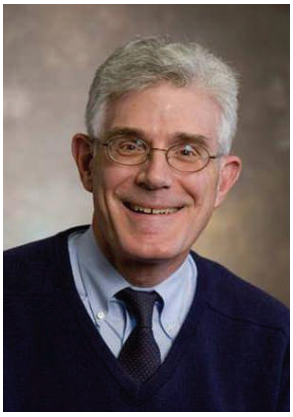
Volume 3 Issue 1

April 2010

YC-XLH News

Newsletter of the Yale Center for X-linked Hypophosphatemia http://www.ycxlh.yale.edu/

LOOKING BACK AT 2009 . . .



Since last year's newsletter, a wide range of YC-XLH activities have progressed or begun, and Dr. Karl Insogna and I are pleased to apprise you of these efforts. The year marked the substantial advancement of the three major research projects (described in pages 5-7), and we are now into the second year of funding for the three pilot and feasibility studies that were begun in September of 2008 (p. 8-10).

The Administrative Core has been particularly busy in its promotion of several outreach projects (p. 2-3), as well as coordinating our successful 2009 Advisory Board Meeting in October and maintaining the YC-XLH website. We have been particularly fortunate to have Ms. Teri Tuma join us as an interim Administrative Coordinator during Danielle Franks' extended leave of absence. Congratulations to Ms. Frank and her husband as they welcome their healthy new twins, Delaney and Briley.

The Research Core, under the direction of Dr. Caren Gundberg, has managed a high volume of samples (>5000) for assay in both human and animal studies. Histologic procedures and histomorphometric analysis have also been a major activity of the Core in the past year.

Finally, we have enhanced our clinical operation with the addition of dentistry consultation, and the expansion of our Metabolic Bone Clinic.

Sincerely,

Thomas O. Carpenter, MD

Thomas O. Carpenter, MD

Who we are:

Thomas Carpenter, MD	Director and Principle Investigator, Project 1
Karl Insogna, MD	Associate Director
Marie Demay, MD	Principle Investigator, Project 2
Joseph Schlessinger, PhD	Principle Investigator, Project 3
Elizabeth Olear, MS, MA	Clinical Research Coordinator
Teri Tuma, BA	Interim Administrative Coordinator

YC XLH Rickets Research

Inside this issue:

Center Outreach

A directed goal of the YC-XLH has been to promote an extended clinical and scientific interest in XLH and in phosphate metabolism in general. These activities have been at the forefront of our mission in 2009 and have taken form in a variety of ways.

Joint sponsorship of Bone Biology Seminars with the Yale Core Center for Musculoskeletal Disorders

Since our last update we have had several nationally known speakers present informative seminars in this venue, including:

- Dr. John Adams, UCLA,, *"Vitamin D and estrogen action in bone: a concerted role for hnRNPs in the C family" (*3/19/09)
- Dr. Michael Levine, Children's Hospital of Philadelphia, *"Gcm2: master gene for parathyroid development" (*4/16/09)
- Dr. José Millán, Burnham Institute for Medical Research, La Jolla, CA, *"The role of phosphatases in skeletal calcification" (*10/15/09)
- Dr. Tom Clemens, Johns Hopkins, *"Insulin signaling in bone regulates bone and body composition"* (12/17/09)

Bone Biology Seminars are generally held on the third Thursday of each month at 11am, at the Boyer Center for Molecular Medicine on the Yale Medical School campus. The schedule is posted on the YCCMD website: http://info.med.yale.edu/intmed/yccmd/.

Fostering the YC-XLH as an institutional model for translational research

Central to the mission of YC-XLH, as an NIH-sponsored Center of Research Translation (CORT), is the promotion of ongoing dialog between basic and clinical researchers and encouraging new investigators to examine phosphate problems. With respect to the latter, Drs. Raghbir Kaur and Suher Baker, members of the Department of Pediatric Dentistry at Yale-New Haven Hospital, have taken a direct interest in the dental aspects of the natural history of XLH and have submitted clinical research protocols that have begun to capture this history, together with dental imaging, in XLH subjects seen at the institution. Plans for interventional trials regarding prevention of dental abscesses are currently being discussed. The participation of pediatric dentistry and orthopedic specialists in related seminars and conferences has occurred, lending a new clinical dimension to many of the related discussions.

