PROSPECTIVE BIOMARKERS OF BONE METABOLISM IN HEMOPHILIA A

Hypothesis

Hemophilia is associated with decreased skeletal health. We hypothesize biomarkers of bone disease in hemophilia A will normalize with factor VIII (FVIII) replacement.

Introduction

The landscape of hemophilia treatment has changed dramatically over the last several decades. We are now in an era where safe factor replacement is widely available for preventing and treating bleeding episodes. Rates of HIV and HCV are essentially absent in those less than 28 years of age. Furthermore people with hemophilia (PwH) have a normal life expectancy [1]. The focus of hemophilia management must now turn to addressing long-term complications of this condition [1,2]. Unfortunately PwH are seldom screened for decreased bone mineral density (BMD) despite having several major risk factors for bone loss.

Decreased BMD and increased risk for fractures has been identified in PwH. Studies in adults showed 70% of patients have low BMD, over 40% had osteopenia and 27% had osteoporosis [3,4]. Several studies have demonstrated that children with hemophilia have significantly lower bone density than age matched control populations [5-7]. All of these studies included patients with HIV and HCV infections and patients had varying degrees of hemophilic arthropathy; all of which are risk factors for decreased BMD [5,8, 9]. Thus it has been generally believed that decreased skeletal health in PwH is secondary to the risk factors of hemarthroses, chronic disease and immobility.

Using a FVIII-deficient mouse model we have recently demonstrated that decreased skeletal health is independent of these risk factors and that bone disease is intrinsic to FVIII deficiency[10]. As shown in Table 1, 20-week old (the age of peak bone mass) mice have decreased BMD and biomechanical strength compared to wild-type control. In a subsequent study we demonstrated through quantitative histology (histomorphometry) that the difference is caused by increased bone resorption in the factor deficient mice[11] (data not shown).

	Femoral BMD (mg/cm^2)	Cortical Thickness (mm)	Ultimate Force (N)	Stiffness (N/mm)
FVIII-deficient (n = 23)	56.49 (2.66)	0.1597 (0.006)	20.08 (1.41)	98.50 (9.83)
WT (n = 20)	58.48 (3.36)	0.1638 (0.006)	22.31 (2.24)	108.2 (11.22)
***p* value**	**0.036**	**0.039**	**0.0003**	**0.005**

Table 1. Femoral bone measurements of 20-week old mice showing statistically significant differences in BMD, cortical thickness, stiffness, and ultimate breaking force between WT and FVIII-deficient animals. Standard deviations are in parenthesis.

We demonstrated no differences in markers of bone formation (osteocalcin and alkaline phosphatase) nor in a key regulatory pathway of bone metabolism (receptor activator of nuclear factor kappa-B ligand [RANKL] and osteoprotegerin [OPG] (figure 1) between the FVIII-deficient and wild-type mice. We did however identify differences in cytokine production, with decreased interferon-β and interleukin-1α in the FVIII-deficient compared to wild-type mice (data not shown).

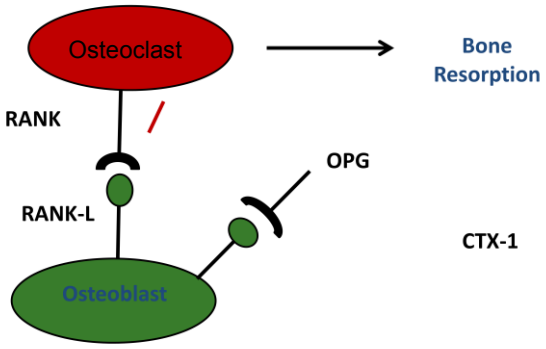

Figure 1: Bone Biomarkers. RANKL binding to the RANK receptor induces the proliferation and differentiation of osteoclasts resulting in increased bone resorption. OPG competitively inhibits RANKL binding to RANK, blocking these osteoclastic effects. CTX-1 is formed through the degradation of type 1 collagen in bone and thus is a direct marker of bone resorption.

