Impact of Funding

The National PKU Alliance has awarded more than $1.3 million in research grants since 2010 to accelerate the science and the treatment of new therapies for PKU. The information below is about the impact of this funding and how our support has led to these new discoveries.

Dr. Michael Allen
University of North Texas Science Center

Recent technological advancements have allowed for the in-depth investigation of the complex microbial communities present in the human gut. It is well known that these microbes, often referred to as the gut 'microbiome', are intricately involved with digestion. More recent research has revealed roles of the microbiome in several human diseases such as Crohn's disease and irritable bowel syndrome, as well as possible links to diabetes, multiple sclerosis, and others. Even more surprising are links between the gut microbiome and the immune system, neurological function, and normal development.

For these reasons we wanted to investigate changes in the bacterial community of patients with PKU. Since the gut microbiome is known to be highly influenced by diet, strict adherence to a low phenylalanine diet might lead to a demonstrably different gut microbiome community, with unknown effects on patient health.

With seed funding from the National PKU Alliance, we first investigated changes in the bacterial community in a mouse model of PKU. Our preliminary results revealed a substantial difference in the gut bacterial communities between PKU and wild type mice. These early findings were presented at the 2014 National PKU Alliance meeting in Salt Lake City, UT. Intriguingly, the bacterial groups identified and the conditions under which they grew suggested that they may preferentially use excess phenylalanine in the gut.

The research is still ongoing, but if the preliminary findings hold true, the identified bacteria might have potential as a probiotic therapy to protect individuals from spikes in phenylalanine levels. Moreover, they might eventually allow the easing of dietary restrictions in human patients. With this in mind and with additional support from the NPKUA, we are also pursuing a parallel line of research to develop a genetically modified bacterial probiotic specifically engineered to degrade phe in the gut. Preliminary results in mice have shown promise, and we are now developing a strain more suitable for human use and could be tested in clinical trials.

Dr. Carey Harding
Oregon Health & Science University

I was fortunate to receive an NPKUA grant in 2011-2013 to support the development and characterization of novel recombinant adeno-associated virus vectors that incorporated the human phenylalanine hydroxylase (PAH) cDNA. The NPKUA funds were used to support part of a lab technician's salary over two years as well as paying for production of the novel vectors.

This funding from NPKUA, in addition to funding from the National Institutes of Health, was critical to completing our research work. I am very grateful for the support from the NPKUA and hope that our work successfully fulfilled NPKUA's research mission.

Katherine Deming
University of North Texas Denton

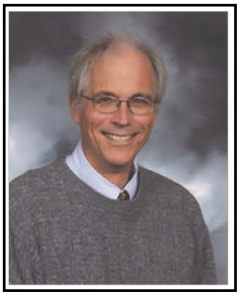
Without funding from the NPKUA, this lab would not have done research on PKU. With the NPKUA funds we have shown proof of concept for a new treatment; that a probiotic with heightened phe catabolism via a PAL enzyme can lower blood phe in mice. With this proof of concept, a few companies and investment firms have made inquiries prior to us developing a human therapeutic based on our research.

Slightly outside of the scope of PKU research, it seems the probiotic strain used can cause permanent colonization of the conventional mouse gut (containing natural Lactobacilli). This has not been demonstrated before. We can perform a plating test to confirm these results if desired, but it is not greatly shocking as this strain will colonize a mouse gut lacking Lactobacilli.

Prior to the Allen lab receiving its own NPKUA funding, we provided fecal samples from our animals demonstrating a striking difference between PKU and healthy littermate gut microbiota. The NPKUA funding at that time paid for the mice, their care, and our equipment, while the Allen lab paid for the microbial analysis.

We additionally found segregation of C57BL6/J and BTBR IGF-1 alleles in the mouse colony. The BTBR allele correlated with undesirable traits in a dose dependent manner. It would appear the BTBR IGF-1 gene causes problems. We have received attention from a probiotics company for further development/clinical trials once we have created the human version and negotiations have begun.

Our work performed with the first two years of funding from the NPKUA including: molecular/DNA manipulation, creation of bacterial cell lines, in-vitro phe catabolism, freeze drying optimization, raising a mouse colony, and preliminary animal studies lasting 4 days in 2 animals attracted the attention of the NSPKU in Great Brittan. Funds from the NSPKU allowed the final stages of our research to be completed by observing 3-4 animals treated for 2 weeks with the engineered probiotic.



Dr. K Michael Gibson
University of North Texas Science Center

Pilot funding from the NPKU Alliance enabled us to attempt novel therapeutic strategies in PKU mice that were not likely to be supported by NIH. Along these lines, we had found that selected, non- physiological amino acids were reasonably selective at blocking entry of phenylalanine into the brain of PKU mice. The funding from the Alliance allowed us to make further progress on this project, enabling us to obtain further pilot data to support our hypothesis.

