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| 14. ABSTRACT<br>Anterolateral tibial bowing is a morbid skeletal manifestation observed in 5% of children with neurofibromatosis type 1 (NF1), typically identified in infancy. The majority of NF1 individuals with tibial bowing will sustain a fracture that will not heal (i.e. pseudarthrosis) resulting in multiple surgeries, poor limb function, and amputation. Some NF1 individuals with tibial bowing, however, do not fracture and the bowing improves over time. Clinical predictors to help drive management are lacking, and the pathophysiology of tibial bowing and pseudarthrosis is not well understood. Our objective is to identify clinical predictors of tibial pseudarthrosis and better understand its pathophysiology. We have begun recruitment and assessed many individuals with NF1 with and with tibial bowing. QUS measurements and osteolytic activities have been assessed in the individuals recruited to date, but numbers are small and hence statistically significant conclusions cannot be made. However, 7/8 individuals with tibial bowing had decreases in speed-of-sound z-scores in the bowed tibia compared to the affected tibia. In addition, we were able to confirm that bone resorption is increased in NF1. |             |                          |                            |                                                  |                                           |
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# **Tibial Bowing and Pseudarthrosis in Neurofibromatosis Type 1**

**(PI: David Stevenson, MD)**

## **Introduction**

Anterolateral tibial bowing is a morbid skeletal manifestation observed in 5% of children with neurofibromatosis type 1 (NF1), typically identified in infancy (Friedman and Birch, 1997). The majority of NF1 individuals with tibial bowing will sustain a fracture that will not heal (i.e. pseudarthrosis) resulting in multiple surgeries, poor limb function, and amputation. Some NF1 individuals with tibial bowing, however, will not fracture and the bowing improves over time (Stevenson et al., 2009). Clinical predictors to help drive management are lacking, and the pathophysiology of tibial bowing and pseudarthrosis is not well understood. Our objective is to identify clinical predictors of tibial pseudarthrosis and better understand its pathophysiology. Our integrative proposal will gain novel information about the pathophysiology of tibial bowing and pseudarthrosis using techniques innovative in their application to NF1 tibial dysplasia. As part of the study we will validate use of an imaging modality for tibial bowing for clinical trials and clinical practice. We will also help in the understanding of osteoclast function in tibial bowing. Additionally we will provide novel information on genetic modifiers and pathophysiology of the skeletal phenotype of NF1. Ultimately, our proposal will help in the development of personalized treatment protocols based on an NF1 individual's quantitative ultrasound measurements, osteolytic activity, and somatic mutation profile.

## **Body**

The following section will describe the research accomplishments associated with each task outlined in the approved Statement of Work in which the time frame of this first annual report pertains. Tasks in the approved Statement of Work that fall outside the time of the first 24 months are not included. The individual tasks are underlined below followed by a description of accomplishments related to the task.

### **Task 1. Plan Development, Patient Recruitment, and Institutional Review (Months 0-6):**

- a. Train a clinical coordinator to identify potential subjects and contact appropriate providers to offer enrollment.

-We have trained Heather Hanson as a clinical coordinator on the project. Heather has met personally with the investigators to discuss the project and critical areas including timing of shipment of blood, consenting, number of participants needed and enrollment criteria.

The clinical coordinator has investigated and made contact with organizations that are likely to have contact with individuals who have neurofibromatosis type 1 and tibial bowing prior to fracture. With her help we have sent out recruitment flyers to NF support groups and orthopedic agencies.

- b. Review current research registries to identify and prioritize individuals for recruitment with primary focus in first 6 months on recruitment of individuals with tibial bowing.

-We have an NF Registry in which individuals with NF1 have been recruited and consented to be contacted for future studies. As part of the NF Registry clinical information is contained in our database. We have searched our NF registries for individuals with long bone bowing and NF1 individuals who could serve as controls who have agreed to be contacted for future research. We have identified these individuals who are potential study participants for future contact. We have focused on those with tibial bowing primarily.

During this time period we recruited individuals coming to the University of Utah for clinical or research purposes for convenience to the family.

- c. Arrange requests, procedures and transfer of prospectively acquired tissue from the NF1 Orthopedic Core Facility (NOCF) for analysis for Specific Aim 3.

-We have received samples from the NOCF that were located at the Shrine Hospital in SLC and the samples are now at the University of Utah.

- d. Assure compliance with USAMRMC and home institutional guidelines on research involving human subjects.

-This has been done.

