenous calcium fails to abrogate the urinary loss of calcium induced by apheresis (35). It is therefore likely that urinary calcium loss is an obligatory side effect of apheresis blood donation. Exogenous 1,25 di-hydroxy-vitamin D increases serum calcium through intestinal absorption and can indirectly modify the set point of parathyroid hormone (95). Exposure to citrate AC during apheresis blood donation results in surges of parathyroid hormone in apheresis blood donors that acutely suppresses serum ionized calcium concentrations. ALTRUYST deliberately recruited healthy volunteers, naïve to apheresis, and their laboratory test results at baseline were within expected ranges. Therefore, it is plausible that high frequency apheresis could have altered the set point of parathyroid hormone with repeated donation during the study. In addition to altering the set point of parathyroid hormone, studies among patients with vitamin D deficiency have indicated

that the kidneys can become more sensitive to parathyroid hormone over time (96). This observation invokes the complimentary possibility that the body copes with high frequency apheresis blood donation by altering the exchange of phosphate in the proximal convoluted tubule and calcium in the distal convoluted tubule of kidney nephrons. Serial serum samples were not obtained from ALTRUYST participants largely due to concerns around anticipated upper limits of allowable volume depletion in volunteer blood donors. The physiological mechanism that permits high frequency apheresis blood donation without alterations to bone mineral density is a provocative question and future studies would need to integrate design features that stimulate repeated surges in parathyroid hormone without losing participants to follow-up whose donation volumes are in excess of accepted standards.

Secondary hyperparathyroidism is a clinical condition that results in sustained elevations in parathyroid hormone. Insufficient excretion of phosphate, an inability to produce 1,25 di-hydroxy vitamin D, vitamin D deficiency, and intestinal malabsorption are among the causes of secondary hyperparathyroidism and the clinical sequelae include decreased BMD. Because parathyroid hormone concentrations surge in the 60-90 minute interval when apheresis is being performed (11, 22, 38, 39), we hypothesized that frequent, repeated surges in parathyroid hormone from apheresis blood donation could reduce BMD through osteoclastic osteolysis. One clinical feature of secondary hyperparathyroidism is the enlargement of the glands themselves (parathyroid gland hyperplasia) that can be reversed with medication (97), illustrating the ability of the parathyroid glands to adapt to chronic stress. In contrast to secondary hyperparathyroidism where parathyroid hormone production is chronically increased, the

recovery of parathyroid hormone to pre-apheresis levels in donors after 120 minutes (11) may suggest that the stress of apheresis is insufficient to produce hyperplasia in the parathyroid glands in apheresis donors. Though high frequency apheresis blood donation does not induce changes in BMD, the possibility that morphological changes in donor parathyroid glands do not occur cannot be ruled out.

Understanding how the stimulus for parathyroid hormone secretion is received by the parathyroid glands could provide another mode for mitigating the large surges in parathyroid hormone experienced by apheresis blood donors. The calcium-sensing receptor (CASR) is a membrane spanning protein that plays a central role in maintaining calcium homeostasis in the blood (37) making it an ideal candidate to consider in the context of personalized apheresis blood donation. Homozygous lack of function mutations in the CASR gene cause neonatal severe hyperparathyroidism, an autosomal dominant heritable disease that can be fatal if the parathyroid gland isn't removed (98-100). Heterozygous lack of function mutations in the CASR gene cause familial hypocalciuric hypercalcemia resulting in altered bone mineralization and increased incidence of kidney stones (101-103). These clinically apparent diseases have led to the careful characterization of the CASR (e.g. (104)), including the assessment of CASR genotype on calcium. A meta-analysis by He and colleagues (105) found that one or two serine substitutions at locus 986 of CASR resulted in significantly higher total (0.028; 0.012-0.045, P=0.001) and ionized calcium (0.016; 0.013-0.020, P<0.0001) as compared to subjects homozygous for alanine. Nevertheless, studies combining clinical outcomes, such as low bone density and kidney stones, with measurements of extracellular calcium were not able to consistently show detrimental effects of CASR mutations on these

measures [e.g. (106)] and it is unlikely that blood collecting agencies would genotype donor polymorphisms for CASR or other potential determinants of mineral homeostasis before permitting apheresis blood donation. Furthermore, results from ALTRUYST indicate that any such efforts would primarily target modest improvements in donor side effects to citrate exposure and not long term health outcomes.

ALTRUYST deliberately studied male blood donors because they constitute the vast majority (approximately 80%) of the apheresis blood donor population at BloodCenter of Wisconsin, with 85% of higher frequency donors (defined as ≥15 apheresis donations within a one year period) also male. Furthermore, the study recruited donors with no more than five lifetime apheresis donations so as to avoid any potential biological adaptation that may occur with repeated exposure to citrate AC. Though we report no change in bone mineral density among men, aged 18-65 years, experiencing high frequency apheresis over a one year period, we are unable to extrapolate these findings to women of any age. There remains the possibility that high frequency apheresis affects women differently than men, particularly during the peri-menopausal period when changes in serum estrogen have been correlated with large fluctuations in BMD with supplemental estrogen improving bone-related health outcomes (107, 108). The scarcity of higher frequency female apheresis donors at the blood center studied indicate that any exploration of the impact of high frequency apheresis on BMD among women would require a multi-center design, and, careful consideration of how to concurrently mitigate the effects of iron deficiency associated with regular platelet apheresis blood donation (109) that disproportionately impact women (110). Furthermore, the high prevalence of low BMD among women, especially that increases

over the life course (111), indicates this would be an ideal group to evaluate the possible benefit of repeated alterations to PTH through apheresis (53, 112) that resemble those of synthetic PTH treatments for osteopenia/osteoporosis with demonstrable improvement in BMD (14).

Before 1988, the US Food and Drug Administration allowed individual volunteer donors to make 12 apheresis platelet donations per year (9). Two studies presented in an AABB Advisory Committee comment made in 2006 (113) predate a policy change increasing the number of donations an individual volunteer donor can make to 24 apheresis platelet donations per rolling 12 month period with no lifetime maximum (114). In one of these studies (6), 335 donors underwent platelet apheresis at frequent, but highly varied collection intervals. Initially, platelet count and yield declined to nadir between the seventh and ninth donation. However, a progressive rise in platelet count was observed over the course of repeated donations and recovery was achieved at the tenth donation for many donors despite continued collections. The safety of repeated collections was illustrated by the fact that at no time a donor exhibited a platelet count putting them at risk for acute bleeding (8), nor did any develop signs of persistent thrombocytopenia. A second study (8) is referenced in the Guidance where 105 platelet apheresis donors of various collection intensities (single, double, and triple product donors) were assessed for acute thrombocytopenia immediately following collections. It was reported that post-donation platelet counts never dropped below 100,000/μL. In all cases of post-donation platelet count falling below 150,000/μL the donor's platelet count recovered within the 2 – 4 weeks following donation. Findings from the ALTRUYST trial extend our understanding of the safety of repeated apheresis platelet collections

beyond the context of these earlier studies documenting that donors are able to maintain platelet counts within the normal biological range with repeated donation. The highest frequency of apheresis blood donation appears safe in terms of platelet recovery and stresses to mineral homeostasis.

CONCLUSIONS

Central to the safety and availability of the global blood supply is the community of volunteer blood donors whose altruism saves the lives of patients in need of transfusion. Though the collection of blood will inherently confer some risk to blood donors, it is essential that these risks are calibrated appropriately. In contrast to whole blood collections, apheresis requires the use of an anticoagulant to prevent extracorporeal coagulation. Citrate is the industry standard anticoagulant and functions by sequestering serum ionized calcium. Apheresis blood donors experience exposure to large quantities of citrate as the mixture of residual blood components and anticoagulant are returned intravenously. This exposure results in dramatic fluctuations in mineral homeostasis including changes serum ionized calcium, parathyroid hormone, vitamin D, and markers of bone metabolism. Cross-sectional studies of bone mineral density among apheresis blood donors drew conflicting conclusions about the impact of citrate exposure and skeletal health. ALTRUYST was a prospective, longitudinal, randomized, controlled trial testing the hypothesis that high frequency apheresis causes declines in bone density. Forty-one donors completed the study and there was no change in bone mineral density at any site measured among donors completing a median of 20 apheresis blood donations in the one year study period. Bone density did not change among members of the control group who did not undergo apheresis blood donation. Despite significant, repeated challenges to mineral homeostasis among apheresis blood donors, we conclude that current collection guidelines adequately protect the skeletal health of adult male, high frequency apheresis blood donors.

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APPENDICES

Appendix 1 – Informed Consent

Medical College of Wisconsin & Froedtert Hospital

EFFECTIVE
04/26/2016
MCW/FH IRB

Informed Consent for Research
Template A-com - Version: March 6, 2015
IRB Protocol Number: 26241
IRB Approval Period: 04/26/2016 – 04/25/2017

Medical College of Wisconsin and Froedtert Hospital
CONSENT TO PARTICIPATE IN RESEARCH

Name of Study Subject: _____

RAndomized Longitudinal STudy of ApheResis VolUntarY Blood DonorS' Bone DensiTy
<<ALTRUYST>>

Paula Papanek, PhD, MPT, LAT, FACSM
Clinical and Translational Rehabilitation Health
(414) 288-5069
Marquette University, Cramer Hall Room 215a, Milwaukee, WI 53201

Thank you for donating blood. You are invited to take part in this research study. This form tells you why this research study is being done, what will happen in the research study, and possible risks and benefits to you. If there is anything you do not understand, please ask questions. Then you can decide if you want to join this study or not.

A1. INTRODUCTION – WHY ARE WE ASKING YOU ABOUT THIS STUDY?

You are invited to participate in this research study because you are male, 18 – 65 years of age, an eligible volunteer blood donor, you have made at least one apheresis blood donation in the past but no more than five, and you are planning to donate apheresis blood products frequently (20 - 26 times) in the next 12 months.

The Director of the study is Dr. Paula Papanek in the Department of Clinical and Translational Rehabilitation Health. A study team works with Dr. Papanek. You can ask who these people are. About 60 people are expected to participate in this study at BloodCenter of Wisconsin. Marquette University and BloodCenter of Wisconsin are funding the study.

A2. DO I HAVE TO BE IN THIS STUDY?

You can decide whether to take part in this study or not. You are free to say yes or no. If you say no, your relationship with BloodCenter of Wisconsin and/or Marquette University will not change. Even if you join this study, you do not have to stay in it. You may stop at any time.

A3. WHY IS THIS RESEARCH STUDY BEING DONE?

The purpose of this study is to investigate the impact of repeated apheresis blood donation on the bone density of donors. Apheresis utilizes citrate, a clear fluid that keeps your blood from clotting in the tubing. Citrate works by binding calcium in your blood. Most of the calcium in your body is stored in bone. We'd like to know if citrate used in apheresis affects the density of your bones.

B1. WHAT WILL HAPPEN IF I TAKE PART IN THE STUDY?

Screening procedures:

If you decide to join the study, you will complete a **questionnaire** where we will ask you about things affecting your risk of a bone fracture like your race, family history of osteoporosis (a condition characterized by a decrease in the density of bone), or taking a daily calcium supplement. If the screening questionnaire shows that you meet the requirements, then you will be able to start the study.

If the screening questionnaire shows that you cannot be in the research study, then you will not complete any further study procedures. You may continue being a blood donor.

Randomization:

We do not know if donating apheresis blood products affects your bones in any way, either positively or negatively. To help us answer this question, you will be "randomized" into one of two study groups. Randomization means that you are put into a group by chance. It is like flipping a coin. Neither you nor the study director can choose what group you will be in.

One group will be encouraged to undergo **regular apheresis** for one year as planned. If you are randomized to this group, then we will ask you to donate apheresis blood products as frequently as possible. Two in three people will be randomly placed in this group.

Another group will be asked to **forgo apheresis** during the study altogether. We will ask you to refrain from donating apheresis blood products for a 12 month period, and whole blood donation is allowable. One in three people will be randomly placed in this group.

Study procedures:

If you are eligible and randomized, then we will perform a suite of **bone density scans** at Marquette University both at the beginning and end of the study:

- it is painless and takes approximately 30 minutes;
- you will be asked to wear comfortable clothing;
- you will be asked to remove any body piercing or other metal or electronic objects from your body as these objects interfere with the scan;
- you will be asked to lie still during your scans;
- you will change positions for each scan: first, you will lie on your back on a table for approximately 7 minutes; then, you will lie on your back on a table for approximately 5 minutes while your legs are turned inward; then you will lie on your back with your legs elevated for approximately 5 minutes;
- and a mechanical arm will pass over your body.

We will **collect procedure information** from each apheresis donation that you make during your time in the study. We will also use your **donation history information** as far back in time as it exists in the blood center records. We will collect **a blood sample** (approximately 1.5 teaspoons) before the start of an apheresis procedure for laboratory testing. Finally, you will be asked to **report any fractures** (broken bones) that you have during your participation in the study.

As part of the procedure for donating a unit of blood, your blood will be tested for diseases that can be passed on to other people by transfusion, including AIDS (the disease caused by the HIV virus), syphilis, hepatitis B, hepatitis C and others. If certain tests are positive, BloodCenter of Wisconsin may inform you, put your name on a list of ineligible donors, and inform certain government health agencies as required by law. Results of your blood test for HIV will be released only to authorized persons as governed by Wisconsin law. A list of persons to be notified and reasons that will cause release of your blood test for HIV is available upon request. Results of the test for HIV will be released to BloodCenter physicians and their assistants. Abnormal test results of active military personnel will be forwarded to the military medical authority of the base to which you are assigned, as required by the Department of Defense.