The funds from the Alliance did indeed pinpoint that two of our NPAA species, 2-aminoisobutyrate and methyl-2-aminoisobutyrate, were very specific in their capacity to retard entry of phenylalanine into the brain of PKU mice. These species were both specific, and did not substantively retard the entry of other key large neutral amino acids into the brain. With the pilot data that was the result of support from the Alliance, we were able to acquire additional funding from the ITHS (Institute of Translational Health Sciences), University of Washington, to further characterize the species described above. We have begun to look at the metabolism, pharmacokinetics and pharmacodynamics of both compounds in preparation for human Phase 0 trials.

The work supported by the NPKU Alliance resulted in two publications (highlighted below) in peer-reviewed journals. A third report is currently under preparation, which has been jointly supported by both the NPKUA Alliance and the ITHS grant.

Citations:

Vogel KR, Arning E, Wasek BL, Bottiglieri T, Gibson KM. Characterization of 2-(methylamino)alkanoic acid capacity to restrict blood-brain phenylalanine transport in Pah enu2 mice: preliminary findings. Mol Genet Metab. 2013;110 Suppl:S71-8. doi: 10.1016/j.ymgme.2013.08.004. Epub 2013 Aug 15.

Vogel KR, Arning E, Wasek BL, Bottiglieri T, Gibson KM. Non-physiological amino acid (NPAA) therapy targeting brain phenylalanine reduction: pilot studies in PAHENU2 mice. J Inherit Metab Dis. 2013 May;36(3):513-23. doi: 10.1007/s10545-012-9524-8. Epub 2012 Sep 14.

Dr. Roberto Gramignolli
University of Pittsburgh

Our group has always been primarily focused in establishing hepatocyte transplantation (HTx) as a clinical therapy to correct liver based metabolic defects. We were the first to transplant human hepatocytes into patients with liver disease and, in collaboration with Dr. Fox, Children's Hospital in Pittsburgh, we received the first, Investigative New Drug (IND) approval from the US Food and Drug Administration (FDA) to conduct hepatocyte Isolation for clinical transplant, including PKU patients.

It is clear from animal and clinical studies that when sufficient numbers of proficient hepatocytes engraft, phenotypic correction of metabolic disease is attained. Since PKU is not normally treated by whole liver transplant, it is not an automatic target for HTx.

Thanks to the support granted by National PKU Alliance in 2010, we were able to achieve important results and identify several additional roadblocks preventing a more successful implementation of clinical HTx. All the preclinical studies resulted in the first two human HTx in PKU patients: one performed in Belgium, Europe, and another one at Children's Hospital in Pittsburgh. Both treatments have been performed based on the novel approach we published in 2013: a study in which we evaluated the clinical potential of hepatocyte infusion using inborn error deficient liver cells, also known as domino liver cell transplantation

Although the outcomes for the two PKU patients have been greatly improved, the lack of human liver tissues remains the major obstacle for this promising treatment.

Consequently, we have been focusing our effort in studying alternative cellular sources for transplant. We begun serious research efforts into trying to generate normal, mature human hepatocytes from adult and fetal stem cells, mainly fetal liver and human amnion, whose importance and peculiar pluripotent characteristics we were the first to describe. Human amnion epithelial cells isolated from term human placenta have been emerging as the most promising tool in cell-based therapy. Our group was the first in isolating and *in vitro* differentiating into hepatocytes-like cells.

During the last years we have been collecting several preclinical evidences on the effects of amnion stem cell transplantation in order to correct liver diseases. We successfully corrected inborn error such as Maple Syrup Urine Disease (MSUD), by injecting human cells and measuring murine mature liver gene expressions and correction. Based on these studies, we have been transferring this approach to evaluate correction on another inborn error animal model: PKU mouse model. Using our previous studies as basis and roadmap for our current investigation, we injected human AE cells in Pah^{enu} animals and we observed correction of Phe level. We analyzed serum and brain Phe levels from transplanted Pah^{enu} mice, observing a Phe and other amino acids level corrections, even higher to the level achieved by transplanting syngeneic hepatocytes.

Thanks to the support provided by PKU Alliance, it was possible to generate a big amount of data, and important new information, concerning the brain amino acids and neurotransmitter levels and their possible correction by HTx, was established.

Strong preclinical data generated so far are encouraging clinicians to consider amnion epithelial cell transplants for orphan diseases, not only liver-related. At Karolinska Institute we are now moving amnion epithelial cell isolation and banking in cGMP conditions, in preparation to phase II clinical trial, probably starting in 2015. And we are very proud to present these data underlying that nothing would have been probably achieved without the generous support of the National PKU Alliance. We all agree that the poor quality of life in association with the problems related to non-compliance to a lifelong unpalatable Phe-restricted diet, are the major issue we need to aggressively address. And all the support we received (not only economic) has been actively pushing our preclinical research forward, allowing us to generate what we believe are important data that increases not only our knowledge on this severe disease, but also moves one step closer everyday to definitely eradicate it.