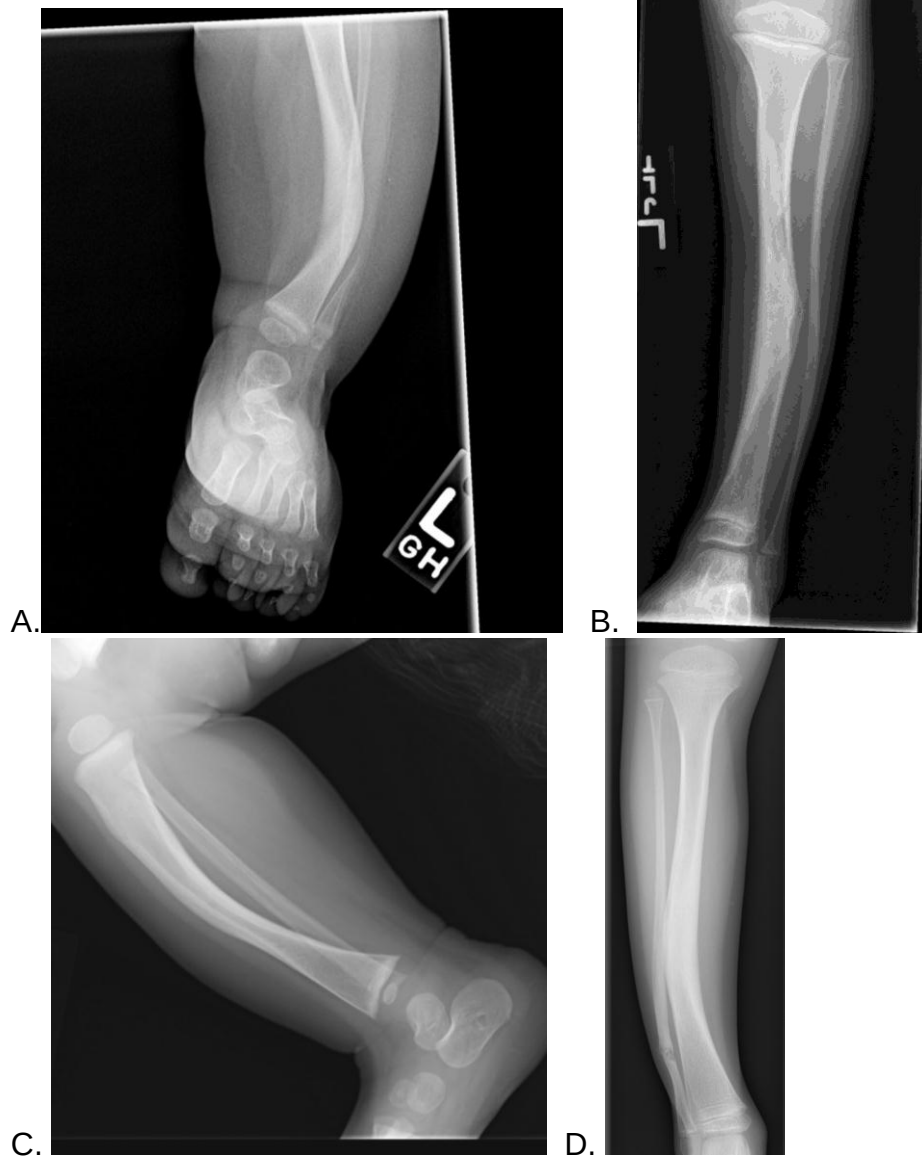
## Task 2. Data Collection, QUS Imaging, and Molecular Analysis (Months 6-42):

- a. Continue to recruit subjects for all specific aims. A projected 150 individuals with NF1 will be recruited over the course of the 3-year period (35 individuals for Specific Aim 1).

-We have enrolled 14 individuals with NF1 with tibial bowing. We have 3 additional individuals with tibial bowing who are scheduled to fly out to be evaluated but we have not yet evaluated them. We have enrolled 83 individuals with NF1 without tibial bowing to function as controls. To help in recruitment primarily of NF1 individuals with bowing, Dr. Stevenson traveled to the Children's Tumor Foundation NF Forum for advertisement to the US clinic coordinators and chapter leaders and is working with the CTF to highlight the study on their website.

Examples of the radiographs of a few of the NF1 individuals with tibia bowing who have enrolled are shown in **Fig.1** and document the various anterolateral bowing that is typically seen in individuals with NF1. However, the radiographs also show that there is variability in the radiographic features of each individual with NF1 in terms of the tibial structure. This suggests that

not all individuals with NF1 who have tibial dysplasia will have the same bone architecture and may result in varying clinical outcomes.





**Figure 1.** Examples of radiographs (A-D) of the bowed leg of different individuals with NF1 with tibial dysplasia who have enrolled.

- b. Document findings from physical examinations and medical histories on NF1 exam forms for data entry upon enrollment.