B2. HOW LONG WILL I BE IN THE STUDY?

You will be in this research study for about one year. After the study is finished, we may want to contact you to follow your health over time. We may contact you by telephone or email to ask you a few questions or explain a follow-up study.

B3. CAN I STOP BEING IN THE STUDY?

You may stop at any time. If you decide to leave the study, please let the study team know. The study investigator may stop your participation in the study at any time for any reason without your consent. She will tell you if this happens.

B4. ARE THERE ANY SPECIAL INSTRUCTIONS WHILE I AM IN THE STUDY?

Please report any fractures (broken bones) to Walt Bialkowski at (414) 937-3851.

C1. WHAT RISKS OR PROBLEMS CAN I EXPECT FROM THE STUDY?

This is an observational study only, meaning that there is no intervention and no change to your medical care. Other than the known risks of blood donation, the additional risks of participation in this research study include those associated with **radiation exposure** and **confidentiality**.

The bone density scanner emits a very low level of radiation. An exposure of 6 microsieverts for four DXA scans is expected. That is less than one-fifteenth of the radiation that you get with one chest x-ray. Typically, persons in the U.S. receive a radiation dose of approximately 8 microsieverts per day from natural sources of radiation, including the sun, air, water and soil. **Total radiation dose from research procedures will be less than the amount of natural background radiation an average person is exposed to each day.** There are no confirmed adverse health effects associated with this amount of radiation exposure and no radiation has been shown to remain in the body after the scan. Such doses of radiation may be harmful, but the risks are so small that they are difficult to measure.

Another risk may be loss of confidentiality. Every effort will be made to keep your study records confidential but we cannot guarantee it.

C4. ARE THERE ANY BENEFITS TO TAKING PART IN THE STUDY?

You may or may not benefit from participating in this study. Upon request, you will be given a summary report of the bone density scan(s) performed as part of this study which may or may not be beneficial. You will only receive these reports at the end of the study. There is the possibility that what is learned in this study could benefit other blood donors in the future.

D1. ARE THERE ANY COSTS TO BEING IN THE STUDY?

There are no costs to you for any of the visits or services you receive in this study.

D2. WILL I BE PAID FOR PARTICIPATING IN THE STUDY?

You will receive $50.00 for each DXA appointment you complete to cover your expenses. There is one appointment at the beginning of the study and another at the end (up to $100.00 total). If it is determined that you are ineligible after your first bone density scan, then you will receive $50.00 (not $100.00). At the end of your participation the study manager will evaluate whether or not you complied with the study guidelines (Section B1). If you adhere to all study guidelines then you will be provided with an additional $100 (no apheresis group) or $250 (regular apheresis group). The maximum amount of remuneration is $200 for donors in the no apheresis group and $350 for donors

in the regular apheresis group. This compensation is not tied to the blood donations you made during your participation in the study, but solely for the clinical assessments and complying with research study guidelines.

D3. WHAT OTHER CHOICES DO I HAVE?

Your decision whether or not to take part in this study is voluntary. It will not change your relationship with BloodCenter of Wisconsin or Marquette University in any way.

D4. WILL I BE GIVEN NEW INFORMATION ABOUT THE STUDY?

Certain unexpected test results we obtain may be important to your health. You will be notified if DXA scan results are out of the normal range and may be of potential medical importance.

A description of this clinical trial is available on *http://www.ClinicalTrials.gov*, as required by U.S. Law. This web site will not include information that can identify you. At most, the web site will include a summary of the results of the entire study. You can search this web site at any time (NCT02655055). You can also contact the study team at any time.

D5. WHAT HAPPENS IF I AM INJURED BECAUSE I TOOK PART IN THE STUDY?

No funds have been set aside to pay any costs if you are harmed because of this study. If you think that you were harmed because of this study, let the study director know right away by calling (414) 288-5069.

D6. WHO CAN ANSWER MY QUESTIONS ABOUT THE STUDY?

If you have more questions about this study, please call Dr. Papanek at (414) 288-5069.

If you have questions about your rights as a study participant, want to report any problems or complaints, obtain information about the study, or offer input, you can call the MCW/Froedtert Hospital Research Subject Advocate at 414-955-8844.

E. PERMISSION TO COLLECT, USE AND SHARE HEALTH INFORMATION

E1. What health information will be collected and used for this study?

To be in this research study, the study team needs your permission to access, collect and use some of your health information. If you say no, you cannot be in the study. This information may come from questions we ask, forms we ask you to fill out, or your medical record, as described below. We will only collect and use information needed for the study.

The protected health information (PHI) originates from services you will or have received at one or more of the following locations: the Medical College of Wisconsin (MCW); BloodCenter of Wisconsin (BCW); any Froedtert Health Affiliate- Froedtert Memorial Lutheran Hospital (FMLH), Inc.; Community Memorial Hospital (CMH) Menomonee Falls, Inc.; St. Joseph's Community Hospital (SJH) West Bend, Inc.; Froedtert & The Medical College of Wisconsin Community Physicians Clinics, Inc. (FMCWCP); the West Bend Surgery Center, LLC; and the Froedtert Surgery Center, LLC.

The health information to be collected and used for this study is:

- ☐ BloodCenter of Wisconsin blood donation records
- ☐ Bone density reports collected as part of this study
- ☐ Questionnaire data collected as part of this study

Medical College of Wisconsin & Froedtert Hospital

EFFECTIVE
04/26/2016
MCW/FH IRB

Informed Consent for Research
Template A-com - Version: March 6, 2015
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E2. Who will see the health information collected for this study?

The only individuals allowed to handle your health information are those on the study team, those on the Institutional Review Board (IRB) and those who check on the research activities to make sure rules are followed.

E3. What are the risks of sharing this health information?

One risk of taking part in a research study is that more people will handle your personal health information collected for this study. The study team will make every effort to protect the information and keep it confidential, but it is possible that an unauthorized person might see it. Depending on the kind of information being collected, it might be used in a way that could embarrass you or affect your ability to get insurance. If you have questions, you can talk to the study director about whether this could apply to you.

E4. How long will you keep the health information for this study?

If you sign this form, we plan to keep your information for up to10 years after the research study ends.

E5. Can I cancel my permission to share this health information?

If you change your mind later and do not want us to collect or share your health information, you need to send a letter to Dr. Papanek. The letter must say that you have changed your mind and do not want the researcher to collect and share your health information. At that time, we may decide that you cannot continue to be part of the study. We may still use the information we have already collected. If your health information is no longer identified as yours, it is not possible to remove it from the study.

CONSENT TO PARTICIPATE IN THE STUDY

By signing my name below, I confirm the following:

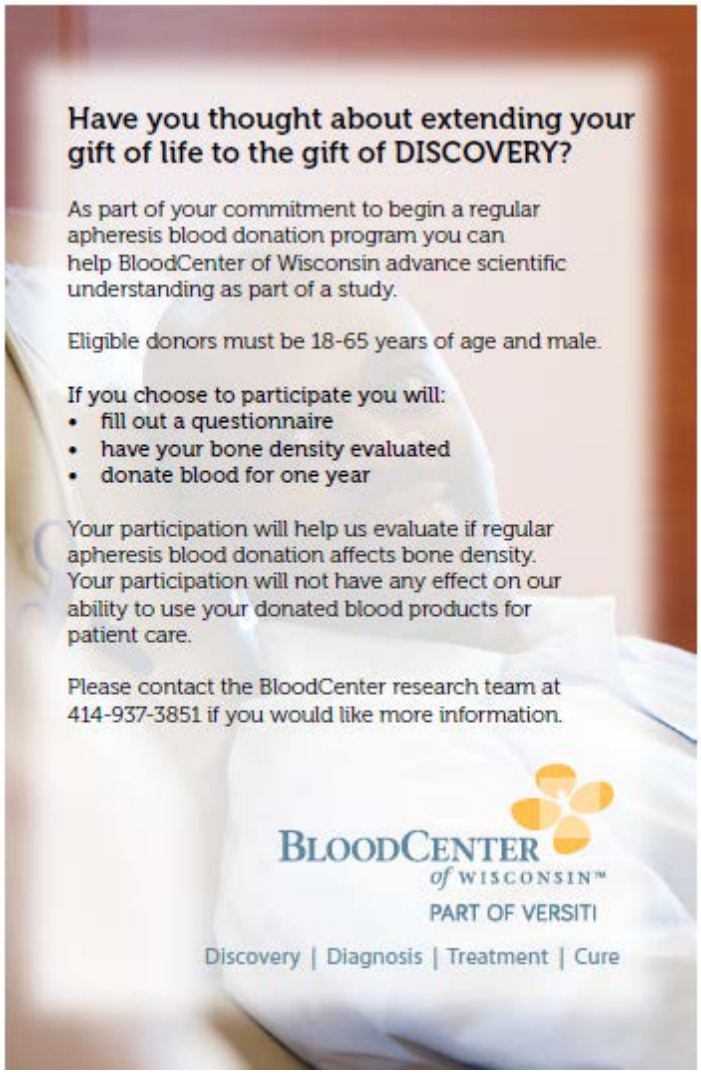
- I have read (or had read to me) this entire consent document. All of my questions have been answered to my satisfaction.
- The study's purpose, procedures, risks and possible benefits have been explained to me.
- I agree to let the study team use and share the information gathered for this study.
- I voluntarily agree to participate in this research study. I agree to follow the study procedures as directed. I have been told that I can stop at any time.

IMPORTANT: You will receive a signed and dated copy of this Consent Form. Please keep it where you can find it easily. It will help you remember what we discussed today.

Subject's Name *please print*	**Subject's Signature**	**Date**

Name of person discussing/ obtaining consent *please print*	**Signature of person discussing/obtaining consent**	**Date**

Appendix 2 – Recruitment Flyer



Appendix 3 – Recruitment Language

[DATE]

Dear [TITLE] [SURNAME],

As part of your commitment to begin a regular apheresis blood donation program you can help BloodCenter of Wisconsin advance scientific understanding as part of a study. Eligible donors must be 18-65 years of age and male. If you choose to participate you will:

- fill out a questionnaire
- have your bone density evaluated
- donate blood for one year

Your participation will help us evaluate if regular apheresis blood donation affects bone density. Your participation will not have any effect on our ability to use your donated blood products for patient care.
Please contact the BloodCenter research team at 414-937-3851 if you would like more information.

Thank you,

[SIGNATORY]

Appendix 4 – Questionnaire

Instructions: *Please fill in the information or place a checkmark in the bubble next to the appropriate answer for each question below.*

DEMOGRAPHIC INFORMATION

Date of Birth *(MM/DD/YYYY):* ____/____/_____ *Age:* _____

Sex:

- ○ Male
- ○ Female

Ethnicity:

- ○ Hispanic / Latino / Spanish
- ○ Not Hispanic / Latino / Spanish
- ○ I am not sure or do not wish to answer

Race (Check more than one if applicable):

- ○ White / Caucasian
- ○ Black / African American
- ○ Asian
- ○ American Indian / Alaska Native
- ○ Native Hawaiian or other Pacific Islander
- ○ I am not sure or do not wish to answer

STUDY ELIGIBILITY

		Yes	No
1.	Are you eligible for whole blood donation?	○	○
2.	Do you have a metal prosthesis in your lumbar spine or hip?	○	○
3.	Have you experienced a bone fracture in your lumbar spine or hip?	○	○
4.	Since you turned 18 years of age, have you experienced a bone fracture as a result from a fall from standing height or less?	○	○
5.	Have you undergone spinal fusion surgery at your lumbar spine?	○	○
6.	Have you been diagnosed with cystic fibrosis, emphysema, celiac disease, and/or Chrohn's disease?	○	○
7.	Are you willing to donate apheresis blood products 20 – 26 times over the next 12 month period?	○	○

PERSONAL MEDICAL INFORMATION

Have you…		Yes	No
1.	Smoked cigarettes at any time in the past 30 days.	○	○
2.	Smoked cigarettes at any time in the past for more than 1 continuous year.	○	○
3.	Exercised for more than 20 minutes three times per week in the past 30 days.	○	○
4.	Significantly increased or decreased your level of physical activity in the past 6 months.	○	○
5.	Significantly changed your diet within the past 12 months.	○	○

FAMILY INFORMATION

Has someone in your family been…	**Yes**	**No**
1. Diagnosed with osteoporosis?	○	○
2. Diagnosed with osteoarthritis?	○	○
3. Diagnosed with rheumatoid arthritis?	○	○
4. Diagnosed with kyphosis or scoliosis?	○	○
5. Treated for a bone fracture - <u>not</u> caused by a trauma (i.e., accident, fall, etc.)?	○	○
6. A parent was treated for a fractured hip - caused by trauma (i.e., accident, fall, etc.)?	○	○

DIAGNOSIS OF MEDICAL CONDITIONS

Have you been <u>diagnosed by a physician</u> with any of the following?	**Yes**	**No**
1. Osteoporosis	○	○
2. Osteogenesis Imperfecta	○	○
3. Osteopenia	○	○
4. Kyphosis	○	○
5. Scoliosis	○	○
6. Malnutrition	○	○
7. Eating Disorder (*Anorexia Nervosa* or *Bulimia*)	○	○
8. Intestinal Disorder	○	○
9. Chronic Liver Disease	○	○
10. Parathyroid Disease	○	○
11. Kidney Disease/Kidney Stones	○	○
12. Thyroid Disease (hyperthyroidism or hypothyroidism)	○	○
13. Diabetes - Type I	○	○
14. Diabetes - Type II	○	○
15. Rheumatoid Arthritis	○	○
16. Hypogonadism	○	○
17. Other Autoimmune Disease If yes, what kind: _____	○	○