Based in part on these findings Baxter Bioscience funded a retrospective study investigating the bone biomarkers of PwH from the biorepository at the University of Colorado. We evaluated the bone biomarkers of 79 males with severe Hemophila A, 20 males with severe Hemophilia B and 51 healthy male controls. The results of the study were presented at the American Society of Hematology meeting in December 2013 in poster form (please see the Appendix for the full poster). There were several key findings in our study. First, biomarkers of bone disease were markedly different in individuals with developing bone (<16 years of age) compared to developed bone (age 16+) for both healthy controls and individuals with hemophilia A. There was increased CTX-1, a biomarker for increased bone resorption, in individuals with hemophilia A and mature bone compared to control. Finally, samples with factor replacement the day prior had

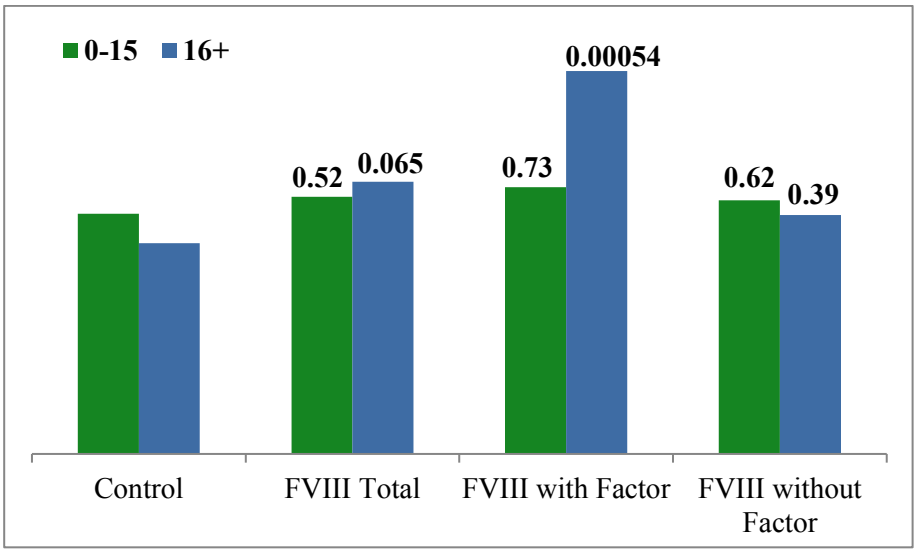

Figure 2. Factor replacement within 1 calendar day prior to blood draw increases OPG in FVIII-deficient subjects with mature bone. *p* values are shown relative to controls.

increased OPG (Figure 2) and normalized CTX-1 (Figure 3) compared to controls. These results suggest that factor replacement ameliorates bone disease in hemophilia A. Supporting the clinical significance of this study, an additional study demonstrated a correlation between bone biomarkers and BMD in individuals with hemophilia A[12].

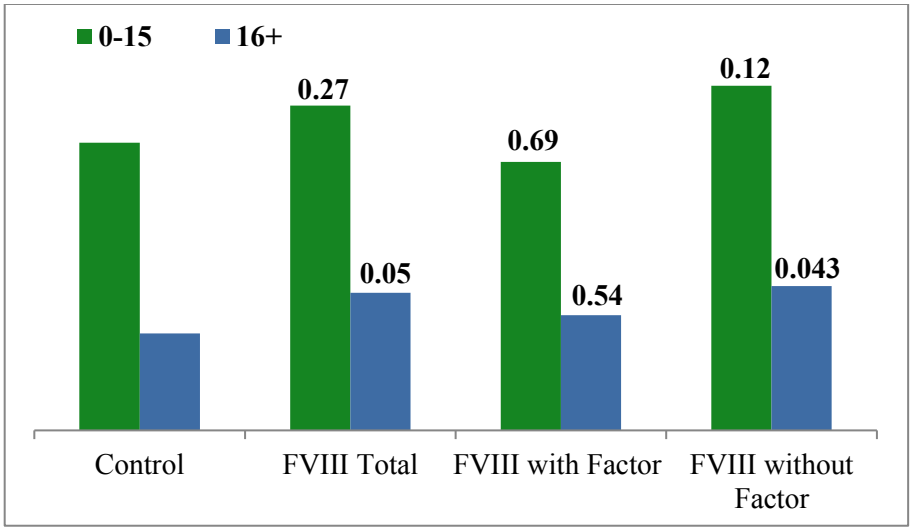

Figure 3. Factor replacement within 1 calendar day prior to blood draw normalizes CTX-1 in FVIII-deficient subjects. *p* values are shown relative the controls.