-All individuals were personally examined by Dr. Stevenson and the subjects filled out releases of information to obtain radiographs and medical reports to ensure appropriate diagnosis and categorization. Findings were documented through standardized exam forms and entered into spreadsheets by the research coordinator.

- c. Biannual phone interviews with individuals with tibial bowing enrolled in Specific Aim 1.

-We have performed biannual phone interviews for individuals with tibial bowing who have reached their required time for phone interviews. In addition we have informed all subjects and their families to contact us for any fracture or surgical intervention.

To date, none of the individuals have sustained a tibial fracture. However, one individual elected to undergo surgical procedures to correct the bowing prior to fracture.

- d. Obtain QUS at baseline on all NF1 individuals with anterolateral tibial bowing (Specific Aim 1; N=35).

-We have obtained quantitative ultrasound measurements on both legs of individuals with tibial bowing of those who have enrolled. Decreased z-scores for speed of sound as measured by the quantitative ultrasound machine were observed in the affected tibia in 12/14 participants (see results in **Table 1**).

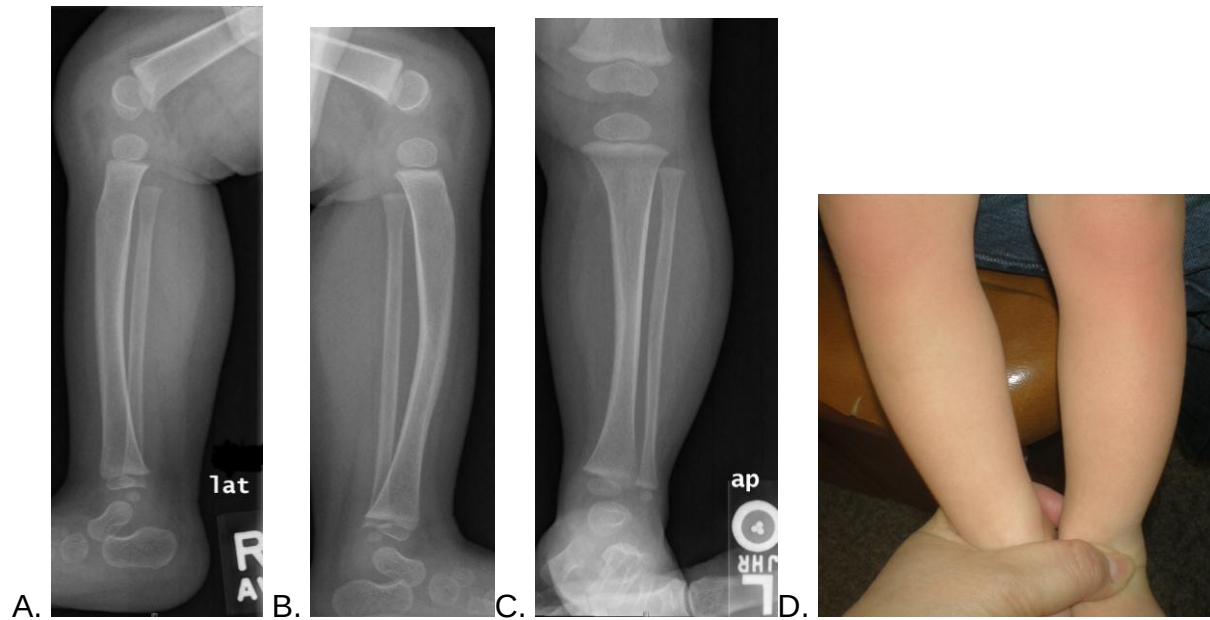
Since none of the individuals have yet fractured, we are unable to determine if the degree of difference in the speed of sound z-scores as measured by quantitative ultrasound between the bowed and non-bowed tibia can help predict who will fracture. We will continue to follow these individuals with our biannual phone interviews to document clinical progression to fracture or continuation of an intact tibia.

**Table 1. Mean Z-scores of Speed of Sound from Quantitative Ultrasound of Bowed and Non-bowed Tibia in NF1 Individuals**