(continued on page 3)

Link for More:

YCCMD
http://info.med.yale.edu/intmed/yccmd/

Pediatric Dentistry, YNHH
http://www.ynhh.org/ynhch/pedi_dentistry.html

NIH-NAIMS
http://www.niams.nih.gov/

XLH Network, Inc.
http://xlhnetwork.org/

ASBMR
http://www.asbmr.org/

Center Outreach *(continued from page 2)*

Interactions with the XLH Network

Established in 1996, the XLH Network, Inc. is an international, all-volunteer organization dedicated to providing up-to-date information on the diagnosis and treatment of XLH and links to the latest research. The Network's web address is http://xlhnetwork.org.

Drs. Carpenter and Insogna, together with Ms. Elizabeth Olear, research associate and study coordinator of the Project 1 studies, regularly correspond with the XLH Network, providing regional physician referral information for patients and responding to queries regarding new developments in XLH research opportunities and clinical updates. At the request of the Network, a brief document providing treatment guidelines for XLH is in preparation, as well.

Multi-center Collaboration / Industry Partnerships

The Center has served as the lead site in a successful collaboration established with Indiana and Duke Universities investigating the first-in-humans trial of KRN 23, a potential new therapy for XLH. This project, under the sponsorship of Kyowa Hakko Kirin Pharma, has been active throughout the year. After considerable effort in protocol development, this Phase I study has progressed in a very timely manner. This past year we have also participated in the design aspects of a second Phase II study, which should begin in the near future.

Highlights from 2009 Meetings

American Society for Bone and Mineral Research (ASBMR)
September 11-15, 2009 Denver, CO

Preliminary data from Project 1, our observational study exploring parathyroid hormone and its significance in XLH was presented at the ASBMR this year (Carpenter TO, Insogna KI, Zhang JH, Ellis B, Simpson C, Olear E, Gundberg C. *Sustained treatment results in increased circulating FGF23, but not Klotho, in XLH.* Program Annual Meeting of the ASBMR, 2009).

Progress on Dr. Macica's pilot project describing the enthesis abnormalities in the Hyp mouse was also presented at the meeting (Macica C, Lam T, Wu T, Carpenter T, Liang G. *Mineralizing enthesopathy of Hyp mice, the murine model of X-linked hypophosphatemia: putative roles of elevated FGF-23 and cell-specific alkaline phosphatase activity.* Program Annual Meeting of the ASBMR, 2009).

(continued on page 4)

Highlights from 2009 Meetings *(continued from page 3)*

The YC-XLH Advisory Board Meeting
October 15, 2009 New Haven, CT

On October 15, 2009 the YC-XLH hosted its third annual Advisory Board meeting. Attendees included Dr. Joseph Craft, Dr. Francis Glorieux, Dr. Ralph Meyer, Mrs. Joan Reed, and Dr. Breanna Winger, who attended in lieu of her father, Dr. Larry Winger.

Dr. José Millán, our guest seminar speaker from the Burnham Institute in La Jolla, spoke on "The Role of Phosphatases in Skeletal Calcification." The Board reviewed the Administrative and Research Cores in a closed session and also reviewed, in detail, the progress of each of the funded projects and the pilot and feasibility program, with consideration for the plans of a competitive renewal application in the next year. The YC-XLH thanks the Board for its efforts in the conscientious oversight of our center.

YC-XLH Advisory Board members met with Center researchers at the Board's annual meeting on October 15, 2009.

From left to right: **Joseph Craft, MD**, Veraragavan Eswarakumar, PhD, Karl Insogna, MD, Breanna Winger, MBBS, (representing **Larry Winger, PhD**), **Ralph Meyer, PhD**, Carolyn Macica, PhD, Thomas Carpenter, MD, ***Joan Reed***, Marie Demay, MD, Caren Gundberg, PhD, Elizabeth Olear, MA, MS, **Francis Glorieux, MD, PhD**. (Advisory Board members in bold print.)

Update of Center Projects

Project 1: The role of parathyroid hormone in the pathogenesis of skeletal disease in XLH

This project, exploring the role of parathyroid hormone (PTH) in XLH, has continued with steady recruitment. The cross-sectional observational study is actively examining the potential correlation of disease severity with circulating levels of PTH, FGF-23, or phosphorus. Disease assessment quantifies skeletal symptomatology, height, fracture history, surgical interventions, and dental abscesses. These studies are beginning their third year; 47 affected subjects and 11 control subjects have been studied. Subjects spend one overnight session in our hospital research unit undergoing serial blood sampling and urine collections. A scintigraphic bone scan, a radiographic skeletal survey and an echocardiogram are performed. We expect to enroll 50 to 60 subjects affected with XLH, and 20 controls.