Preliminary (unpublished) data

Since the American Society of Hematology annual meeting additional data has been obtained from the biorepository samples. We found no difference in osteocalcin (a biomarker for bone formation) between the various groups. Several cytokines are known to play a significant role bone metabolism (Table 2). Using a multiplex system we simultaneously measured the plasma levels of key cytokines including: IFNγ, TNFα, MSCF, IL-10, IL6, IL-12, IL-17α, IL-1α, IL-1β. There was significant decrease in IL-10, IL-12 and TNFα in the plasma of patients with hemophilia A compared to controls ($P < .01$), which normalized with recent factor infusion. Since IL-10 and IL-12 have opposing effects on osteoclast function compared to TNFα, it is difficult to interpret the results. Further evaluation of how cytokines vary with factor use and other variables in hemophilia is needed.

Cytokine	Osteoclast Activation
IL-1	++++
MCSF	+++
TNF-α	+++
IL-6	+++
IL-10	Inhibitory
IL-12	Inhibitory
IL-17	+++
IFN-γ	Inhibitory
IFN-β	Inhibitory

Table 2: Known effects of cytokines on osteoclast activation and bone resorption (adapted from [13]). Plus signs indicate relative activation potential of the cytokine

One of the major shortcomings in studying bone disease in hemophilia is the lack of fracture outcome data demonstrating the clinical significance of decreased BMD and altered bone biomarkers in the hemophilia population. We undertook a retrospective analysis of the fracture rate of all PwH at our hemophilia treatment center over a 10-year period ending in December 2012. A total of 47 fractures in 387 subjects over 738,000 subject days were identified for a PwH fracture rate of 23.2 fractures per 1000 subject years, over twice the rate of 9.58 fractures per 1000 subject years in the general population[14]. As demonstrated in the figure, the fracture rate in PwH also increased with age. Subjects with hemophilia A and hemophilia B had similar fracture rates (23.2 v 23.7 fractures per 1000 subject years). This study demonstrates that PwH have an increased risk of fracture compared to the general population and that the issue of bone health will increase in importance as the PwH population ages.

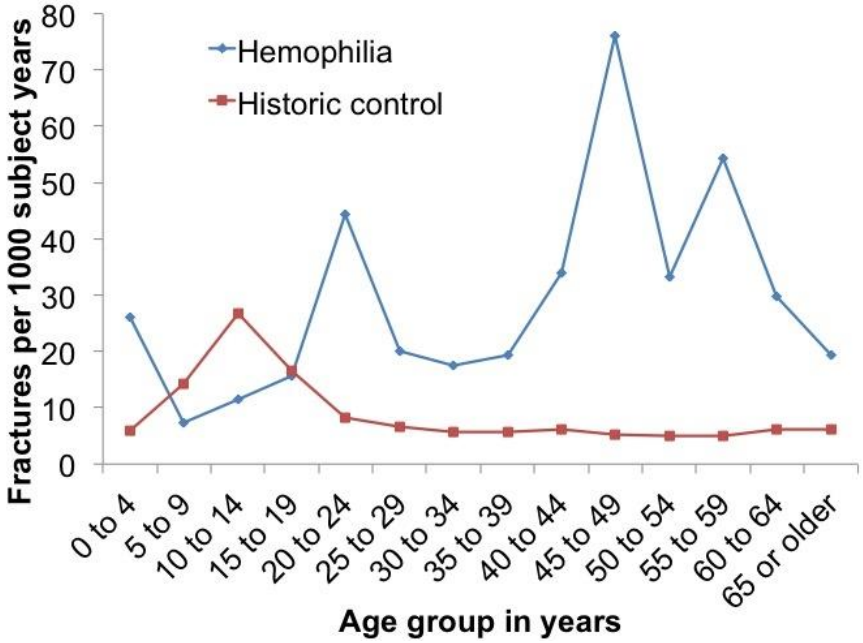

Figure 4. Fracture rates in PwH compared to historic controls.

Specific Aim

1. Characterize the impact of FVIII replacement on the levels of biomarkers of bone metabolism in subjects with severe Hemophilia A.
2. Correlate the levels of biomarkers of bone metabolism with subject history, physical examination and bone mineral density.

Study Design

This is a pilot study to determine the impact of factor replacement on bone biomarkers in up to 20 hemophilia A subjects. Subjects will be recruited over 1 year for the 5-day protocol.