MEDICATION USE

Have you ever taken any of the following medications?	Yes, Currently using	Yes, used in the past	No
1. Steroids or glucocorticoids (prednisone, cortisone, etc.) for more than three months	○	○	○
2. Seizure medications (Dilantin, etc.) for chronic management	○	○	○
3. Thyroid Hormone (Synthroid, Levothroid, Lexoxyl, etc.)	○	○	○
4. Etidronate (Didronel, Didrocal)	○	○	○
5. Alendronate (Fosamax)	○	○	○
6. Risedronate (Actonel)	○	○	○
7. Tamoxifen (Nolvadex, Istubal, Valodex)	○	○	○
8. Testosterone (Androderm, Delatestryl)	○	○	○
9. Nasal Calcitonin (Miacalcin)	○	○	○
10. Raloxifene (Evista)	○	○	○
11. Parathyroid Hormone (PTH, Forteo)	○	○	○
12. Pamidronate (Aredia)	○	○	○
13. Zoledronic Acid (Zometa, Reclast)	○	○	○
14. Ibandronate (Boniva)	○	○	○
15. Clodronate (Bonefos, Ostac)	○	○	○
16. Sodium Fluoride (Fluotic)	○	○	○
17. Estrogen	○	○	○
18. Diabetes medication (insulin, metformin, etc.)	○	○	○
19. Other Osteoporosis Medication If yes, what kind: _____	○	○	○

DIET & SUPPLEMENT USE

Which of the following do you generally consume?	Yes	No
1. I generally consume less than 2 servings of dairy per day.	○	○
2. I generally consume less than 2 servings of green, leafy vegetables per day.	○	○
3. I generally consume less than 2 servings of calcium fortified food (such as fortified orange juice or fortified soy milk) per day.	○	○
4. I generally consume less than 4 servings of vegetables per day.	○	○
5. I drink more than 2 servings of coffee or soda per day.	○	○
6. I drink more than 8 servings of tea per day.	○	○
7. I drink 3 or more servings of alcohol per day.	○	○
8. I take a vitamin D supplement (includes MVIT and liver oil). If yes, how many IU per day: _____	○	○
9. I take a daily calcium supplement (includes TUMS). If yes, how many milligrams per day: _____	○	○

Appendix 5 – DXA Appointment Letter

[BCW Branding] [Marquette University Branding]
Dear [Title] [Surname]:
Your bone density appointment is scheduled for [TIME] on [DATE]. The Marquette University bone density scanner is located at **604 N. 16th St** in the Department of Physical Therapy. Entrance to this building is on 16th street and just a few stairs up from the sidewalk. Please go to the second floor.

You will be asked to remove any body piercing or other metal or electronic objects from your body as these objects interfere with the quality of the images. Please wear comfortable clothing that does **not** contain metal – not even a metal eye loop for drawstring shorts.

First, you will lie on your back on a table for approximately 5 – 10 minutes. Then you will lie on your back while your legs are turned inward for 5 minutes. Finally, you will lie on your back with your legs elevated for 1 minute. This last scan will be repeated three times.

You will be asked to lie still during your scans. You will change positions for each scan. During the scan, a mechanical device (the scanner) passes over your body.

We may need to reschedule your appointment if:

- you have a **barium x-ray** in the two weeks before your appointment, or;
- you have a **nuclear medicine scan** in the week before your appointment, or;
- you have an **injection of x-ray dye** in the week before your appointment, or;
- you are unable to make your appointment for any reason.

Please avoid taking **any calcium supplement** (including TUMS or multiple vitamins) in the two hours before your appointment. Please feel free to call 414-881-2130 if you have any questions.

Thank you,

[SIGNATORY]

Appendix 6 – DXA Notification Letter

[DATE]

Dear [TITLE] [SURNAME],

Thank you for enrolling in the ALTRUYST study. We have reviewed the results from your bone density scans taken on [DATE OF SCAN]. The purpose of this letter is to inform you that we observed **bone density outside the expected range** at one or more of the sites measured. Your individual results are indicated below.

Measurement Site

□ femoral neck □ total hip □ lumbar spine (L1-L4)

Z-Score

□ < (-2.0) □ > (2.0)

DXA Machine

□ Marquette University's Lunar iDXA

These test results are not cause for immediate concern. Bone density scans performed as part of a research study are not intended to make clinical diagnoses. Nevertheless, we encourage you to contact your primary care physician before your next routine clinical examination. **Please share this notice with your primary care physician.** You and your physician are welcome to contact me to discuss these results.

Sincerely,

Robert D. Blank, MD, PhD
Chief, Endocrinology, Metabolism, and Clinical Nutrition
Medical College of Wisconsin
(414) 955-6722

Appendix 7 – Final Appointment Letter

[BCW Branding] [Marquette University Branding]

Dear [Title] [Surname]:

Thank you for participating in the ALTRUYST study!! Your **final study visit** will include a suite of bone density scans and completion of a final questionnaire. Your bone density appointment is scheduled for [TIME] on [DATE]. The Marquette University bone density scanner is located at **604 N. 16th St** in the Department of Physical Therapy. Entrance to this building is on 16th street and just a few stairs up from the sidewalk. Please go to the second floor.

You will be asked to remove any body piercing or other metal or electronic objects from your body as these objects interfere with the quality of the images. Please wear comfortable clothing that does not contain metal – not even a metal eye loop for drawstring shorts.

We may need to reschedule your appointment if:

- you have a barium x-ray in the two weeks before your appointment, or;
- you have a nuclear medicine scan in the week before your appointment, or;
- you have an injection of x-ray dye in the week before your appointment, or;
- you are unable to make your appointment for any reason.

Please avoid taking any calcium supplement (including TUMS or multiple vitamins) in the two hours before your appointment. Please feel free to call 414-881-2130 if you have any questions.

You will receive $50 for each of your two completed bone density visits, $100 total, to cover costs associated with travel. You are also eligible to receive the results of your bone density scans (both from enrollment and final visits) free of charge.

Study Compliance: On [ENROLLMENT DATE] you were randomly assigned to the [GROUP NAME] group. You were then asked to [GROUP INSTRUCTIONS]. Our records indicate that you underwent apheresis [# APH] during the one year period from [ENROLLMENT DATE] through [FINAL DATE]. If you complete your final visit and have otherwise complied with this study's protocol, then you will also receive [GROUP RATE]. This compensation is not tied to the blood donations you made during your participation in the study, but solely for the clinical assessments and complying with research study guidelines.

We encourage study participants to continue following the progress of this study at the clinicaltrials.gov website: https://www.clinicaltrials.gov/ct2/show/NCT02655055.

Again, thank you for your valued contribution to this research study. Please contact me with any questions that you may have.

Sincerely,

[SIGNATORY]

Appendix 8 – FDA Notice

From: Miles, Kimberly [mailto:Kimberly.Miles@fda.hhs.gov]
Sent: Friday, February 19, 2016 10:56 AM
To: Robertson, Maura
Subject: RE: Question from BloodCenter of Wisconsin - Volunteer donor classification review requested

Good Morning Maura,

This is a response to your donor incentive communicated to the Food and Drug Administration (FDA) on February 10, 2016. The incentive, as proposed to the FDA, offers remuneration to eligible, volunteer apheresis donors for participating in a research study. Your incentive describes apheresis donors will be clinically evaluated before and after the donation process with no compensation at the time of apheresis donation(s). Donors who choose to complete a clinical assessment outside of the donation process, which includes a comprehensive questionnaire and bone density scans, will become study participants. Monetary compensation for study participation includes $50 for clinic visits and an additional $100 or $250 for completing questionnaires, follow-up appointments, and adhering to all protocol guidelines.

The proposed donor incentive does not constitute monetary payment within the meaning of 21 CFR 606.121(c)(8)(v) and Compliance Policy Guide 230.150, Blood Donor Classification; therefore, units collected under this incentive can be labeled as "volunteer". We strongly recommend that all study participation material(s) clearly state compensation is not tied to blood donation but solely for the clinical assessment and completing research study protocol(s).

I have attached a direct link to the Donor Incentive Compliance Policy Guide, (CPG): 230.150, for your review. Hopefully, it will clarify CBER's position on donor incentives you may have going forward.
http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm122798.htm

I received your second donor incentive request and should have a response for you soon.

Thank you,
Kimberly T. Miles
Blood and Tissue Compliance Branch (BTCB)
Division of Case Management (DCM)
Office of Compliance and Biologics Quality (OCBQ)
CBER/FDA
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From: Robertson, Maura [mailto:Maura.Robertson@BCW.edu]
Sent: Wednesday, February 10, 2016 4:55 PM
To: Miles, Kimberly
Cc: Bialkowski, Walter; Marriott, Robert
Subject: Question from BloodCenter of Wisconsin - Volunteer donor classification review requested

Hello Kimberly,

We received your contact information from Sharon O'Callaghan for review of blood donor incentive questions from BloodCenter of Wisconsin. We would like to confirm that it is possible to use the Volunteer Donor statement for blood products collected during the research study described below:

BloodCenter of Wisconsin (BCW) would like to offer remuneration to eligible, voluntary apheresis donors participating in a research study. Donors in the study will be clinically evaluated before and after a high-frequency apheresis donation period (20 times or more in approximately one year), with no compensation at the time of the donations. The blood component products collected would be processed as normal products and released into inventory. Remuneration would instead be tied to the clinical assessments of the donors, including bone density scans and a comprehensive questionnaire, completed in a clinic outside of the donation process. BCW would offer study participants $50 each for the two clinic visits, at the initiation and completion of the study. For completing the questionnaires, follow-up appointments, and adhering to all protocol guidelines, participants will also receive either an additional $100 or $250 in compensation.

More information on this study's rationale, design, and analysis plan can be found on the clinicaltrials.gov registration page: https://www.clinicaltrials.gov/ct2/show/NCT02655055. We have attached a *draft appointment letter* that research participants would receive upon scheduling their final visit.

Please let me know if you have any questions or if you need any additional information. Thank you in advance for your advice.

Best regards,
Maura Robertson

Maura Robertson
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Appendix 9 – Syntax for NHANES Extract

```
> rm(list=ls())
> library(Hmisc)
Loading required package: grid
Loading required package: lattice
Loading required package: survival
Loading required package: Formula
Loading required package: ggplot2
Attaching package: 'Hmisc'
The following objects are masked from 'package:base':
format.pval, round.POSIXt, trunc.POSIXt, units
> ###extract SAS files from NHANES directory
> ###modify location as needed
> spinedata<-sasxport.get("C://NHANES/DATA/DXXSPN_F.XPT")
Processing SAS dataset DXXSPN_F           ..
> demodata<-sasxport.get("C://NHANES/DATA/DEMO_F.XPT")
Processing SAS dataset DEMO_F             ..
> ###visualize the variables extracted
> names(spinedata)
 [1] "seqn"    "dxaspnst" "dxxosbcc" "dxxl1bcc" "dxxl2bcc" "dxxl3bcc" "dxxl4bcc" "dxxosbmd" "dxxosbmc"
"dxxosa"   "dxxl1bmd"
[12] "dxxl1bmc" "dxxl1a"   "dxxl2bmd" "dxxl2bmc" "dxxl2a"   "dxxl3bmd" "dxxl3bmc" "dxxl3a"   "dxxl4bmd"
"dxxl4bmc" "dxxl4a"
[23] "dxaspnk"  "dxaspnd0"
> names(demodata)
 [1] "seqn"    "sddsrvyr" "ridstatr" "ridexmon" "riagendr" "ridageyr" "ridagemn" "ridageex" "ridreth1"
"dmqmilit" "dmdborn2"
[12] "dmdcitzn" "dmdyrsus" "dmdeduc3" "dmdeduc2" "dmdschol" "dmdmartl" "dmdhhsiz" "dmdfmsiz"
"indhhin2" "indfmin2" "indfmpir"
[23] "ridexprg" "dmdhrgnd" "dmdhrage" "dmdhrbr2" "dmdhredu" "dmdhrmar" "dmdhsedu" "sialang"
"siaproxy" "siaintrp" "fialang"
[34] "fiaproxy" "fiaintrp" "mialang"  "miaproxy" "miaintrp" "aialang"  "wtint2yr" "wtmec2yr" "sdmvpsu"
"sdmvstra"
> ###merge the two data files
> m<-merge(spinedata,demodata)
> ###visualize the merged variables
> names(m)
 [1] "seqn"    "dxaspnst" "dxxosbcc" "dxxl1bcc" "dxxl2bcc" "dxxl3bcc" "dxxl4bcc" "dxxosbmd" "dxxosbmc"
"dxxosa"   "dxxl1bmd"
[12] "dxxl1bmc" "dxxl1a"   "dxxl2bmd" "dxxl2bmc" "dxxl2a"   "dxxl3bmd" "dxxl3bmc" "dxxl3a"   "dxxl4bmd"
"dxxl4bmc" "dxxl4a"
[23] "dxaspnk"  "dxaspnd0" "sddsrvyr" "ridstatr" "ridexmon" "riagendr" "ridageyr" "ridagemn" "ridageex"
"ridreth1" "dmqmilit"
[34] "dmdborn2" "dmdcitzn" "dmdyrsus" "dmdeduc3" "dmdeduc2" "dmdschol" "dmdmartl" "dmdhhsiz"
"dmdfmsiz" "indhhin2" "indfmin2"
[45] "indfmpir" "ridexprg" "dmdhrgnd" "dmdhrage" "dmdhrbr2" "dmdhredu" "dmdhrmar" "dmdhsedu"
"sialang"  "siaproxy" "siaintrp"
[56] "fialang"  "fiaproxy" "fiaintrp" "mialang"  "miaproxy" "miaintrp" "aialang"  "wtint2yr" "wtmec2yr"
"sdmvpsu"  "sdmvstra"
> ###isolate variables of interest
> ###seqn is the participant sequence number (linking variable)
> ###riagendr=1 for male
> ###ridreth1=3 for non-latino caucasian
> ###ridageyr is age
```