|                 | Tibia Affected | Z-score Right Tibia | Z-score Left Tibia | Difference between bowed and non-bowed tibia |
|-----------------|----------------|---------------------|--------------------|----------------------------------------------|
| Participant #1  | Left           | -0.7                | -1.0               | -0.3                                         |
| Participant #2  | Left           | -3.3                | -2.4               | +0.9                                         |
| Participant #3  | Left           | +1.3                | -1.0               | -2.3                                         |
| Participant #4  | Right          | -3.7                | -0.7               | -3.0                                         |
| Participant #5  | Right          | -0.5                | +0.3               | -0.7                                         |
| Participant #6  | Right          | -4.2                | -1.7               | -2.5                                         |
| Participant #7  | Left           | -0.3                | -1.0               | -0.7                                         |
| Participant #8  | Left           | -0.3                | -3.9               | -3.6                                         |
| Participant #9  | Right          | -7.5                | +0.5               | -8.0                                         |
| Participant #10 | Right          | -4.5                | -0.7               | -3.2                                         |
| Participant #11 | Right          | -3.2                | -2.4               | -0.8                                         |
| Participant #12 | Left           | +3.2                | -5.2               | -8.4                                         |
| Participant #13 | Left           | 0                   | +2.6               | +2.6*                                        |
| Participant #14 | Right          | -5.2                | -0.2               | -5.0                                         |

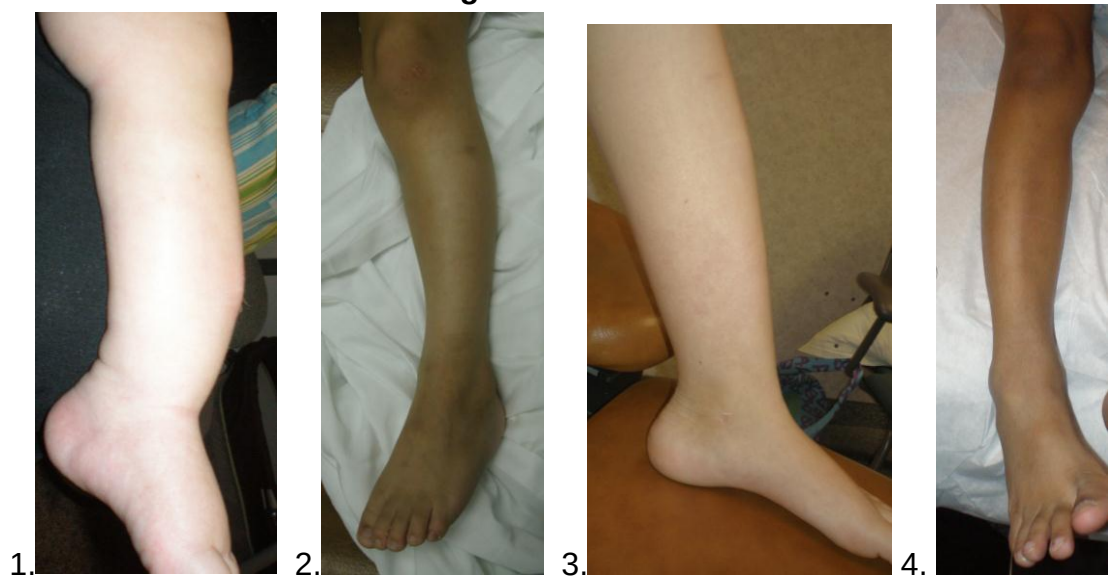
\*This patient had very minimal anterolateral bowing without radiographic findings of tibial dysplasia (ie. cortical thickening and medullary canal narrowing) – see **Figure 2** and although the patient was included in the study, we think given the young age of the child that this is probably physiologic bowing of infancy and hence an indicator that bone ultrasound is helpful in differentiating pathologic tibial bowing vs. physiologic bowing. Longitudinal follow-up will help clarify this question.





**Fig. 2.** Radiograph of Participant #13 with minimal anterior bowing on left (B,C) compared to right (A), and no significant lateral bowing without cortical thickening or medullary canal narrowing. This was very difficult to discern on examination (D).

Examples of photos of the bowed tibia of each NF1 individual with tibial bowing that have been enrolled are shown below in **Fig.3.**



5.



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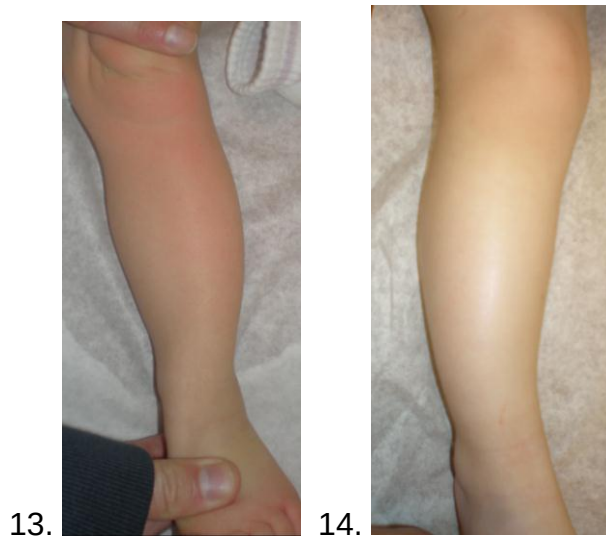


11.