Following a 72-hour washout period, factor levels and bone biomarkers will be followed before and after 50 units/kg replacement on Day 1 and 20 units/kg replacement on Day 3. Each subject can serve as their

own control using baseline washout biomarker levels to compare with subsequent values after factor replacement. The subjects will also undergo two DEXA scans (spine and hip) and answer several survey questions about their history and factor use.

Recruitment Methods

Subjects who are currently using any factor FVIII replacement therapy will be recruited from the Hemophilia Center at Oregon Health & Science University. Subjects will be approached during a scheduled clinic visit, via email or over the phone and a research visit will be scheduled.

The age of bone maturation depends on what criteria used but generally occur occurs around 16 years of age[15]. Biomarkers of bone metabolism display a significant difference during development compared to bone that is more mature (Figure 2-3). To verify that 16 years is an appropriate cutoff age to compare biomarkers in the hemophilia A population subanalysis CTX-1 levels from biorepository data was performed. CTX-1 showed significant different between plasma samples in subjects $\geq$ 16 compared to < 16 (Figure 3). Furthermore to prevent potential confounding effects of recent factor replacement samples from subjects that received factor replacement the day prior were removed from this analysis. There was a highly significant statistical difference in CTX-1 plasma concentration in the severe hemophilia A population comparing 14-15 to 16-17 year old subjects ($p = 0.009$), while there was no statistical difference in CTX-1 plasma concentration comparing16 to 17-19 year old subjects with severe hemophilia A. Thus 16 will be the cutoff age for enrollment in this study.

Consent Process

All participants will be both verbally instructed and provided with a written description of how the data will be collected, stored, used for future study, and how their privacy and confidentiality with be protected by de-indentifying the data. If consent is obtained, we will ask the subjects to have at least a 72-hour washout.

Methods

Inclusion criteria:

1. Males with a diagnosis of hemophilia A with a historic baseline FVIII level $\leq$ 2%.
2. Age $\geq$ 16 years old
3. Currently using any FVIII replacement therapy

Exclusion criteria:

1. Subject or guardian is unwilling or unable to give written informed consent and/or assent
2. Joint or muscle bleeding within 2 weeks of Study Day 1
3. Presence of a current factor inhibitor (>0.6 BU/mL via Nijmegan-modified Bethesda assay)
4. Known collagen vascular bone disease.
5. Current acute infection, per investigator discretion

Subjects will be withdrawn from the study if a bleeding event occurs during the 5-day study visit period.

Procedures

The study will take place over two weeks and each subject will return daily to OHSU to complete study Visits 2-6. The study staff will call the subjects the day before study Visit 2 to confirm eligibility. If subject has a current acute infection or has had a bleed in the last two weeks.

Blood draws

As outlined in Table 2, time points for blood draws include: time 0, 0.5, 4, 24, 48, 48.5, 72, and 96 hours. At each time point two tubes of citrated blood will be drawn. One tube will be sent to the clinical laboratory to determine FVIII levels. The second tube will be centrifuged to obtain platelet poor plasma, divided into 400 μl aliquots and placed into a -80 degree freezer within 30 minutes of the blood draw. A third tube will be drawn at time 0 to measure Vitamin D and C-reactive protein. FVIII inhibitor assay, von Willebrand Factor (vWF) antigen, and vWF activity will also be performed at the time 0 timepoint.

Bone biomarker assays to be run at each timepoint include: OPG and RANKL (see Figure 1); CTX-1 and TRAP 5b (markers of bone resorption); osteocalcin and bone-specific alkaline phosphatase (markers of bone formation); and cytokine multiplex (see Table 2 and Preliminary Data).

Factor infusion

ADVATE [Antihemophilic Factor (Recombinant), Plasma/Albumin-Free Method] is an antihemophilic factor approved by the Food & Drug Administration for control and prevention of bleeding episodes in adults and children with Hemophilia A. Subjects who are currently using any factor VIII replacement therapy will be eligible for enrollment in this study. Subjects will receive ADVATE on Day 1 Hour 0 at a dose of 50 units/kg replacement. Subjects will subsequently receive ADVATE on Day 3 Hour 0 at a dose of 20 units/kg factor replacement. The participants in this study are have all seen exogenous factor VIII previously and data demonstrates that in this population the risk of these serious side effects, especially in a short term study is <1%."