Appendix 10 – Syntax for Power

```
> ###Appendix: Proposal Simulations 1: Comparison change of
 outcome from
> ###before to after for two groups.
> rm(list=ls())
> library(MASS)
> ###simulate test statistic under H0
> ###set population parameters
> ###specify muXbefore, muXafter, muYbefore and muYafter
> ###such that muXafter-muXbefore=muYafter-muYbefore
> muXbefore<-1.054925
> muYbefore<-1.054925
> muXafter<-1.054925
> muYafter<-1.054925
> ###Set parameters for standard error and correlation
> sigmaXbefore<-0.1345117
> sigmaYbefore<-0.1345117
> sigmaXafter<-0.1345117
> sigmaYafter<-0.1345117
> rhoX<-0.95
> rhoY<-0.95
> ###Generate covariance matrix for (Xafter, Xbefore) and (
Yafter, Ybefore)
> VX<-matrix(data=c(sigmaXafter^2,sigmaXafter*sigmaXbefore*
rhoX,sigmaXbefore*sigmaXafter*rhoX,sigmaXbefore^2),ncol=2,n
row=2)
> VY<-matrix(data=c(sigmaYafter^2,sigmaYafter*sigmaYbefore*
rhoY,sigmaYbefore*sigmaYafter*rhoY,sigmaYbefore^2),ncol=2,n
row=2)
> nx<-15
> ny<-45
> m<-10000
> alpha<-0.05
> par(mfrow=c(2,1))
> ###simulate test statistic under H0
> Z0<-rep(NA,m)
> for (i in 1:m){
+     ###Sample multivariate normal variables Xbefore and X
after
+     X<-mvrnorm(nx,mu=c(muXafter,muXbefore),Sigma=VX)
+     Y<-mvrnorm(ny,mu=c(muYafter,muYbefore),Sigma=VY)
+     ###Begin an experiment and sample data with related v
ariables
+     Xbefore<-X[,2]
+     Ybefore<-Y[,2]
+     Xafter<-X[,1]
+     Yafter<-Y[,1]
+     ###Compute change in X and change in Y
+     XD<-Xafter-Xbefore
+     YD<-Yafter-Ybefore
+     ###Compute sample standard deviation of XD and YD
+     se<-sqrt(var(XD)/nx+var(YD)/ny)
+
```

```
+     ###Compute the statistic as a function of the sample
+     Z0[i]<-(mean(XD)-mean(YD))/se
+ }
> ###show distribution of the test statistic under H0
> hist(Z0,main="Distribution under H0",xlim=c(-10,10))
>
> ###simulate test statistic under Ha
> ###set population parameters
> ###specify muXbefore, muXafter, muYbefore and muYafter
> ###such that muXafter-muXbefore=muYafter-muYbefore
> muXbefore<-1.054925
> muYbefore<-1.054925
> muXafter<-1.054925
> muYafter<-1.02327725
> ###Set parameters for standard error and correlation
> sigmaXbefore<-0.1345117
> sigmaYbefore<-0.1345117
> sigmaXafter<-0.1345117
> sigmaYafter<-0.1345117
> rhoX<-0.95
> rhoY<-0.92
> ####Generate covariance matrix for (Xafter, Xbefore) and
(Yafter, Ybefore)
> VX<-matrix(data=c(sigmaXafter^2,sigmaXafter*sigmaXbefore*
rhoX,sigmaXbefore*sigmaXafter*rhoX,sigmaXbefore^2),ncol=2,n
row=2)
> VY<-matrix(data=c(sigmaYafter^2,sigmaYafter*sigmaYbefore*
rhoY,sigmaYbefore*sigmaYafter*rhoY,sigmaYbefore^2),ncol=2,n
row=2)
> nx<-15
> ny<-45
> m<-10000
> alpha<-0.05
> ###simulate test statistic under Ha
> Z1<-rep(NA,m)
> for (i in 1:m){
+     X<-mvrnorm(nx,mu=c(muXafter,muXbefore),Sigma=VX)
+     Y<-mvrnorm(ny,mu=c(muYafter,muYbefore),Sigma=VY)
+     ###Begin an experiment and sample data with related v
ariables
+     Xbefore<-X[,2]
+     Ybefore<-Y[,2]
+     Xafter<-X[,1]
+     Yafter<-Y[,1]
+     ###Compute change in X and change in Y
+     XD<-Xafter-Xbefore
+     YD<-Yafter-Ybefore
+     ###Compute sample standard deviation of XD and YD
+     se<-sqrt(var(XD)/nx+var(YD)/ny)
+
+     ###Compute the statistic as a function of the sample
+     Z1[i]<-(mean(XD)-mean(YD))/se
+ }
> ###show distribution of the test statistic under Ha
```

```
> hist(Z1,main="Distribution under Ha",xlim=c(-10,10))
>
> ###obtain critical value
> c<-quantile(Z0,probs=1-alpha)
> quantile(Z0,probs=1-alpha)
     95%
1.720837
>
> ###obtain power
> power<-sum(Z1>c)/m
> power
[1] 0.7362
```