12.





**Figure 3.** Photographs of the bowed leg of the 14 individuals. Participants #1-14 with NF1 with tibial dysplasia.

- e. Obtain urine samples for urinary crosslink measurements to be performed at the University of Utah (Specific Aim 2; N=150).

-We have obtained urine samples from NF1 individuals who have enrolled and the urine was frozen prior to being sent for analysis. All urine samples have been sent to Dr. Pasquali's laboratory for pyridinium crosslink analysis. Pyridinium crosslinks are currently being run in a stepwise approach in batches starting first with individuals with tibial bowing. The first batch has been processed and consists of 12 NF1 individuals with tibial bowing (Table 2). The average DPD/PYD ratio was 0.31 which is well above the mean that we have previously reported in children without NF1 (i.e. approximately 0.22) (Stevenson et al. 2010), but we are awaiting results of the individuals without bowing for comparison.

**Table 2. Urine Pyridinium Crosslink Measurements in individuals with Tibial Bowing.**

| NF1 individuals with Tibial Bowing | Pyridinoline (PYD)<br>umol/mol creatinine | Deoxy-pyridinoline (DPD)<br>umol/mol creatinine | DPD/PYD Ratio |
|------------------------------------|-------------------------------------------|-------------------------------------------------|---------------|
| NF1-07                             | 408                                       | 141                                             | 0.35          |
| NF1-211                            | 524                                       | 150                                             | 0.29          |
| NF1-217                            | 127                                       | 33                                              | 0.26          |
| NF1-223                            | 250                                       | 65                                              | 0.26          |
| NF1-225                            | 211                                       | 54                                              | 0.26          |
| NF1-229                            | 508                                       | 128                                             | 0.25          |
| NF1-231                            | 290                                       | 93                                              | 0.32          |

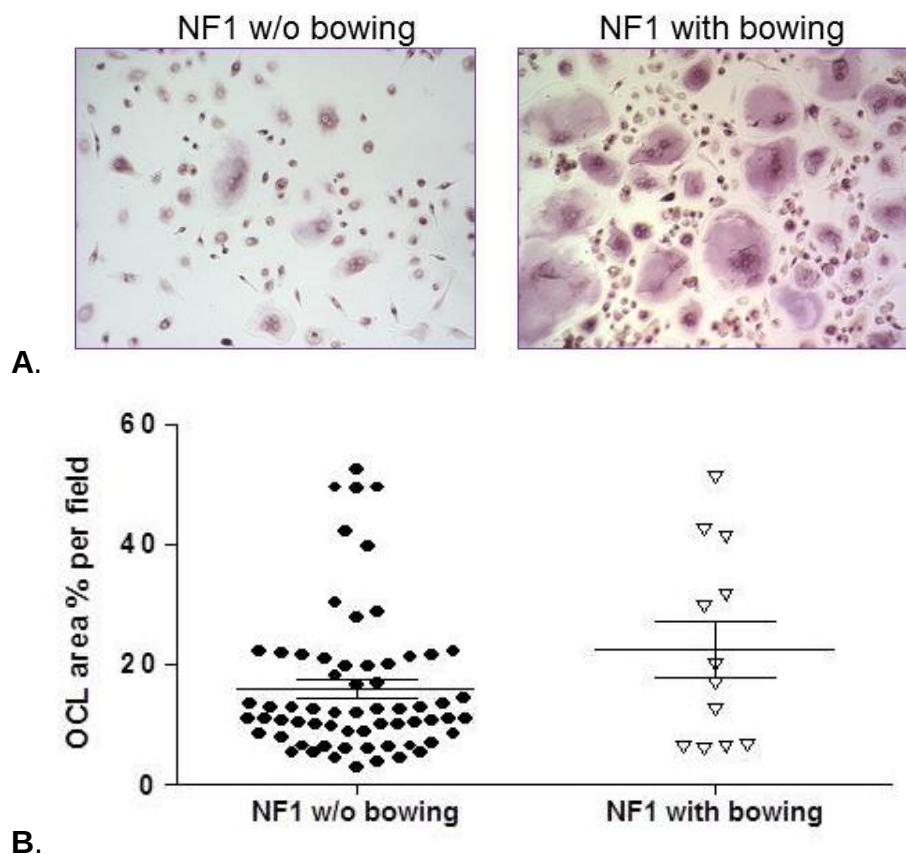
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| NF1-232 | 290 | 90  | 0.32 |
| NF1-243 | 412 | 147 | 0.36 |
| NF1-246 | 357 | 107 | 0.30 |
| NF1-254 | 358 | 147 | 0.41 |
| NF1-257 | 507 | 174 | 0.35 |

\*Measurements are average of two consecutive first morning voids.