Two separate factor dosing levels will be used to help determine dose response of biomarkers to factor replacement and assess the level of factor replacement needed for normalization of biomarkers

Subject Questionnaires

On Day 1 subjects will answer questionnaires to obtain medications, past medical history and a complete bleeding history. Addition questionnaires will be given on quality of life and factor compliance. For visits 3-6, the subjects will be given a short daily questionnaire asking about recent bleeding, changes in health and physical activity. [Appendix A-G]

List of questionnaires:

- Quality of life (Haemo-QoL)
- Hemophilia Activities List (HAL)
- Bleeding history
- Factor compliance (VERITAS-PRN)
- Daily health and pain assessment (EQ-5D-3L)
- Daily protocol specific questionnaires (Day 1 and Day 2-5)

Physical examination

One study Visit 2, subjects will have a physical exam by a physician. A joint assessment by a physical therapist using the Hemophilia Joint Health evaluation (Version 2.1) will be completed within one week of Visit 2. The joint assessment score will be correlated with health history, BMD and bone biomarkers. [Appendix H]

Gait Map Assessment

Subjects will undergo a gait map evaluation for gait abnormality within 1 week of Study Day 1. Subjects will be asked to walk the full length of the mat 4 to 12 times, at least 2 twice with shoes on and at least 2 twice with shoes off. This assessment will help identify abnormalities that may impact the BMD and physical activity.

Dual-energy X-ray Absorptiometry (DEXA)

Two DEXA scans will be performed on each subject within 1 week of the Study Day 1. DEXA scans are being performed in order to correlate BMD with the bone biomarkers. The imaging will be done on the spine and hip.

Table 2: Study Procedure Chart

	Consenting Visit	Visit 2 Day 1			Visit 3 Day 2	Visit 4 Day 3		Visit 5 Day 4	Visit 6 Day 5
		0 hours	30 min	4 hours	24 hours	48 hours	48.5 hours	72 hours	96 hours
Informed consent review	x								
Physical Exam		x							
Weight	x	x							
Factor Replacement		50 units/kg				20 units/kg			
Biomarkers		x	x	x	x	x	x	x	x
Factor Level		x	x	x	x	x	x	x	x
vWF antigen and activity		x							
Gait Map		Must be completed within one week of Day 1							
DEXA scans		Must be completed within one week of Day 1							
Questionnaires		x			x	x		x	x

Compensation

Subjects will be compensated $500 to complete the entire 5study visit protocol, DEXA scans and gait mapping.

Institutional approval

This protocol will obtain full institutional approval, including the Institutional Review Board, prior to initiation of the study.

Data Analysis and Evaluation
A statistician will evaluate the data using multivariable analysis to compare baseline bone biomarkers with those following FVIII infusion as well as to patient history, joint health and bone mineral density. For instance, repeated measures ANOVA and survival analysis will test for differences in biomarker levels in response to factor replacement therapy.

RedCap
A portion of the data for this project will be stored in OCTRI's installation of REDCap, a highly secure and robust web-based research data collection and management system.

Features of REDCap that protect participants' privacy and data security include:

- Physical Security: OCTRI's REDCap software is housed on servers located in ITG's Advanced Computing Center providing locked physical security.
- Electronic Security: The REDCap servers are housed behind both the OHSU firewall and a second ACC firewall. All web-based data transmissions are encrypted with industry-standard SSL methods.
- Controlled User Access: REDCap is employs a robust multi-level security system that enables researchers to easily implement "minimum necessary" data access for their research staff, including specification of data fields that are identifiers. This feature includes “single click” ability to provide completely deidentified (removing all identified data fields and shifting dates) for analysis or other purposes. User activities are logged to enable auditing of all data access. Access is integrated with OHSU's network such that users who are also OHSU employees are authenticated against their OHSU network credentials.
- Data Integrity: REDCap is jointly managed in accordance with OHSU Information Security Directives by ACC staff and members of OCTRI's Biomedical Informatics Program, ensuring fidelity of database configuration and back-ups. User activities are logged to enable auditing of all data changes.

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Appendix A: Quality of life questionnaire (Haemo-QoL)

Appendix B: Hemophilia Activities List (HAL)

Appendix C: Bleeding history

Appendix D: Factor compliance questionnaire (VERITAS-PRN)

Appendix E: Daily health and pain questionnaire (EQ-5D-3L)

Appendix F: Day 1 Questionnaire

Appendix G: Days 2-5 Questionnaires

Appendix H: Hemophilia Joint Health Assessment