Appendix 11 – Syntax for Analysis

```
##Clear Directory
rm(list=ls())
##Load Libraries
library(xlsx)
library(MASS)
library(ggplot2)

##Identify Working Directory
data<-read.xlsx2("M:/Staff/W Bialkowski/PhD
Marquette/ALTRUYST/Analysis/ALTRUYST Analytic Data
Set.xlsx",
                 2,
                 as.data.frame=TRUE,
                 header=TRUE,
                 keepFormulas=FALSE,
                 colClasses=c(rep("numeric",55)))

##Designate Treatment versus Control Groups
msubC<-subset(data,apheresis==0)
msubT<-subset(data,apheresis==1)

##Descriptive table of study subjects at baseline (Table 1
in manuscript)
##Total
summary(data)
sd(data$age,na.rm=TRUE)
sd(data$height,na.rm=TRUE)
sd(data$weight,na.rm=TRUE)
sd(data$BMI,na.rm=TRUE)
sd(data$Na,na.rm=TRUE)
sd(data$K,na.rm=TRUE)
sd(data$Cl,na.rm=TRUE)
sd(data$CO2,na.rm=TRUE)
sd(data$anion,na.rm=TRUE)
sd(data$urea,na.rm=TRUE)
sd(data$creat,na.rm=TRUE)
sd(data$gluc,na.rm=TRUE)
sd(data$alkphos,na.rm=TRUE)
sd(data$AST,na.rm=TRUE)
sd(data$ALT,na.rm=TRUE)
sd(data$Ca,na.rm=TRUE)
sd(data$P,na.rm=TRUE)
sd(data$prot,na.rm=TRUE)
sd(data$alb,na.rm=TRUE)
sd(data$bili,na.rm=TRUE)
sd(data$test,na.rm=TRUE)
sd(data$pre_L1_4,na.rm=TRUE)
sd(data$pre_TH,na.rm=TRUE)
##control
summary(msubC)
sd(msubC$age,na.rm=TRUE)
sd(msubC$height,na.rm=TRUE)
```

```
sd(msubC$weight,na.rm=TRUE)
sd(msubC$BMI,na.rm=TRUE)
sd(msubC$Na,na.rm=TRUE)
sd(msubC$K,na.rm=TRUE)
sd(msubC$Cl,na.rm=TRUE)
sd(msubC$CO2,na.rm=TRUE)
sd(msubC$anion,na.rm=TRUE)
sd(msubC$urea,na.rm=TRUE)
sd(msubC$creat,na.rm=TRUE)
sd(msubC$gluc,na.rm=TRUE)
sd(msubC$alkphos,na.rm=TRUE)
sd(msubC$AST,na.rm=TRUE)
sd(msubC$ALT,na.rm=TRUE)
sd(msubC$Ca,na.rm=TRUE)
sd(msubC$P,na.rm=TRUE)
sd(msubC$prot,na.rm=TRUE)
sd(msubC$alb,na.rm=TRUE)
sd(msubC$bili,na.rm=TRUE)
sd(msubC$test,na.rm=TRUE)
sd(msubC$pre_L1_4,na.rm=TRUE)
sd(msubC$pre_TH,na.rm=TRUE)
##treatment
summary(msubT)
sd(msubT$age,na.rm=TRUE)
sd(msubT$height,na.rm=TRUE)
sd(msubT$weight,na.rm=TRUE)
sd(msubT$BMI,na.rm=TRUE)
sd(msubT$Na,na.rm=TRUE)
sd(msubT$K,na.rm=TRUE)
sd(msubT$Cl,na.rm=TRUE)
sd(msubT$CO2,na.rm=TRUE)
sd(msubT$anion,na.rm=TRUE)
sd(msubT$urea,na.rm=TRUE)
sd(msubT$creat,na.rm=TRUE)
sd(msubT$gluc,na.rm=TRUE)
sd(msubT$alkphos,na.rm=TRUE)
sd(msubT$AST,na.rm=TRUE)
sd(msubT$ALT,na.rm=TRUE)
sd(msubT$Ca,na.rm=TRUE)
sd(msubT$P,na.rm=TRUE)
sd(msubT$prot,na.rm=TRUE)
sd(msubT$alb,na.rm=TRUE)
sd(msubT$bili,na.rm=TRUE)
sd(msubT$test,na.rm=TRUE)
sd(msubT$pre_L1_4,na.rm=TRUE)
sd(msubT$pre_TH,na.rm=TRUE)

##view distributions
par(mfrow=c(2,2))
hist.default(data$age, xlab="Age (years)",
main="Distribution of Age")
hist.default(data$height, xlab="Height (inches)",
main="Distribution of Height")
```

```
hist.default(data$weight, xlab="Weight (pounds)",
main="Distribution of Weight")
hist.default(data$BMI, xlab="BMI (%)", main="Distribution
of BMI")

par(mfrow=c(2,2))
hist.default(data$Na, xlab="Serum Sodium (mmol/L)",
main="Distribution of Serum Sodium")
hist.default(data$K, xlab="Serum Potassium (mmol/L)",
main="Distribution of Serum Potassium")
hist.default(data$Cl, xlab="Serum Chloride (mmol/L))",
main="Distribution of Serum Chloride")
hist.default(data$CO2, xlab="Serum CO2 (mmol/L)",
main="Distribution of Serum CO2")

par(mfrow=c(2,2))
hist.default(data$anion, xlab="Anion Gap (mmol/L)",
main="Distribution of Anion Gap")
hist.default(data$urea, xlab="Urea (mg/dL)",
main="Distribution of Urea")
hist.default(data$creat, xlab="Serum Creatinine (mg/dL)",
main="Distribution of Serum Creatinine")
hist.default(data$gluc, xlab="Serum Glucose (mg/dL)",
main="Distribution of Serum Glucose")

par(mfrow=c(2,2))
hist.default(data$alkphos, xlab="Alkaline Phosphatase
(U/L)", main="Distribution of Alkaline Phosphatase")
hist.default(data$ALT, xlab="ALT (U/L)", main="Distribution
of ALT")
hist.default(data$AST, xlab="AST (U/L)", main="Distribution
of AST")
hist.default(data$Ca, xlab="Serum Calcium (mg/dL)",
main="Distribution of Serum Calcium")

par(mfrow=c(2,2))
hist.default(data$P, xlab="Serum Phosphorous (mg/dL)",
main="Distribution of Serum Phosphorous")
hist.default(data$prot, xlab="Serum Protein (g/dL)",
main="Distribution of Serum Protein")
hist.default(data$alb, xlab="Serum Albumin (g/dL)",
main="Distribution of Serum Albumin")
hist.default(data$bili, xlab="Serum Bilirubin (mg/dL)",
main="Distribution of Serum Bilirubin")

par(mfrow=c(2,2))
hist.default(data$test, xlab="Male Adult Testosterone
(ng/dL)", main="Distribution of Testosterone")
hist.default(data$pre_L1_4, xlab="Pre Lumbar Spine
(g/cm^2)", main="Distribution of Pre Lumbar Spine")
hist.default(data$pre_TH, xlab="Pre Total Hip (g/cm^2)",
main="Distribution of Pre Total Hip")

##side-by-side transformations
```

```
par(mfrow=c(3,2))
hist.default(data$anion, xlab="Anion Gap before
transformation", main="Distribution of Anion Gap")
hist.default(log(data$anion), xlab="Anion Gap after
transformation", main="Distribution of Anion Gap")
hist.default(data$alkphos, xlab="Alkaline Phosphatase
before transformation", main="Distribution of Alkaline
Phosphatase")
hist.default(log(data$alkphos), xlab="Alkaline Phosphatase
after transformation", main="Distribution of Alkaline
Phosphatase")
hist.default(data$bili, xlab="Bilirubin before
transformation", main="Distribution of Bilirubin")
hist.default(log(data$bili), xlab="Bilirubin after
transformation", main="Distribution of Bilirubin")

##log transform anion, alkphos, and bili
loganion<-log10(data$anion)
logalkphos<-log10(data$alkphos)
logbili<-log10(data$bili)

##Test for differences
##Fishers Exact for Categorical; T-Test for Continuous
fisher.test(msubC$race,msubT$race)
fisher.test(msubC$ethn,msubT$ethn)
t.test(msubC$age,msubT$age)
t.test(msubC$height,msubT$height)
t.test(msubC$weight,msubT$weight)
t.test(msubC$BMI,msubT$BMI)
t.test(msubC$Na,msubT$Na)
t.test(msubC$K,msubT$K)
t.test(msubC$Cl,msubT$Cl)
t.test(msubC$CO2,msubT$CO2)
t.test(msubC$loganion,msubT$loganion)
t.test(msubC$urea,msubT$urea)
t.test(msubC$creat,msubT$creat)
t.test(msubC$gluc,msubT$gluc)
t.test(msubC$logalkphos,msubT$logalkphos)
t.test(msubC$AST,msubT$AST)
t.test(msubC$ALT,msubT$ALT)
t.test(msubC$Ca,msubT$Ca)
t.test(msubC$P,msubT$P)
t.test(msubC$prot,msubT$prot)
t.test(msubC$alb,msubT$alb)
t.test(msubC$logbili,msubT$logbili)
t.test(msubC$test,msubT$test)
t.test(msubC$pre_L1_4,msubT$pre_L1_4)
t.test(msubC$pre_TH,msubT$pre_TH)

##visualize distributions of BMD at enrollment
par(mfrow=c(3,2))
hist(data$pre_L1_4,xlim = c(0.9,1.5),ylim=c(0,12))
hist(data$pre_TH,xlim = c(0.8,1.5),ylim=c(0,12))
hist(msubC$pre_L1_4,xlim = c(0.9,1.5),ylim=c(0,6))
```

```
hist(msubC$pre_TH,xlim = c(0.8,1.5),ylim=c(0,6))
hist(msubT$pre_L1_4,xlim = c(0.9,1.5),ylim=c(0,12))
hist(msubT$pre_TH,xlim = c(0.8,1.5),ylim=c(0,12))

##Visualize BMD at enrollment for Both Groups
##there's a number of ways I'm considering illustrating
these
##this is an example
ggplot(data, aes(x=test)) +
  geom_density(aes(stat="density"),
                 binwidth=0.5,
                 colour="black",
                 fill="white") +
  geom_density(alpha=.2, fill="#FF6666")

par(mfrow=c(2,1))
ggplot(data, aes(x=SubjectID,
                 y=pre_TH))+
       geom_point(size=1.5, shape=21, fill="white")+
       labs(title="Baseline TH BMD, Both Groups")

ggplot(data, aes(x=SubjectID,
                 y=pre_L1_4))
       + ggtitle("Baseline L Spine BMD, Both Groups")

##Visualize BMD at enrollment for Controls
##Visualize BMD at enrollment for Treatment
##Visualize Final BMD for Both Groups
##Visualize Final BMD for Controls
##Visualize Final BMD for Treatment

##compute mean at enrollment
mean(data$pre_TH)
sd(data$pre_TH)
mean(data$pre_L1_4)
sd(data$pre_L1_4)

mean(msubC$pre_TH)
sd(msubC$pre_TH)
mean(msubT$pre_TH)
sd(msubT$pre_TH)

mean(msubC$pre_L1_4)
sd(msubC$pre_L1_4)
mean(msubT$pre_L1_4)
sd(msubT$pre_L1_4)

mean(msubC$pre_TBS,na.rm=TRUE)
sd(msubC$pre_TBS,na.rm=TRUE)
mean(msubT$pre_TBS,na.rm=TRUE)
sd(msubT$pre_TBS,na.rm=TRUE)

mean(msubC$pre_neck)
sd(msubC$pre_neck)
```

```
mean(msubT$pre_neck)
sd(msubT$pre_neck)

##compute mean at follow-up
mean(msubC$post_TH)
sd(msubC$post_TH)
mean(msubT$post_TH)
sd(msubT$post_TH)

mean(msubC$post_L1_4)
sd(msubC$post_L1_4)
mean(msubT$post_L1_4)
sd(msubT$post_L1_4)

mean(msubC$post_TBS,na.rm=TRUE)
sd(msubC$post_TBS,na.rm=TRUE)
mean(msubT$post_TBS,na.rm=TRUE)
sd(msubT$post_TBS,na.rm=TRUE)

mean(msubC$post_neck)
sd(msubC$post_neck)
mean(msubT$post_neck)
sd(msubT$post_neck)

##create derived 'change in BMD' variables
deltaTH<-(data$post_TH-data$pre_TH)
deltaL1_4<-(data$post_L1_4-data$pre_L1_4)

deltaTHC<-(msubC$post_TH-msubC$pre_TH)
deltaL1_4C<-(msubC$post_L1_4-msubC$pre_L1_4)
deltaTBSC<-(msubC$post_TBS-msubC$pre_TBS)
deltaneckC<-(msubC$post_neck-msubC$pre_neck)

deltaTHT<-(msubT$post_TH-msubT$pre_TH)
deltaL1_4T<-(msubT$post_L1_4-msubT$pre_L1_4)
deltaTBST<-(msubT$post_TBS-msubT$pre_TBS)
deltaneckT<-(msubT$post_neck-msubT$pre_neck)

##Compute mean change
mean(deltaTHC)
sd(deltaTHC)
mean(deltaTHT)
sd (deltaTHT)

mean(deltaL1_4C)
sd(deltaL1_4C)
mean(deltaL1_4T)
sd(deltaL1_4T)

mean(deltaTBSC,na.rm=TRUE)
sd(deltaTBSC,na.rm=TRUE)
mean(deltaTBST)
sd(deltaTBST)
```

```
mean(deltaneckC)
sd(deltaneckC)
mean(deltaneckT)
sd(deltaneckT)

##95% CIs and p values
t.test(deltaTHC)
t.test(deltaL1_4C)
t.test(deltaTHT)
t.test(deltaL1_4T)
t.test(deltaTBSC,na.rm=TRUE)
t.test(deltaTBST)
t.test(deltaneckC)
t.test(deltaneckT)

##hists
par(mfrow=c(2,2))
hist(deltaL1_4C,xlim=c(-0.1,0.1),ylim=c(0,15),main="Delta L
Spine Control",nclass=5)
hist(deltaL1_4T,xlim=c(-0.1,0.1),ylim=c(0,15),main="Delta L
Spine Apheresis",nclass=10)
hist(deltaTHC,xlim=c(-0.1,0.1),ylim=c(0,15),main="Delta TH
Controls",nclass=5)
hist(deltaTHT,xlim=c(-0.1,0.1),ylim=c(0,15),main="Delta TH
Apheresis",nclass=10)


##compare mean change to LSC in treated
##LSC at lumbar spine is 0.00743
if((mean(deltaL1_4T))>0.00743)
  print("mean change among treated subjects exceeds LSC for
lumbar spine in the positive direction")
if((mean(deltaL1_4T))<(-0.00743))
  print("mean change among treated subjects exceeds LSC for
lumbar spine in the negative direction")

##LSC at TH is 0.00671
if((mean(deltaTHT))>0.00671)
  print("mean change for treated subjects exceeds LSC for
total hip in the positive direction")
if((mean(deltaTHT))<(-0.00671))
  print("mean change for treated subjects exceeds LSC for
total hip in the negative direction")

##compare mean change to LSC in controls
##LSC at lumbar spine is 0.00743
if((mean(deltaL1_4C))>0.00743)
  print("mean change among control subjects exceeds LSC for
lumbar spine in the positive direction")
if((mean(deltaL1_4C))<(-0.00743))
  print("mean change among control subjects exceeds LSC for
lumbar spine in the negative direction")

##LSC at TH is 0.00671
```

```
if((mean(deltaTHC))>0.00671)
  print("mean change for control subjects exceeds LSC for
total hip in the positive direction")
if((mean(deltaTHC))<(-0.00671))
  print("mean change for control subjects exceeds LSC for
total hip in the negative direction")

##plain t test for differences
t.test(deltaL1_4C,deltaL1_4T)
t.test(deltaTHC,deltaTHT)

##Visualize change in BMD for both groups
par(mfrow=c(2,2))
plot(deltaL1_4C, ylim=c(-0.17,0.17))
plot(deltaL1_4T, ylim=c(-0.17,0.17))
plot(deltaTHC, ylim=c(-0.17,0.17))
plot(deltaTHT, ylim=c(-0.17,0.17))

##Boxplots change in BMD
par(mfrow=c(2,2))
boxplot(deltaL1_4C, ylim=c(-0.1,0.1),main="L spine
control")
boxplot(deltaL1_4T, ylim=c(-0.1,0.1),main="L spine
treated")
boxplot(deltaTHC, ylim=c(-0.05,0.05),main="hip control")
boxplot(deltaTHT, ylim=c(-0.05,0.05),main="hip treated")

##create Figure 3 for main paper - box plots
ggplot(data, aes(x=factor(apheresis), y=deltaL1_4))+
  ylim(-0.06,0.06)+
  geom_boxplot()+
  theme_bw()+
  stat_summary(fun.y="mean", geom="point", shape=23,
size=3, fill="black")+
  annotate("rect",xmin=0.5,xmax=2.5,alpha=0.1,ymin=-
0.00743,ymax=0.00743,fill="blue")+
  theme(panel.border = element_blank(),
        panel.grid.major = element_blank(),
        panel.grid.major.y = element_line(linetype =
"dashed", colour = "black"),
        panel.grid.minor = element_blank(),
        panel.background = element_blank(),
        axis.line = element_line(colour = "white"))

ggplot(data, aes(x=factor(apheresis), y=deltaTH))+
  ylim(-0.06,0.06)+
  geom_boxplot()+
  theme_bw()+
  stat_summary(fun.y="mean", geom="point", shape=23,
size=3, fill="black")+
  annotate("rect",xmin=0.5,xmax=2.5,alpha=0.1,ymin=-
0.00743,ymax=0.00743,fill="blue")+
  theme(panel.border = element_blank(),
        panel.grid.major = element_blank(),
```

```
        panel.grid.major.y = element_line(linetype =
"dashed", colour = "black"),
        panel.grid.minor = element_blank(),
        panel.background = element_blank(),
        axis.line = element_line(colour = "white"))

##Define Number of Subjects Analyzed in Each Group
nC<-15
nT<-26

##BEGIN: Unadjusted Analysis
##Compute and Show Standard Errors
vardeltaTHC<-var(deltaTHC)
vardeltaTHC
vardeltaL1_4C<-var(deltaL1_4C)
vardeltaL1_4C
vardeltaTHT<-var(deltaTHT)
vardeltaTHT
vardeltaL1_4T<-var(deltaL1_4T)
vardeltaL1_4T

##Test for Differences in Baseline BMD by treatment arm
TTH<-(mean(deltaTHC)-
mean(deltaTHT))/sqrt((vardeltaTHC/nC)+(vardeltaTHT/nT))
TTH
TL1_4<-(mean(deltaL1_4C)-
mean(deltaL1_4T))/sqrt((vardeltaL1_4C/nC)+(vardeltaL1_4T/nT
))
TL1_4

##compute degrees of freedom
vTH<-((vardeltaTHC/nC)+(vardeltaTHT/nT))/
  ((((vardeltaTHC/nC)^2)/(nC-1))+(((vardeltaTHT/nT)^2)/(nT-
1)))
VTH
vL1_4<-((vardeltaL1_4C/nC)+(vardeltaL1_4T/nT))/
  ((((vardeltaL1_4C/nC)^2)/(nC-
1))+(((vardeltaL1_4T/nT)^2)/(nT-1)))
vL1_4

##derive critical value
qtTH<-qt(c(0.95),df=vTH)
qtTH
qtL1_4<-qt(c(0.95),df=vL1_4)
qtL1_4

##output messaging
if((TTH-qtTH)>0)
  print("reject the null hypothesis for total hip")
if((TTH-qtTH)<=0)
  print("you are not able to reject the null hypothesis for
total hip")

if((TL1_4-qtL1_4)>0)
```

```
  print("reject the null hypothesis for lumbar spine")
if((TL1_4-qtL1_4)<=0)
  print("you are not able to reject the null hypothesis for
lumbar spine")

##test for difference in proportions
LspineI<-matrix(c(8,7,13,13),byrow=TRUE, 2, 2)
LspineI
chisq.test(LspineI, correct = FALSE)
LspineD<-matrix(c(5,10,8,18),byrow=TRUE, 2, 2)
LspineD
chisq.test(LspineD, correct = FALSE)
THI<-matrix(c(6,9,6,20),byrow=TRUE, 2, 2)
THI
chisq.test(THI, correct = FALSE)
THD<-matrix(c(3,12,11,15),byrow=TRUE, 2, 2)
THD
chisq.test(THD, correct = FALSE)

##END: Unadjusted Analysis