Our initial analysis of limited data has revealed that FGF23 levels in treated XLH patients are greater than in those who are untreated. Moreover, there appears to be a resistance to the normal suppressive effects of FGF23 on the parathyroid gland, which may account for the tendency of XLH patients to develop hyperparathyroidism. Data from this study remains blinded to the investigator until its completion. We thank all who have participated to date, and invite interested potential subjects to contact us regarding participation.

A second study assesses whether correction of secondary hyperparathyroidism is accompanied by reduction in severity of skeletal disease in XLH. This double-blind placebo-controlled study uses the vitamin D analog, paricalcitol, to suppress PTH levels in subjects with XLH who have elevated levels of circulating parathyroid hormone. Initial dose of this drug is 2 mcg per day, with upward titration based on success of suppression of PTH levels to a maximum of 4 mcg per day. We have recruited 22 affected subjects; 15 have completed the study and 7 are currently receiving the randomized therapy. We plan to study a total of 30 subjects in this interventional study.

To learn more about participating in our research, visit www.ycxlh.yale.edu/, send email to teri.tuma@yale.edu, or call (203) 785-2215.

Project 2: Phosphate, PTH and FGF23 as mediators of the rachitic growth plate

The goal of this project is to determine how low circulating phosphate levels lead to the development of rickets. Although vitamin D deficiency leads to rickets, our previous studies demonstrate that disruption of vitamin D activity does not lead to rickets when blood phosphate levels are normal. Mice which lack the kidney Npt2a phosphate transporter (and thereby have low blood phosphate levels) have rickets as young animals, however spontaneous resolution of the rickets occurs as the mice mature. Studies of mice with the combined absence of both the vitamin D receptor and the Npt2a transporter show that the actions of the vitamin D receptor can compensate somewhat for the low blood phosphate level. Similarly, mice lacking Npt2a

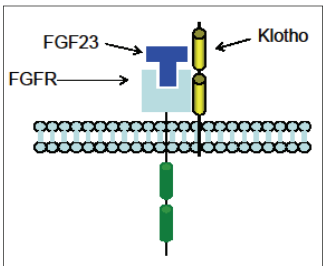
(continued on page 6)

Update of Center Projects *(continued from page 5)*

who are rendered vitamin D deficient are unable to maintain a normal growth plate phenotype when fed a diet that prevents the development of hyperparathyroidism. Thus, these studies point to a critical role of vitamin D in compensating for the growth plate consequences of low phosphate. Current investigations are directed at identifying the molecular mechanism by which this compensation occurs, using cultured embryonic bones and cartilage cells.

In parallel, we are examining cultured cartilage cells to determine how phosphate activates programmed cell death (apoptosis). These investigations point to an association of a decrease in mitochondrial membrane potential (MMP) and increased susceptibility to apoptosis during cartilage cells maturation, consistent with the notion that decreased MMP is a predisposing factor for programmed cell death. Furthermore, this decrease in MMP is further accentuated by exposure to phosphate. Activation of a specific signaling pathway which results in Erk 1/2 activation in mature growth plate chondrocytes is required for this programmed cell death. Inhibition of this pathway in growing mice leads to a phenotype reminiscent of rickets, and mice with low blood phosphate levels exhibit a decrease in Erk 1/2 activation in their growth plates. Thus, targeting this pathway may be a way to prevent or attenuate the development of rickets. If our studies demonstrate that activation of this pathway by hormones or other agents promotes chondrocyte apoptosis, and is essential for the prevention of rickets caused by low phosphate, then early treatment with such agents could help prevent the rickets observed in Hyp mice, an animal model of XLH.