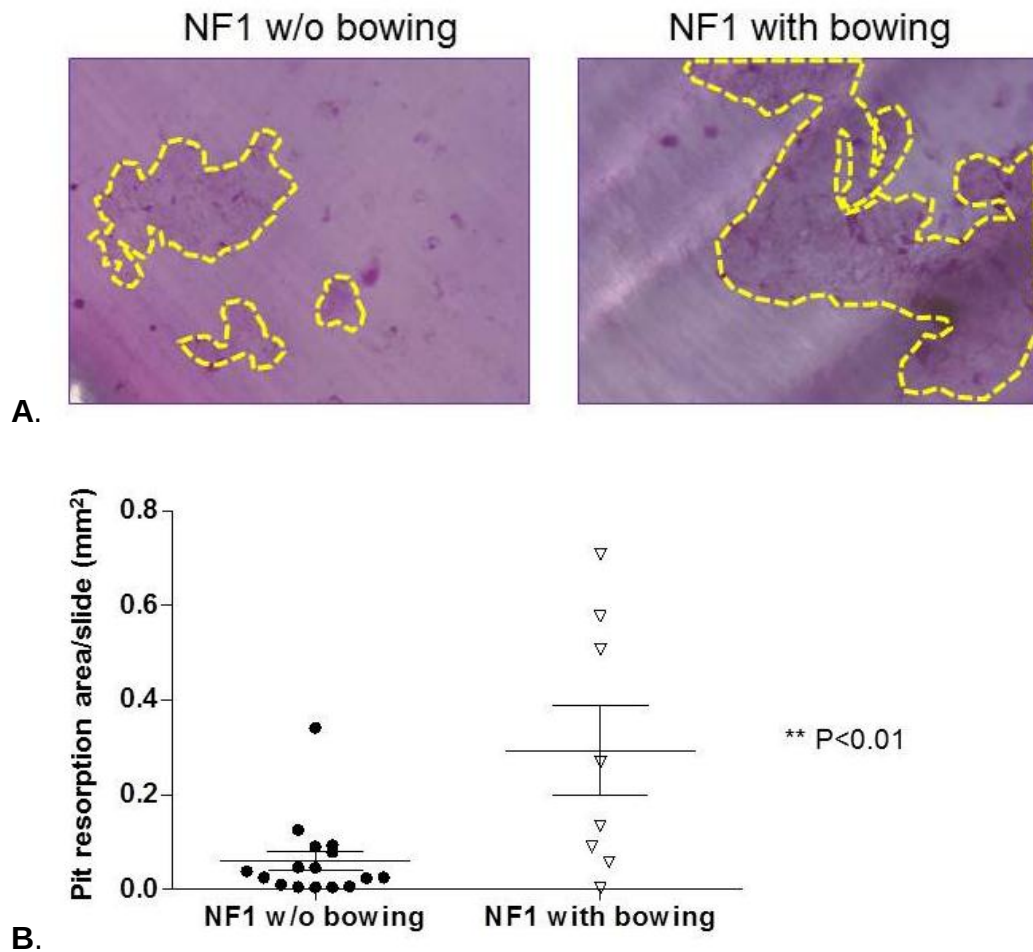
- f. Obtain blood samples for pit resorption assays to be shipped and performed at Indiana University (Specific Aim 2; N=150).

-Blood samples for pit resorption assays have been obtained on individuals with tibial bowing and NF1 individuals without tibial bowing. Samples are shipped via FedEx to Dr. Yang at Indiana University.

In **Figures 4 and 5** we show data that osteoclast activity is increased in NF1 as we have previously described (Stevenson et al., 2011), and that there is a trend toward increased osteoclast formation and pit resorption in individuals with NF1 with bowing compared to NF1 individuals without bowing.



**Figure 4:** **A.** Example of osteoclast (OCL) formation in an individual with NF1 with bowing and an NF1 individual without bowing in which there appears to be increased percent of osteoclast area per field. **B.** There is a slight increase in osteoclast area % per field in individuals with NF1 with bowing but has not reached statistical significance.



**Figure 5: A.** Example of resorption area (outlined in dotted yellow line) of an individual with NF1 with bowing and an individual with NF1 without bowing. **B.** Percent of pit resorption area per low power field is increased in the NF1 individuals with bowing compared to NF1 individuals without bowing.