##BEGIN Adjusted Analysis Using Multivariable Linear
Regression
##visualize distributions of participant demographic and
behavioral characteristics
##can also do str function for each predictor to examine
potential extreme values
##can also do stem and leaf plots
##can do scatterplots to evaluate need for transformations
##can also test for correlations using Pearsons/Spearmans
##can test linearity using Shapiro-Wilk's
##can evaluate leverage values
##can do QQ plots
##can do Jackknife Residuals
##can do Cook's Distance
##can evaluate collinearity with rcorr function
##can do Variance Inflation Factors (VIF>10 interrogation)
##can evaluate multicollinearity with Tolerance Values,
Eigenvalues, Condition Indices/Numbers
par(mfrow=c(3,2))
hist(data$age)
hist(data$risks)
hist(data$family)
hist(data$conditions)
hist(data$medications)
hist(data$diet)

##negative change in L spine
fitnL1_4<-lm(data$bigndeltaL1_4~
          (data$apheresis)+
          (data$age)+
          (as.factor(data$risks))+
          (as.factor(data$family))+
          (as.factor(data$conditions))+
```

```
            (as.factor(data$medications))+
            (as.factor(data$diet))+
            (data$BMI)+
            (data$pre_L1_4)+
            (data$Na)+
            (data$K)+
            (data$Cl)+
            (data$CO2)+
            (data$anion)+
            (data$urea)+
            (data$creat)+
            (data$gluc)+
            (data$alkphos)+
            (data$AST)+
            (data$ALT)+
            (data$Ca)+
            (data$P)+
            (data$prot)+
            (data$alb)+
            (data$bili)+
            (data$test))
summary(fitnL1_4)
anova(fitnL1_4)
##stepwise backwards elimination for negative change in L
spine
stepnL1_4<-stepAIC(fitnL1_4,direction="backward")
stepnL1_4$anova

##positive change in L spine
fitpL1_4<-lm(data$bigpdeltaL1_4~
               (data$apheresis)+
               (data$age)+
               (as.factor(data$risks))+
               (as.factor(data$family))+
               (as.factor(data$conditions))+
               (as.factor(data$medications))+
               (as.factor(data$diet))+
               (data$BMI)+
               (data$pre_L1_4)+
               (data$Na)+
               (data$K)+
               (data$Cl)+
               (data$CO2)+
               (data$anion)+
               (data$urea)+
               (data$creat)+
               (data$gluc)+
               (data$alkphos)+
               (data$AST)+
               (data$ALT)+
               (data$Ca)+
               (data$P)+
               (data$prot)+
               (data$alb)+
```

```
                (data$bili)+
                (data$test))
summary(fitpL1_4)
anova(fitpL1_4)
##stepwise backwards elimination for positive change in L
spine
steppL1_4<-stepAIC(fitpL1_4,direction="backward")
steppL1_4$anova

##negative change in total hip
fitnTH<-lm(data$bigndeltaTH~
                (data$apheresis)+
                (data$age)+
                (as.factor(data$risks))+
                (as.factor(data$family))+
                (as.factor(data$conditions))+
                (as.factor(data$medications))+
                (as.factor(data$diet))+
                (data$BMI)+
                (data$pre_TH)+
                (data$Na)+
                (data$K)+
                (data$Cl)+
                (data$CO2)+
                (data$anion)+
                (data$urea)+
                (data$creat)+
                (data$gluc)+
                (data$alkphos)+
                (data$AST)+
                (data$ALT)+
                (data$Ca)+
                (data$P)+
                (data$prot)+
                (data$alb)+
                (data$bili)+
                (data$test))
summary(fitnTH)
anova(fitnTH)
##stepwise backwards elimination for negative change in L
spine
stepnTH<-stepAIC(fitnTH,direction="backward")
stepnTH$anova

##positive change in TH
fitpTH<-lm(data$bigpdeltaTH~
             (data$apheresis)+
             (data$age)+
             (as.factor(data$risks))+
             (as.factor(data$family))+
             (as.factor(data$conditions))+
             (as.factor(data$medications))+
             (as.factor(data$diet))+
             (data$BMI)+
```

```
              (data$pre_TH)+
              (data$Na)+
              (data$K)+
              (data$Cl)+
              (data$CO2)+
              (data$anion)+
              (data$urea)+
              (data$creat)+
              (data$gluc)+
              (data$alkphos)+
              (data$AST)+
              (data$ALT)+
              (data$Ca)+
              (data$P)+
              (data$prot)+
              (data$alb)+
              (data$bili)+
              (data$test))
summary(fitpTH)
anova(fitpTH)

##stepwise backwards elimination for positive change in L
spine
steppTH<-stepAIC(fitpTH,direction="backward")
steppTH$anova



##Everything prior was the intention to treat analysis
##we can now perform the same analyses limited to protocol
compliers
##i.e. ITT=1 in the data set
##see programming for "ALRUYST Coding compliers" and .xlsx
file ...compliers.xlsx

wb<-c(6,4,5,4,5,5,4,2,1,1,1,0,0,0,0)
median(wb)
mean(wb)
sd(wb)


##Clear Directory
rm(list=ls())
##Load Libraries
library(xlsx)
library(MASS)
library(ggplot2)

##Identify Working Directory
data<-read.xlsx2("M:/Staff/W Bialkowski/PhD
Marquette/ALTRUYST/Analysis/ALTRUYST Analytic Data Set
compliers.xlsx",
                 2,
                 as.data.frame=TRUE,
```

```
                header=TRUE,
                keepFormulas=FALSE,
                colClasses=c(rep("numeric",68)))

##Designate Treatment versus Control Groups
msubC<-subset(data,apheresis==0)
msubT<-subset(data,apheresis==1)

##Descriptive table of study subjects at baseline (Table 1
in manuscript)
##Total
summary(data)
sd(data$age,na.rm=TRUE)
sd(data$height,na.rm=TRUE)
sd(data$weight,na.rm=TRUE)
sd(data$BMI,na.rm=TRUE)
sd(data$Na,na.rm=TRUE)
sd(data$K,na.rm=TRUE)
sd(data$Cl,na.rm=TRUE)
sd(data$CO2,na.rm=TRUE)
sd(data$anion,na.rm=TRUE)
sd(data$urea,na.rm=TRUE)
sd(data$creat,na.rm=TRUE)
sd(data$gluc,na.rm=TRUE)
sd(data$alkphos,na.rm=TRUE)
sd(data$ALT,na.rm=TRUE)
sd(data$AST,na.rm=TRUE)
sd(data$Ca,na.rm=TRUE)
sd(data$P,na.rm=TRUE)
sd(data$prot,na.rm=TRUE)
sd(data$alb,na.rm=TRUE)
sd(data$bili,na.rm=TRUE)
sd(data$test,na.rm=TRUE)
sd(data$pre_L1_4,na.rm=TRUE)
sd(data$pre_TH,na.rm=TRUE)
##control
summary(msubC)
sd(msubC$age,na.rm=TRUE)
sd(msubC$height,na.rm=TRUE)
sd(msubC$weight,na.rm=TRUE)
sd(msubC$BMI,na.rm=TRUE)
sd(msubC$Na,na.rm=TRUE)
sd(msubC$K,na.rm=TRUE)
sd(msubC$Cl,na.rm=TRUE)
sd(msubC$CO2,na.rm=TRUE)
sd(msubC$anion,na.rm=TRUE)
sd(msubC$urea,na.rm=TRUE)
sd(msubC$creat,na.rm=TRUE)
sd(msubC$gluc,na.rm=TRUE)
sd(msubC$alkphos,na.rm=TRUE)
sd(msubC$ALT,na.rm=TRUE)
sd(msubC$AST,na.rm=TRUE)
sd(msubC$Ca,na.rm=TRUE)
sd(msubC$P,na.rm=TRUE)
```

```
sd(msubC$prot,na.rm=TRUE)
sd(msubC$alb,na.rm=TRUE)
sd(msubC$bili,na.rm=TRUE)
sd(msubC$test,na.rm=TRUE)
sd(msubC$pre_L1_4,na.rm=TRUE)
sd(msubC$pre_TH,na.rm=TRUE)
##treatment
summary(msubT)
sd(msubT$age,na.rm=TRUE)
sd(msubT$height,na.rm=TRUE)
sd(msubT$weight,na.rm=TRUE)
sd(msubT$BMI,na.rm=TRUE)
sd(msubT$Na,na.rm=TRUE)
sd(msubT$K,na.rm=TRUE)
sd(msubT$Cl,na.rm=TRUE)
sd(msubT$CO2,na.rm=TRUE)
sd(msubT$anion,na.rm=TRUE)
sd(msubT$urea,na.rm=TRUE)
sd(msubT$creat,na.rm=TRUE)
sd(msubT$gluc,na.rm=TRUE)
sd(msubT$alkphos,na.rm=TRUE)
sd(msubT$ALT,na.rm=TRUE)
sd(msubT$AST,na.rm=TRUE)
sd(msubT$Ca,na.rm=TRUE)
sd(msubT$P,na.rm=TRUE)
sd(msubT$prot,na.rm=TRUE)
sd(msubT$alb,na.rm=TRUE)
sd(msubT$bili,na.rm=TRUE)
sd(msubT$test,na.rm=TRUE)
sd(msubT$pre_L1_4,na.rm=TRUE)
sd(msubT$pre_TH,na.rm=TRUE)

##Test for differences
##Fishers Exact for Categorical; T-Test for Continuous
fisher.test(msubC$race,msubT$race)
fisher.test(msubC$ethn,msubT$ethn)
t.test(msubC$age,msubT$age)
t.test(msubC$height,msubT$height)
t.test(msubC$weight,msubT$weight)
t.test(msubC$BMI,msubT$BMI)
t.test(msubC$prev_WB,msubT$prev_WB)
t.test(msubC$prev_Aph,msubT$prev_Aph)
t.test(msubC$Na,msubT$Na)
t.test(msubC$K,msubT$K)
t.test(msubC$Cl,msubT$Cl)
t.test(msubC$CO2,msubT$CO2)
t.test(msubC$anion,msubT$anion)
t.test(msubC$urea,msubT$urea)
t.test(msubC$creat,msubT$creat)
t.test(msubC$gluc,msubT$gluc)
t.test(msubC$alkphos,msubT$alkphos)
t.test(msubC$AST,msubT$AST)
t.test(msubC$ALT,msubT$ALT)
t.test(msubC$Ca,msubT$Ca)
```

```
t.test(msubC$P,msubT$P)
t.test(msubC$prot,msubT$prot)
t.test(msubC$alb,msubT$alb)
t.test(msubC$bili,msubT$bili)
t.test(msubC$test,msubT$test)
t.test(msubC$pre_L1_4,msubT$pre_L1_4)
t.test(msubC$pre_TH,msubT$pre_TH)

##visualize distributions of BMD at enrollment
par(mfrow=c(3,2))
hist(data$pre_L1_4)
hist(data$pre_TH)
hist(msubC$pre_L1_4)
hist(msubC$pre_TH)
hist(msubT$pre_L1_4)
hist(msubT$pre_TH)

##Visualize BMD at enrollment for Both Groups
##there's a number of ways I'm considering illustrating
these
##this is an example
ggplot(data, aes(x=test)) +
  geom_density(aes(stat="density"),
                 binwidth=0.5,
                 colour="black",
                 fill="white") +
  geom_density(alpha=.2, fill="#FF6666")

par(mfrow=c(2,1))
ggplot(data, aes(x=SubjectID,
                 y=pre_TH))+
       geom_point(size=1.5, shape=21, fill="white")+
       labs(title="Baseline TH BMD, Both Groups")

ggplot(data, aes(x=SubjectID,
                 y=pre_L1_4))
       + ggtitle("Baseline L Spine BMD, Both Groups")

##Visualize BMD at enrollment for Controls
##Visualize BMD at enrollment for Treatment
##Visualize Final BMD for Both Groups
##Visualize Final BMD for Controls
##Visualize Final BMD for Treatment

##create derived 'change in BMD' variables
deltaTH<-(data$post_TH-data$pre_TH)
deltaL1_4<-(data$post_L1_4-data$pre_L1_4)

deltaTHC<-(msubC$post_TH-msubC$pre_TH)
deltaTHC
deltaL1_4C<-(msubC$post_L1_4-msubC$pre_L1_4)
deltaL1_4C

deltaTHT<-(msubT$post_TH-msubT$pre_TH)
```

```
deltaTHT
deltaL1_4T<-(msubT$post_L1_4-msubT$pre_L1_4)
deltaL1_4T

##compute mean at enrollment
mean(msubC$pre_TH)
sd(msubC$pre_TH)
mean(msubT$pre_TH)
sd(msubT$pre_TH)

mean(msubC$pre_L1_4)
sd(msubC$pre_L1_4)
mean(msubT$pre_L1_4)
sd(msubT$pre_L1_4)

##compute mean at follow-up
mean(msubC$post_TH)
sd(msubC$post_TH)
mean(msubT$post_TH)
sd(msubT$post_TH)

mean(msubC$post_L1_4)
sd(msubC$post_L1_4)
mean(msubT$post_L1_4)
sd(msubT$post_L1_4)

##Compute mean change
mean(deltaTHC)
sd(deltaTHC)
mean(deltaTHT)
sd (deltaTHT)

mean(deltaL1_4C)
sd(deltaL1_4C)
mean(deltaL1_4T)
sd(deltaL1_4T)

##hists
par(mfrow=c(2,2))
hist(deltaL1_4C,xlim=c(-0.1,0.1),ylim=c(0,15),main="Delta L
Spine Control",nclass=5)
hist(deltaL1_4T,xlim=c(-0.1,0.1),ylim=c(0,15),main="Delta L
Spine Apheresis",nclass=10)
hist(deltaTHC,xlim=c(-0.1,0.1),ylim=c(0,15),main="Delta TH
Controls",nclass=5)
hist(deltaTHT,xlim=c(-0.1,0.1),ylim=c(0,15),main="Delta TH
Apheresis",nclass=10)

##plain t test for differences
t.test(deltaL1_4C,deltaL1_4T)
t.test(deltaTHC,deltaTHT)

##Visualize change in BMD for both groups
par(mfrow=c(2,2))
```

```
plot(deltaL1_4C, ylim=c(-0.17,0.17))
plot(deltaL1_4T, ylim=c(-0.17,0.17))
plot(deltaTHC, ylim=c(-0.17,0.17))
plot(deltaTHT, ylim=c(-0.17,0.17))

##Boxplots change in BMD
par(mfrow=c(2,2))
boxplot(deltaL1_4C, ylim=c(-0.1,0.1),main="L spine
control")
boxplot(deltaL1_4T, ylim=c(-0.1,0.1),main="L spine
treated")
boxplot(deltaTHC, ylim=c(-0.05,0.05),main="hip control")
boxplot(deltaTHT, ylim=c(-0.05,0.05),main="hip treated")

##create Figure 3 for main paper - box plots
ggplot(data, aes(x=factor(apheresis), y=deltaL1_4))+
  ylim(-0.06,0.06)+
  geom_boxplot()+
  theme_bw()+
  stat_summary(fun.y="mean", geom="point", shape=23,
size=3, fill="black")+
  annotate("rect",xmin=0.5,xmax=2.5,alpha=0.1,ymin=-
0.00743,ymax=0.00743,fill="blue")+
  theme(panel.border = element_blank(),
        panel.grid.major = element_blank(),
        panel.grid.major.y = element_line(linetype =
"dashed", colour = "black"),
        panel.grid.minor = element_blank(),
        panel.background = element_blank(),
        axis.line = element_line(colour = "white"))

ggplot(data, aes(x=factor(apheresis), y=deltaTH))+
  ylim(-0.06,0.06)+
  geom_boxplot()+
  theme_bw()+
  stat_summary(fun.y="mean", geom="point", shape=23,
size=3, fill="black")+
  annotate("rect",xmin=0.5,xmax=2.5,alpha=0.1,ymin=-
0.00743,ymax=0.00743,fill="blue")+
  theme(panel.border = element_blank(),
        panel.grid.major = element_blank(),
        panel.grid.major.y = element_line(linetype =
"dashed", colour = "black"),
        panel.grid.minor = element_blank(),
        panel.background = element_blank(),
        axis.line = element_line(colour = "white"))

##compare mean change to LSC in treated
##LSC at lumbar spine is 0.00743
if((mean(deltaL1_4T))>0.00743)
  print("mean change among treated subjects exceeds LSC for
lumbar spine in the positive direction")
if((mean(deltaL1_4T))<(-0.00743))
```

```
  print("mean change among treated subjects exceeds LSC for
lumbar spine in the negative direction")

##LSC at TH is 0.00671
if((mean(deltaTHT))>0.00671)
  print("mean change for treated subjects exceeds LSC for
total hip in the positive direction")
if((mean(deltaTHT))<(-0.00671))
  print("mean change for treated subjects exceeds LSC for
total hip in the negative direction")

##compare mean change to LSC in controls
##LSC at lumbar spine is 0.00743
if((mean(deltaL1_4C))>0.00743)
  print("mean change among control subjects exceeds LSC for
lumbar spine in the positive direction")
if((mean(deltaL1_4C))<(-0.00743))
  print("mean change among control subjects exceeds LSC for
lumbar spine in the negative direction")

##LSC at TH is 0.00671
if((mean(deltaTHC))>0.00671)
  print("mean change for control subjects exceeds LSC for
total hip in the positive direction")
if((mean(deltaTHC))<(-0.00671))
  print("mean change for control subjects exceeds LSC for
total hip in the negative direction")

##Define Number of Subjects Analyzed in Each Group
nC<-15
nT<-21

##BEGIN: Unadjusted Analysis
##Compute and Show Standard Errors
vardeltaTHC<-var(deltaTHC)
vardeltaTHC
vardeltaL1_4C<-var(deltaL1_4C)
vardeltaL1_4C
vardeltaTHT<-var(deltaTHT)
vardeltaTHT
vardeltaL1_4T<-var(deltaL1_4T)
vardeltaL1_4T

##Test for Differences in Baseline BMD by treatment arm
TTH<-(mean(deltaTHC)-
mean(deltaTHT))/sqrt((vardeltaTHC/nC)+(vardeltaTHT/nT))
TTH
TL1_4<-(mean(deltaL1_4C)-
mean(deltaL1_4T))/sqrt((vardeltaL1_4C/nC)+(vardeltaL1_4T/nT
))
TL1_4

##compute degrees of freedom
vTH<-((vardeltaTHC/nC)+(vardeltaTHT/nT))/
```