Project 3: The structure, function and pharmacologic inhibition of FGF23



Project 3
J. Schlessinger, PhD and
V. Eswarakumar, PhD

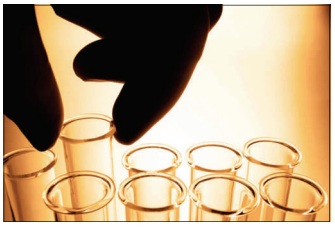
Klotho is a type I membrane protein highly expressed in the distal convoluted tubules of the kidney. Mice deficient in Klotho are hyperphosphatemic and hypercalcemic, however Klotho's role in regulating phosphate/calcium homeostasis is not fully understood. Strikingly, mice deficient for FGF23 or Klotho are phenotypically similar to each other (hyperphosphatemic and hypercalcemic) suggesting that these two molecules may regulate a common signaling pathway involved in mineral homeostasis. We have initially mapped the Klotho binding sites on the c-isoform of FGFR2 using a series of alanine scan mutational analyses. We have narrowed the Klotho interaction region of FGFR2 to three critical residues of the second half of the third Ig-like domain of c-isoforms. Since recent publications demonstrate a more direct role of FGFR1 in mediating Klotho-FGF23 signals in the kidney, we expanded our search for Klotho-interacting sites on the FGFR1c-isoform. For this purpose, we created a series of FGFR1 chimeric constructs in pCDNA3 expression vectors, where portions of FGFR1c-isoform-specific sequences in the second half of the third Ig-like domain were replaced with the corresponding FGFR1b-isoform sequences. We examined the ability of the chimeric protein to associate with Klotho as compared to both the wild-type c-, and b-isoforms, by transient co-transfection into Cos-7 cells, and subsequent immunoprecipitation with a cus-

(continued on page 7)

Update of Center Projects *(continued from page 6)*

tom antibody raised against the c-terminus of FGFR1, and immunoblotting with a custom-made anti-Klotho antibody. The results show that the introduction of the b-isoform-specific sequences located at F and G strands of the Ig-like domain 3 reduce the FGFR1c-isoform's ability to associate with Klotho.

Multiple isoforms of FGFRs are expressed in renal tubules. However, which of the FGFR isoforms Klotho converts in kidney to signal via FGF23 is not known. Further, Klotho is expressed as membrane-bound and soluble forms. The distinct function of these two forms is not known. To address these issues we are in the process of creating a genetic tool using one of two approaches to delete FGFRs, specifically in cells where Klotho is co-expressed, without affecting the expression of membrane-bound or soluble Klotho. Our first approach was to make a transgenic mouse that expresses Cre recombinase gene under control of the Klotho promoter. For this purpose we constructed a 17.2 kb transgene that has the Klotho promoter fused with the EGFPCre gene. We then performed pronuclear microinjection of (C57BL/6 X SJL/J) F2 mouse embryos. We had five germlines transmitting mice positive for the Cre gene, however, none of them expressed Cre recombinase. We have considered that the construct is too long for proper integration, or that it may lack certain enhancer binding elements critical for expression. To overcome these problems, we are now using bacterial artificial chromosome (BAC) engineering technology to express Cre under the endogenous promoter of the Klotho gene. The Klotho-Cre mice, once produced, will be used to inactivate one or more isoforms of FGFRs in the Klotho expressing cells of kidney to determine the *in vivo* mediator of FGF23-Klotho signaling.



The Research Core

The overall goal of the Research Core is to facilitate our basic and translational research projects by supporting the detailed assessment of musculoskeletal homeostasis. Specifically, the Research Core 1) provides histological preparation and analysis of mouse tissue for our basic research projects; 2) performs mutational analysis of the PHEX gene in patients with XLH followed in Project 1; and 3) measures markers of bone turnover, FGF23, and calciotropic hormones in human and mouse samples using purchased kits and in-house assays for clinical and basic YC-XLH projects. To date we have performed more than 3,500 biochemical measurements in human samples for Project 1, and 2,000 measurements in mouse samples for Projects 2 and 3. We have also processed 265 mouse bones for histology, histomorphometry, and special staining.

In the past year we have evaluated and optimized a new kit for the measurement of Klotho. We performed cross-sectional analysis of serum Klotho in 24 subjects by age and gender in both affected subjects and in controls. In longitudinal analysis, we identified a circadian variation in both subjects and controls. These findings were presented at the annual meeting of the American Society for Bone and Mineral Research in Denver in September 2009.

Pilot and Feasibility Program Update

The Yale Center for X-Linked Hypophosphatemia Pilot and Feasibility program entered its second year of activity in 2009. Based on successful progress in the initial year of funding, all three pilot projects were successfully funded for a second year after administrative review at the National Institutes of Health. The YC-XLH is delighted to have Drs. Eswarakumar, Macica and Shulman as pilot project awardees, and will be enthusiastically following the progress of their research over the next few years.