- g. Perform genetic analyses (next-generation sequencing and confirmation Sanger sequencing) and histologic evaluations on osseous tissue specimens obtained at the University of Utah. These analyses will take place with prioritized fashion with first analyses on prospectively acquired tissue and subsequently analyze archived tissues beginning in the second year of the proposal (Specific Aim 3).

-We have continued to collect tissue samples from individuals with tibial pseudarthrosis. None of the individuals with tibial bowing have proceeded to fracture and have non-union and hence we do not have bone tissue from any of these individuals yet. We have extracted DNA from peripheral blood of all individuals with tibial bowing and wait to see if they will fracture. Whole



genome amplification has been performed on the DNA extracted from the pseudarthrosis tissue in two individuals. Given the advances in next generation sequencing we have elected to utilize exome sequencing for our analyses. As mentioned in the task above these analyses will take place in a prioritized fashion given the cost of next generation sequencing which is even more important now that we are utilizing exome sequencing and will be performed primarily in the third year.

Work in progress [DNA extraction from bone and whole genome amplification (WGA)]: DNA extraction from bone is challenging. There are three key facts which affect the yield of the DNA extraction; 1) There are low cell numbers (e.g. osteocytes) in bone, 2) release of DNA from osteocytes nucleus is difficult, 3) calcium precipitation. A Qiagene kit was used for DNA extraction from bone tissue. Figure 6 is gel picture for the DNA extractions from four different pieces of the bone from tibial dysplasia. 10 ug of bone has been used for each extraction. The yield of DNA is between 50ng and 1000ng/ per sample. Due to the low yield of DNA whole genome amplification was pursued to meet the requirement for exome sequencing ( 200 ug of DNA required from Illumina TrueSeq Exome capture, 1.5ug of DNA for Nimblegen Exome and 3ug of DNA for Agilent Exome capture). Whole genome amplification has been performed on some of the samples with low yield of DNA using GenomePlex® ( Sigma-Aldrich Co.). Please see the gel pic show below showing post amplification. The first WGA yielded 981.3ng/ul in 100 ul of DNA and the second WGA (lane 2) yielded 983.7 ng/ul in 100 ul of DNA, see Figure 7.

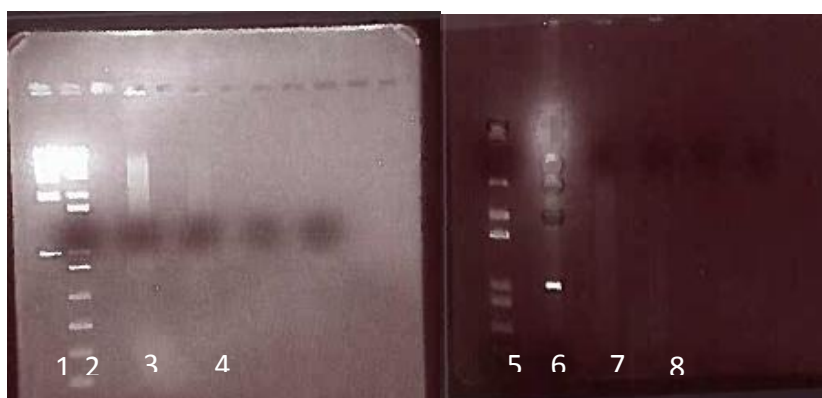


Figure 6. DNA extractions from four pieces of bone tissue from a patient. Lane 1,2,5,6 are the molecular weight markers. Lane 3,4,7,8 are DNA from different extractions.

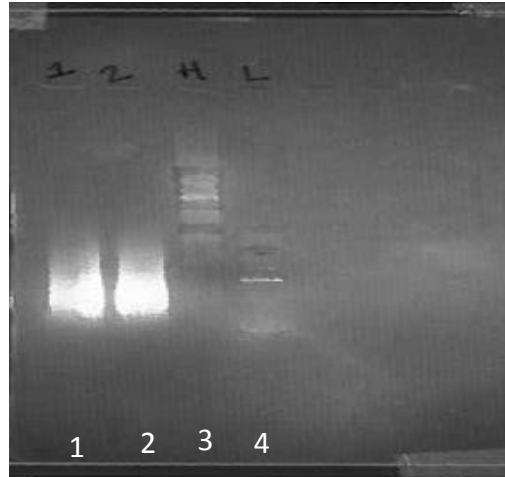


Figure 7. Lane 1 and 2 are whole genome amplifications from the same sample. Lane 3 is high molecular weight marker, Lane 4 is 50bp molecular weight marker.