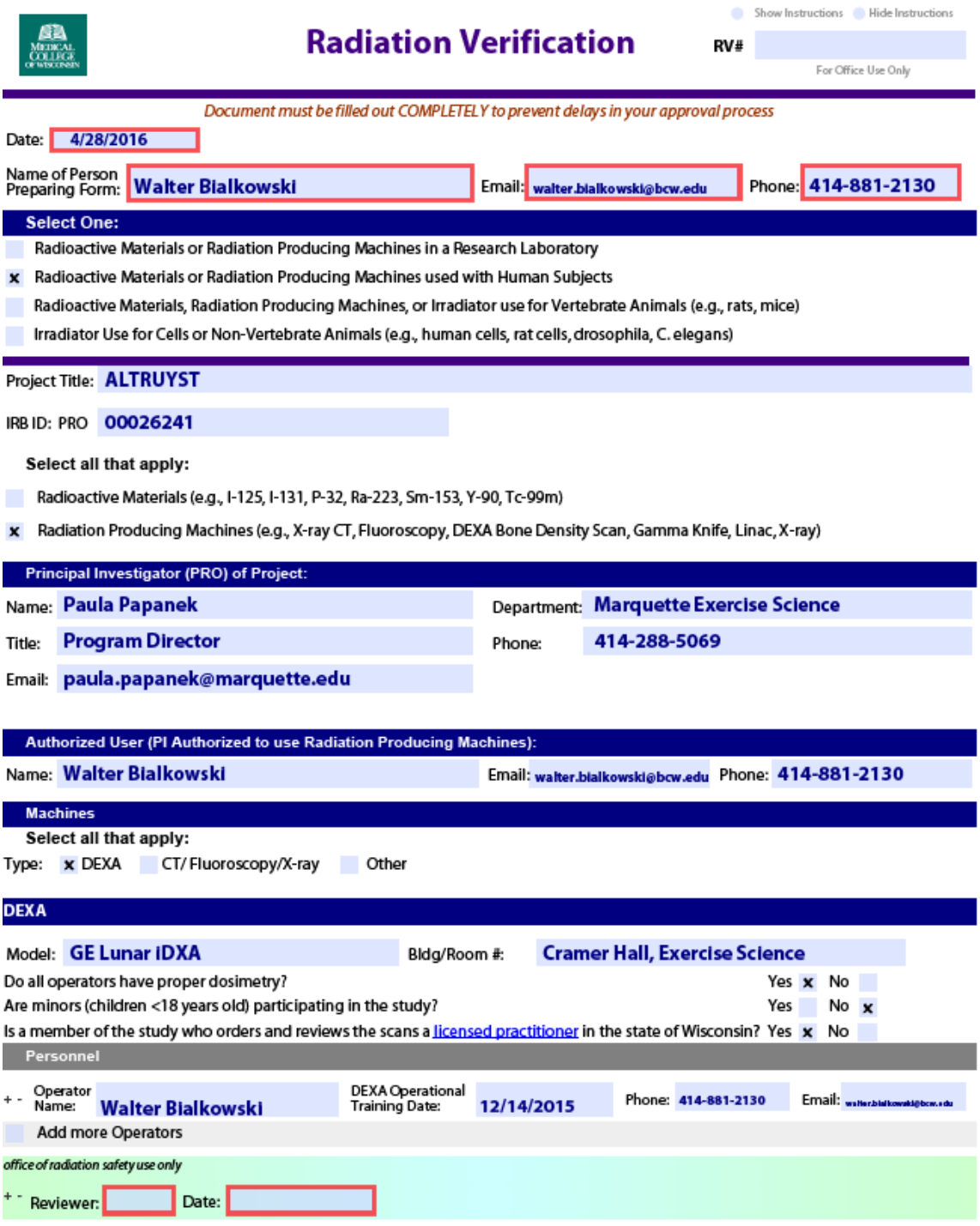
```
  ((((vardeltaTHC/nC)^2)/(nC-1))+(((vardeltaTHT/nT)^2)/(nT-
1)))
VTH
vL1_4<-((vardeltaL1_4C/nC)+(vardeltaL1_4T/nT))/
  ((((vardeltaL1_4C/nC)^2)/(nC-
1))+(((vardeltaL1_4T/nT)^2)/(nT-1)))
vL1_4

##derive critical value
qtTH<-qt(c(0.95),df=vTH)
qtTH
qtL1_4<-qt(c(0.95),df=vL1_4)
qtL1_4

##output messaging
if((TTH-qtTH)>0)
  print("reject the null hypothesis for total hip")
if((TTH-qtTH)<=0)
  print("you are not able to reject the null hypothesis for
total hip")

if((TL1_4-qtL1_4)>0)
  print("reject the null hypothesis for lumbar spine")
if((TL1_4-qtL1_4)<=0)
  print("you are not able to reject the null hypothesis for
lumbar spine")

##END: Unadjusted Analysis

##BEGIN Adjusted Analysis Using Multivariable Linear
Regression
##visualize distributions of participant demographic and
behavioral characteristics
##can also do str function for each predictor to examine
potential extreme values
##can also do stem and leaf plots
##can do scatterplots to evaluate need for transformations
##can also test for correlations using Pearsons/Spearmans
##can test linearity using Shapiro-Wilk's
##can evaluate leverage values
##can do QQ plots
##can do Jackknife Residuals
##can do Cook's Distance
##can evaluate collinearity with rcorr function
##can do Variance Inflation Factors (VIF>10 interrogation)
##can evaluate multicollinearity with Tolerance Values,
Eigenvalues, Condition Indices/Numbers
par(mfrow=c(3,2))
hist(data$age)
hist(data$risks)
hist(data$family)
hist(data$conditions)
hist(data$medications)
hist(data$diet)
```

```
##negative change in L spine
fitnL1_4<-lm(data$bigndeltaL1_4~
               (data$apheresis)+
               (data$age)+
               (as.factor(data$risks))+
               (as.factor(data$family))+
               (as.factor(data$conditions))+
               (as.factor(data$medications))+
               (as.factor(data$diet))+
               (data$BMI)+
               (data$pre_L1_4)+
               (data$Na)+
               (data$K)+
               (data$Cl)+
               (data$CO2)+
               (data$anion)+
               (data$urea)+
               (data$creat)+
               (data$gluc)+
               (data$alkphos)+
               (data$AST)+
               (data$ALT)+
               (data$Ca)+
               (data$P)+
               (data$prot)+
               (data$alb)+
               (data$bili)+
               (data$test))
summary(fitnL1_4)
anova(fitnL1_4)
##stepwise backwards elimination for negative change in L
spine
stepnL1_4<-stepAIC(fitnL1_4,direction="backward")
stepnL1_4$anova

##positive change in L spine
fitpL1_4<-lm(data$bigpdeltaL1_4~
               (data$apheresis)+
               (data$age)+
               (as.factor(data$risks))+
               (as.factor(data$family))+
               (as.factor(data$conditions))+
               (as.factor(data$medications))+
               (as.factor(data$diet))+
               (data$BMI)+
               (data$pre_L1_4)+
               (data$Na)+
               (data$K)+
               (data$Cl)+
               (data$CO2)+
               (data$anion)+
               (data$urea)+
```

```
                (data$creat)+
                (data$gluc)+
                (data$alkphos)+
                (data$AST)+
                (data$ALT)+
                (data$Ca)+
                (data$P)+
                (data$prot)+
                (data$alb)+
                (data$bili)+
                (data$test))
summary(fitpL1_4)
anova(fitpL1_4)
##stepwise backwards elimination for positive change in L
spine
steppL1_4<-stepAIC(fitpL1_4,direction="backward")
steppL1_4$anova

##negative change in total hip
fitnTH<-lm(data$bigndeltaTH~
             (data$apheresis)+
             (data$age)+
             (as.factor(data$risks))+
             (as.factor(data$family))+
             (as.factor(data$conditions))+
             (as.factor(data$medications))+
             (as.factor(data$diet))+
             (data$BMI)+
             (data$pre_TH)+
             (data$Na)+
             (data$K)+
             (data$Cl)+
             (data$CO2)+
             (data$anion)+
             (data$urea)+
             (data$creat)+
             (data$gluc)+
             (data$alkphos)+
             (data$AST)+
             (data$ALT)+
             (data$Ca)+
             (data$P)+
             (data$prot)+
             (data$alb)+
             (data$bili)+
             (data$test))
summary(fitnTH)
anova(fitnTH)
##stepwise backwards elimination for negative change in L
spine
stepnTH<-stepAIC(fitnTH,direction="backward")
stepnTH$anova

##positive change in TH
```

```
fitpTH<-lm(data$bigpdeltaTH~
             (data$apheresis)+
             (data$age)+
             (as.factor(data$risks))+
             (as.factor(data$family))+
             (as.factor(data$conditions))+
             (as.factor(data$medications))+
             (as.factor(data$diet))+
             (data$BMI)+
             (data$pre_TH)+
             (data$Na)+
             (data$K)+
             (data$Cl)+
             (data$CO2)+
             (data$anion)+
             (data$urea)+
             (data$creat)+
             (data$gluc)+
             (data$alkphos)+
             (data$AST)+
             (data$ALT)+
             (data$Ca)+
             (data$P)+
             (data$prot)+
             (data$alb)+
             (data$bili)+
             (data$test))
summary(fitpTH)
anova(fitpTH)

##stepwise backwards elimination for positive change in L
spine
steppTH<-stepAIC(fitpTH,direction="backward")
steppTH$anova
```