Veraragavan Eswarakumar, PhD

Yale School of Medicine, Department of Orthopaedics & Rehabilitation and Department of Pharmacy

Use of an animal model of Epidermal Nevus Syndrome to understand the mechanism of FGFR signaling in renal phosphate wasting syndromes

Epidermal Nevus Syndrome (ENS) is a congenital skin lesion disease that occurs in 1 in 1,000 people. Skeletal abnormalities are present in 15–70% of ENS patients, and many ENS patients have low serum phosphate levels due to renal wasting of inorganic phosphate (Pi). Recent studies indicate that 33% of ENS cases are caused by a mosaicism of an FGFR3 activating mutation at codon 248 (Arg248Cys) in the epidermis of human skin. When present in the germline in humans, this same mutation causes lethal thanatrophic dysplasia type I (TDI). These findings suggest that this FGFR3 mutation may cause the skeletal defects and phosphate wasting associated with ENS. Because ENS is due to mosaicism resulting from a postzygotic mutation of the FGFR3 gene, we hypothesized that cells other than keratinocytes harbor this mutation and thus contribute to the skeletal abnormalities and phosphate wasting observed in ENS patients. Our primary goal is to create an animal model for Epidermal Nevus Syndrome by introducing the human FGFR3-R248C equivalent mutation into the mouse germline and then to selectively activate the mutation in specific tissues such as skin, bone, and kidney to explore the mechanism of FGFR3 signaling in phosphate homeostasis. To this end, we have generated the knock-in targeting vector for the ENS causing FGFR3 activating mutation and subsequently produced targeted mouse embryonic stem cells by homologous recombination. These cells will be used to produce mutant mouse model for ENS syndrome. However, the mutation is kept in dormant stage by the insertion of stop signals flanked by two directly repeated *loxP* (locus of crossover [x] in P1) elements. Tissue specific Cre-mediated recombination will result in the permanent removal of the stop signals, leading to constitutive activation of the mutant receptor in the respective tissues such as osteoblasts, epidermis or renal tubules. We anticipate that these genetic experiments will lay the groundwork for identification of novel cellular signaling pathways activated by mutant FGFR3 that cause ENS and the associated phosphate wasting.

(continued on page 9)

Pilot and Feasibility Program Update *(continued from page 8)*

A study of enthesopathy in X-Linked Hypophosphatemia

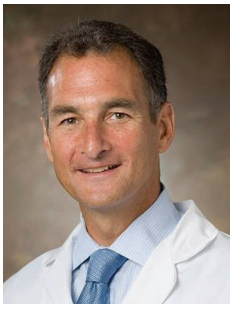
Carolyn Macica, PhD

Yale School of Medicine, Department of Internal Medicine, Endocrinology

This pilot is focused on a debilitating complication of XLH, in which paradoxical mineralization of tendon and ligament insertion sites occur (enthesopathy). Additionally, patients with XLH are afflicted with formation of osteophytes (lateral mineralized outgrowths of bone at the margin of the articular surface of synovial joints) which occur secondary to degenerative osteoarthritis (OA). Degenerative OA precedes osteophyte formation, and are thought to occur as a functional adaptation to joint damage. The evolution of degenerative OA and osteophyte formation occurs with high frequency in patients with XLH regardless of treatment for the underlying disease. These two complications dominate the clinical picture in adults and account for a great deal of the disease's morbidity in adulthood.

Our data, obtained from Hyp mice (murine XLH), show down-regulation of several articular cartilage extracellular matrix (ECM) proteins, including matrix metalloproteinase-13 (MMP13) and osteopontin (OPN). MMP13 and OPN are expressed in the hypertrophic zone of calcified cartilage and both have been implicated in the progression of degenerative OA. Formation of mineralized cartilage involves the remodeling of matrix by extensive denaturation of type II collagen by MMP13 along with up-regulation of non-fibrillar type X collagen prior to mineralization. The biochemical changes we observe in articular cartilage from ten-week-old Hyp mice suggest that normal ECM turnover, in which resident ECM is enzymatically removed and replaced by newly synthesized ECM, is defective in Hyp mice. These findings recapitulate the events of early OA and suggest that defective ECM turnover ultimately results in loss of the mineralized zone of articular chondrocytes. Indeed, the calcified zone of articular cartilage is decreased in Hyp mice. We are currently conducting studies to determine if the cellular changes in articular cartilage of Hyp mice are resistant to treatment of the underlying rickets, and to determine if high circulating FGF23, as seen in XLH, play a role in the evolution of this process.