Work in progress for exome sequencing: Our new approach is to use exome sequencing to exam the entire coding region of the genome. A tier analysis has been designed to first analyze the 16 genes in RAS/MAPK Pathway as initially proposed, although exome sequencing will now allow for a much broader approach to look at a multitude of other genes that can be identified on exome. Using a variant analysis for the whole blood sample compared to bone tissue, the mutations in only bone tissue are considered as secondary events and likely play a role as genetic modifiers of pseudarthrosis. As proof of principal, exome sequencing was performed on seven samples. Of those, two samples are paired blood and bone extraction, two samples are paired of blood and whole genome amplification and three samples are known germline NF1 mutations. Illumina TruSeq Exome Enrichment kit is an in-solution sequence capture method for isolating regions of 62Mb of exomic sequences, 20,000-25,000 gene coding regions, exon/intron boundaries and 5'- and 3'-UTRs. The capture exome DNAs were further processed with Illumina HiSeq2000 100X2 paired end sequencing following manufacture protocol. With the first experiment with the Illumina TrueSeq Exome capture, the hybridization temperature did not hold steady through the 16 hours of incubation. The yield of the captured DNA was low. Therefore, the sequence obtained from Illumina HiSeq was low. The 16 RAS pathway genes of interest are covered with a mean coverage of 5-10X. The known mutations in the NF1 gene were detected in one sample and missed by the other one due to low coverage for the mutation region (2X). The paired blood and bone tissue samples showed a mean coverage of 5-10x for the 16 gene panel. There were no mutations in the 16 gene panel found to date. In addition, variants in *NF1*, *SPRED1*, *SPRED2* and *TP53* were consistently seen on both specimens. A secondary variant in *SOS1*, c.3812G>T, p.R1271M was seen only in bone tissue, but not blood. These set of experiments showed exome

sequencing was suitable for the tier of the 16 RAS gene panel using whole exome sequencing.

h. Interim analyses and manuscripts.

-Interim analyses are described above respectively for each aspect of the study. To date we do not have enough data to generate a manuscript.

i. Annual reports will be written.

-This has been performed herein.

## **Key Research Accomplishments**

- Enrollment of **97** individuals with NF1.
- Fourteen individuals with NF1 with tibial bowing without fracture have been recruited and medical histories and examinations documented and followed prospectively. We have identified an additional 3 individuals with tibial bowing who are scheduled to be evaluated
- Quantitative ultrasound measurements show decreases in the speed of sound of the affected leg compared to the unaffected leg in 12/14 individuals. This is a key finding as it will allow for future surrogate marker for clinical trials focused on therapeutics to improve bone quality prior to fracture in a non-invasive and age specific manner
- DNA extraction from peripheral blood for somatic mutation comparison in tibial tissue.
- Whole genome amplification of pseudarthrosis tissue
- Confirmation of increased bone resorption in NF1.

## **Reportable Outcomes**

Given that this proposal is primarily prospective in which we are following NF1 individuals with tibial bowing over time to see who will fracture, reportable outcomes and research accomplishments will be limited in the first two years of the study. Hence in this annual report, outcomes are minimal.

The following abstracts and presentations were given in which aspects of the current study supported some of the rationale for discussion:

Stevenson DA, Allen S, Tidyman WE, Carey JC, Viskochil DH, Stevens A, Hanson H, Sheng X, Thompson GA, Okumura M, Reinker K, Johnson B, Rauen KA. Peripheral muscle weakness in RASopathies. Oral presentation



at the Western Society for Pediatric Research, Carmel, California, January, 2012.

*Bone Health in NF1.* Invited speaker at the Children's Tumor Foundation NF Forum. New Orleans, Louisiana (June, 2012)

*Physical Fitness and Muscle in NF1.* Invited speaker at the Children's Tumor Foundation NF Forum. Nashville, TN, April, 2013.

*Comparison of the Musculoskeletal Findings in RASopathies.* Invited speaker at the Bone Series, Vanderbilt University, Nashville, TN, April, 2013.

## **Conclusion**

Our integrative proposal will gain novel information about the pathophysiology of tibial bowing and pseudarthrosis. At this point in time we are currently still in the phase of collecting data on individuals with tibial bowing and following them over the course of the study to see if quantitative ultrasound measurements, and osteolytic activity from cultured osteoclasts and urine crosslinks can be used as a predictor of fracture. Our data to date suggest that the bowed tibia has increased porosity based on the decrease in speed of sound z-scores in the affected limb. Ultimately, our proposal will help in the development of personalized treatment protocols based on an NF1 individual's QUS measurements, osteolytic activity, and somatic mutation profile.

## **References**

1. Friedman JM, Birch PH. Type 1 Neurofibromatosis: A descriptive analysis of the disorder in 1728 patients. *Am J Med Genet* 1997;70:138-143.
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## **Appendices**

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