Appendix 12 – IBC Approval



Show Instructions Hide Instructions

Radiation Verification

RV# For Office Use Only

Document must be filled out COMPLETELY to prevent delays in your approval process

Date: 4/28/2016

Name of Person Preparing Form: Walter Bialkowski Email: walter.bialkowski@bcw.edu Phone: 414-881-2130

Select One:

- [ ] Radioactive Materials or Radiation Producing Machines in a Research Laboratory
- [x] Radioactive Materials or Radiation Producing Machines used with Human Subjects
- [ ] Radioactive Materials, Radiation Producing Machines, or Irradiator use for Vertebrate Animals (e.g., rats, mice)
- [ ] Irradiator Use for Cells or Non-Vertebrate Animals (e.g., human cells, rat cells, drosophila, C. elegans)

Project Title: ALTRUYST

IRB ID: PRO 00026241

Select all that apply:

- [ ] Radioactive Materials (e.g., I-125, I-131, P-32, Ra-223, Sm-153, Y-90, Tc-99m)
- [x] Radiation Producing Machines (e.g., X-ray CT, Fluoroscopy, DEXA Bone Density Scan, Gamma Knife, Linac, X-ray)

Principal Investigator (PRO) of Project:

Name: Paula Papanek Department: Marquette Exercise Science

Title: Program Director Phone: 414-288-5069

Email: paula.papanek@marquette.edu

Authorized User (PI Authorized to use Radiation Producing Machines):

Name: Walter Bialkowski Email: walter.bialkowski@bcw.edu Phone: 414-881-2130

Machines

Select all that apply:

Type: [x] DEXA [ ] CT/ Fluoroscopy/X-ray [ ] Other

DEXA

Model: GE Lunar iDXA Bldg/Room #: Cramer Hall, Exercise Science

Do all operators have proper dosimetry? Yes [x] No [ ]

Are minors (children <18 years old) participating in the study? Yes [ ] No [x]

Is a member of the study who orders and reviews the scans a licensed practitioner in the state of Wisconsin? Yes [x] No [ ]

Personnel

+ - Operator Name: Walter Bialkowski DEXA Operational Training Date: 12/14/2015 Phone: 414-881-2130 Email: walter.bialkowski@bcw.edu

[ ] Add more Operators

office of radiation safety use only

+ - Reviewer: Date:

Appendix 13 – Radiation Safety Approval

MEDICAL COLLEGE OF WISCONSIN

Radiation Safety
Office of Research

RADIATION VERIFICATION

RV ID:	RV462
PI:	Paula Papanek, PhD
PRO:	26241
Project Title:	ALTRUYST
Approval Date:	April 28, 2016

The Radiation Verification Form for the study indicated above was reviewed by the Office of Radiation Safety for the following items, as applicable:

- Properly Registered Radiation Producing Machine(s)
- Personnel/Operators Properly Trained
- Appropriate Dosimeters Issued
- Radioactive Material(s) Approved

The study is in compliance with the conditions of the Froedtert and Medical College of Wisconsin State of Wisconsin Department of Health Services Broad Scope Radioactive Material License and X-ray Device Registrations.

Leo Kaiser

Digitally signed by Leo Kaiser
DN: cn=Leo Kaiser, o=Medical College of Wisconsin, ou=Office of Research, email=lkaiser@mcw.edu, c=US
Date: 2016.04.28 09:43:48 -05'00'

Office of Radiation Safety

Radiation Verification

RV# 462

For Office Use Only

Document must be filled out COMPLETELY to prevent delays in your approval process

Date: 4/28/2016

Name of Person Preparing Form: Walter Bialkowski Email: walter.bialkowski@bcw.edu Phone: 414-881-2130

Select One:

- [ ] Radioactive Materials or Radiation Producing Machines in a Research Laboratory
- [x] Radioactive Materials or Radiation Producing Machines used with Human Subjects
- [ ] Radioactive Materials, Radiation Producing Machines, or Irradiator use for Vertebrate Animals (e.g., rats, mice)
- [ ] Irradiator Use for Cells or Non-Vertebrate Animals (e.g., human cells, rat cells, drosophila, C. elegans)

Project Title: ALTRUYST

IRB ID: PRO 00026241

Select all that apply:

- [ ] Radioactive Materials (e.g., I-125, I-131, P-32, Ra-223, Sm-153, Y-90, Tc-99m)
- [x] Radiation Producing Machines (e.g., X-ray CT, Fluoroscopy, DEXA Bone Density Scan, Gamma Knife, Linac, X-ray)

Principal Investigator (PRO) of Project:

Name: Paula Papanek Department: Marquette Exercise Science

Title: Program Director Phone: 414-288-5069

Email: paula.papanek@marquette.edu

Authorized User (PI Authorized to use Radiation Producing Machines):

Name: Walter Bialkowski Email: walter.bialkowski@bcw.edu Phone: 414-881-2130

Machines

Select all that apply:

Type: [x] DEXA [ ] CT/ Fluoroscopy/X-ray [ ] Other

DEXA

Model: GE Lunar iDXA Bldg/Room #: Cramer Hall, Exercise Science

Do all operators have proper dosimetry? Yes [x] No [ ]

Are minors (children <18 years old) participating in the study? Yes [ ] No [x]

Is a member of the study who orders and reviews the scans a licensed practitioner in the state of Wisconsin? Yes [x] No [ ]

Personnel

Operator Name: Walter Bialkowski DEXA Operational Training Date: 12/14/2015 Phone: 414-881-2130 Email: [redacted]

office of radiation safety use only

Reviewer: LK Date: 4/28/2016

Appendix 14 – MCW IRB Approval

Medical College of Wisconsin /
Froedtert Hospital
Institutional Review Board

To: Paula Papanek, PhD, MPT
Walter Bialkowski
CC: Jerome Gottschall, MD
Robert Blank, MD, PhD
Cheng Zheng

Date:**5/6/2016**

Re: **Project Title**:RAndomized Longitudinal STudy of ApheResis VolUntarY Blood DonorS' Bone DensiTy
PRO ID: PRO00026241

IRB Approval Date: 4/26/2016
IRB Expiration Date: 4/25/2017

The MCW/FH Institutional Review Board #3 reviewed this submission. At the IRB meeting on 4/26/2016, the Committee determined that the submission met all criteria at 21 CFR 56.111, but that modifications were required before approval. The required modifications have been reviewed and found satisfactory; therefore approval of this submission is granted.

Approval has been granted for the following institutions:

Marquette University†
BloodCenter of Wisconsin and Blood Research Institute

The consent form and related HIPAA authorization are effective as of 4/26/2016. Signed consent forms for each subject must be kept on file as part of the project records.

The items listed below were submitted and reviewed when the IRB approved this submission. Research must be

conducted according to the IRB approved document listed below:

Attachment 1 ALTRUYST Informed Consent v1.4
Attachment 2 ALTRUYST Informational Flyer
ALTRUYST Protocol Final MAR2016

The MCW/FH Institutional Review Board #3 also granted approval of a waiver of HIPAA authorization requirements at 45 CFR 164 and a waiver of informed consent requirements at 45 CFR 46.116 for the purpose of Records Review for Potential Subjects.

Any and all proposed changes to this submission must be reviewed and approved by the IRB prior to implementation. When necessary to eliminate hazards to subjects, changes may be made first. This should be followed promptly by a protocol deviation and amendment.

In accordance with federal regulations, continuing approval for this submission is required prior to 4/25/2017. The Continuing Progress Report (CPR) must be received by the IRB with enough time to allow for review and approval prior to the expiration date. Failure to submit the CPR in a timely manner may result in the expiration of IRB approval.

A Final Report must be submitted to the IRB within 30 days of when all project activities and data analysis have been completed.

All Unanticipated Problems Involving Risks to Subjects or Others (UPIRSO) must be reported promptly to the MCW/FH IRB according to IRB Standard Operating Procedures (SOPs).

If your project involves the use of any Froedtert Health resource such as, space, staff services, supplies/equipment or any ancillary services - lab, pharmacy, radiology, protected health/billing information or specimen requests, OCRICC approval is required before beginning any research activity at those sites.

If you have any questions, please contact the IRB Coordinator II for this IRB Committee, Sara Miller, at 414-955-4196 or ssmiller@mcw.edu.

Sincerely,

Ryan Spellecy, PhD
IRB Chair
MCW/FH Institutional Review Board #3

Appendix 15 – DXA Quality Control Records

BONE & BODY COMPOSITION LABORATORY

MARQUETTE UNIVERSITY
MILWAUKEE, WI 53201

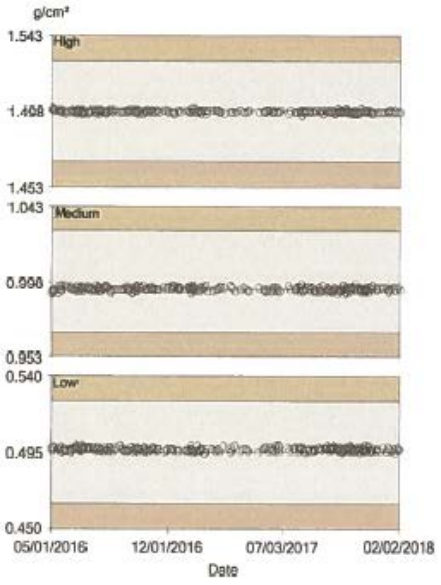
QA Phantom Report

03/23/2018
7:34:13 AM

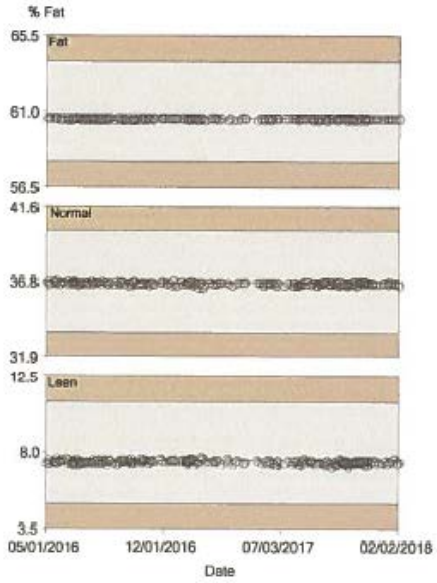
Lunar iDXA
ME+210352
(14.10)

QA Phantom

BMD Calibration



Composition Calibration



QA Phantom

BMD	0.993 g/cm²	Pass
BMC	23.55 g	Pass
Area	23.71 cm²	Pass

QC Tests

X-ray and Detector Status	Pass
Mechanical Tests	Pass
Calibration Status	Pass

Precision

BMD CV	0.24%

BONE & BODY COMPOSITION LABORATORY

MARQUETTE UNIVERSITY
MILWAUKEE, WI 53201

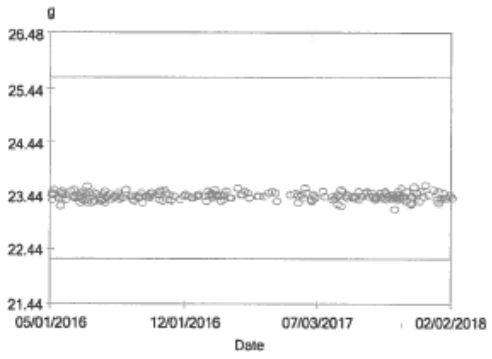
QA Phantom Ancillary Report

03/23/2018
7:34:13 AM

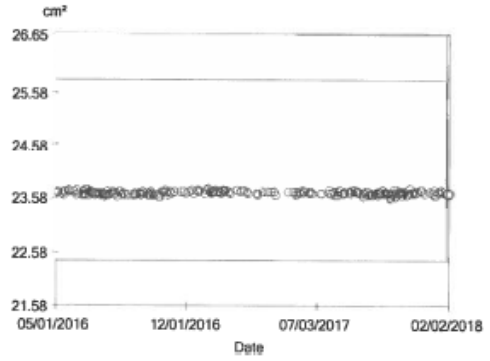
Lunar iDXA
ME+210352
(14.10)

QA Phantom

BMC



Area



Functional Tests	Value	Status
Peaking	1,585	Pass
Beam Stop	0.25 / 0.25	Pass
Mechanical Test		
Transverse	678.82	Pass
Longitudinal	1,979.80	Pass
Spillover Test		
Mean %	9.37%	Pass
Stability	-0.01%	Pass
Reference Counts		
High mA	155,236 / 225,479	Pass
Ratio at High mA	0.69	Pass

Test	Mean	Precision	Status
BMD		% CV	
High	1.497	0.3%	Pass
Medium	0.995	0.6%	Pass
Low	0.497	1.3%	Pass
Tissue		SD	
Lean	7.3%	0.2%	Pass
Normal	36.5%	0.4%	Pass
Fat	61.2%	0.2%	Pass