In vivo ^{31}P NMR studies of intracellular phosphate, mitochondrial and whole body energy metabolism in normal and hypophosphatemic mice



Gerald Shulman, MD, PhD

Yale School of Medicine, Department of Internal Medicine, Endocrinology

This pilot study examines the role of intracellular phosphate as pertains to energy metabolism. Using ^{31}P magnetic resonance spectroscopy to non-invasively measure rates of ATP synthesis in skeletal muscle, our lab has recently found that a ~65% decrease in plasma concentrations of Pi in mice, induced by feeding mice a Pi-deficient diet, promoted a ~50% reduction of the ATP synthesis flux in skeletal muscle. These

(continued on page 10)

Pilot and Feasibility Program Update *(continued from page 9)*

results support a potential role of intracellular Pi on the regulation of ATP synthesis. We are now examining this hypothesis in a mouse model with a loss of function of the PHEX gene. These mice have very low Pi concentrations in plasma due to an excessive renal Pi leak. The results of these studies have important implications in understanding the potential role of intracellular phosphate in the regulation of muscle mitochondrial ATP production as well as understanding alterations in muscle energy metabolism in patients with hypophosphatemia.

Raghbir Kaur, DMD

Brian Smith, MD

Laleh Ardeshirpour, MD

Clinical Services

Our clinical operations have expanded considerably. Many research subjects have raised questions about their need for therapy as they age and have requested ongoing clinical consultation. Because of the frequent complication of dental abnormalities that occur in XLH, we have brought Dr. Raghbir Kaur, a pediatric dental resident at Yale-New Haven Hospital, into the fold. Dr. Kaur has developed primary interests in both the clinical and research aspects of the disorder. Dr. Laleh Ardeshirpour, a junior member of the Section of Pediatric Endocrinology at Yale has developed an interest in bone diseases and co-attends the clinic with Dr. Carpenter. The recruitment of Dr. Brian Smith as the Director of Pediatric Orthopaedics at Yale has also provided an opportunity to expand our multi-disciplinary clinical goals—Dr. Smith, together with Christina Rao, an experienced pediatric physical therapist, co-attend a monthly metabolic bone clinic where comprehensive orthopaedic and medical care are provided at a single clinic visit. This effort, which also includes the above-mentioned dentistry services, has proven to be an ideal model in which to provide care for children affected with the disorder. Dr. Karl Insogna, together with members of the Endocrine Section in Internal Medicine at Yale, provides the mainstay of clinical care for adults.

Looking Ahead...

In the coming year we aim to complete our current clinical studies and to continue the development of many of the outreach projects described in these pages. We expect that our main projects will be generating new publications and look forward to the translation of their basic findings into potential clinical tools for the affected population. We aim to fully incorporate our dental department into the research aspects of the Center, and to explore the construction of an international registry of individuals with XLH, in cooperation with the XLH Network, Inc. As interest in the field continues to grow, we are enthusiastic about the potential of new approaches and improved outcomes in the understanding and management of XLH and its complications.

The Yale Center for X-linked Hypophosphatemia is dedicated to improving the health, and alleviating the suffering of patients with disorders of phosphate metabolism, especially X-linked hypophosphatemic rickets.

The Center supports research specifically focused on developing new and more effective treatments for these diseases. We are also committed to partnering with the pharmaceutical industry to develop and test novel therapies. Through educational programs, the Center informs health professionals and the public at large about diagnosing and correctly managing these diseases. For more information about the Yale Center for X-linked Hypophosphatemia, please contact Teri Tuma at (203) 785-2115 or teri.tuma@yale.edu.

Yale Center for X-linked Hypophosphatemia

333 Cedar Street, LMP 3093
PO Box 208064
New Haven, CT 06520-8064

Phone: (203) 785-2215
Fax: (203) 737-4290
E-mail: teri.tuma@yale.edu

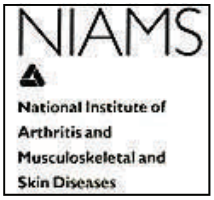
ON THE WEB: http://www.ycxlh.yale.edu/

Yale School of Medicine

YC XLH

Rickets Research

The Yale Center for X-linked Hypophosphatemia is supported by a grant from the National Institutes of Health through the National Institute of Arthritis and Musculoskeletal and Skin Diseases.



Yale Center for XLH
Yale School of Medicine
P.O. Box 208064
New Haven, CT 06520-8064