Publications achieved thanks to NPKUA support:

Gramignoli R, Vosough M, Kannisto K, Raghuraman CS, Strom SC. Clinical Hepatocyte Transplantation: Practical Limits and Possible Solution (invited review). European Surgical Research 2014 [in Press]

Hansel MC, Gramignoli R, Skvorak KJ, Dorko K, Marongiu F, Blake W, Davila J, and Strom SC. The History and Use of Human Hepatocytes for the Study and Treatment of Liver Metabolic Diseases. Curr. Protoc. Toxicol. 2014 [in Press]; 62:14.12.1–14.12.x

Gramignoli R, Tahan V, Dorko K, Skvorak K, Zhao W, Venkataramanan R, Geller D, Fox IJ, Ellis ECS, Strom SC. Rapid-and-sensitive assessment of human hepatocyte function. Cell Transplant 2013 [in press] doi: 10.3727/096368914X680064

Strom SC, Skvorak KJ, Gramignoli R, Marongiu F, Miki T. Translation of amnion stem cells to the clinic. Stem Cells and Development 2013; 22(Suppl.1):96-102

Gramignoli R, Tahan V, Dorko K, Skvorak KJ, Hansel MC, Zao W, Venkataramanan R, Ellis EC, Jorns C, Ericzon BG, Rosenborg S, Soltys K, Mazariegos G.V, Fox IJ, Wilson M, Grompe M, Strom SC. New potential cell source for hepatocyte transplantation: discarded livers from metabolic disease liver transplants. Stem Cell Research 2013; 11(1):563-573

Gramignoli R, Dorko K, Tahan V, Skvorak KJ, Ellis EC, Jorns C, Ericzon BG, Fox IJ, Strom S. Hypothermic storage of human hepatocytes for transplantation. Cell Transplant 2013 [in Press] doi:10.3727/096368913X668627

Skvorak KJ, Dorko K, Marongiu F, Tahan V, Hansel MC, Gramignoli R, Arning E, Bottiglieri T, Gibson KM, Strom SC. Improved Amino Acid, Bioenergetic Metabolite and Neurotransmitter Profiles following Human Amnion Epithelial Cell Transplant in Intermediate Maple Syrup Urine Disease Mice. Mol Genet Metab 2013;109(2):132-138

Skvorak KJ, Dorko K, Marongiu F, Tahan V, Hansel MC, Gramignoli R, Gibson KM, Strom SC. Placental stem cell correction of murine intermediate maple syrup urine disease. Hepatology 2013; 57(3):1017-23

Gramignoli R, Marongiu F, Miki T, Skvorak KJ, Tahan V, Dorko K, Hansel MC, Davila JC, Ellis ECS, Strom SC. Human Placenta Cell Isolation. Methods in Bioengineering: Cell Transplantation (2011). Edited by: Alejandro Soto-Gutierrez, Nalu Navarro-Alvarez, Ira J. Fox. ISBN 978-1-60807-015-2; Chapter 12; pp183-98

Denise M. Ney, PhD, RN
University of Wisconsin-Madison

The cornerstone of management of PKU is lifelong adherence to a low-phenylalanine (phe) diet implemented shortly after birth to prevent permanent cognitive impairment. The diet has two components: severe limitation of natural protein intake (only 5-10g protein/ day from fruits and vegetables is allowed) and consumption of a phe-free amino acid (AA) formula (24-32 oz formula/day). Lifelong compliance with the low-phe diet, especially consumption of the AA formula, is very poor.

The goal of our research program is to improve the nutritional management of PKU (the mainstay of PKU metabolic control) using low-phe medical foods made with glycomacropeptide (GMP), a whey protein produced during cheese making that contains a trace of phe. Since 2003 and with a variety of funding sources, including NIH, University of Wisconsin-Madison, Michaux Foundation, MACPAD, and Tennessee PKU, we have developed GMP medical foods that in 2010 became commercially available. Thus, our research has resulted in a new dietary option that is an alternative to AA formula for the essential nutritional management of PKU.

Skeletal fragility is a common but poorly understood chronic complication of PKU. Using NPKUA funding ($80,000) and the PKU (*Pah*^{enu2}) mouse model we were able to answer an important question that cannot be studied in human PKU. Is skeletal fragility inherent to the PKU genotype or the requisite AA-based diet? The answer is that both the PKU genotype and the AA diet contribute to impaired bone development in PKU. Moreover, a GMP diet improves the bone phenotype resulting in bigger and stronger bones compared to the AA diet in mice.

This NPKUA-funded research has resulted in three research publications cited below, numerous invited seminars in the United States, Canada and Europe to extend understanding of the nutritional management of PKU, and the submission of several research funding applications. Most important, the NPKUA funding helped to garner a 1.4 million dollar grant from the FDA Orphan Products Development Grants Program entitled “Phase 2 Study of Glycomacropeptide vs Amino Acid Diet for Management of PKU” www.clinicaltrials.gov NCT 01428258. This clinical trial extends murine studies of the impact of the GMP and AA diets on bone health and other endpoints in PKU patients living at home. Thus, with NPKUA funding we were able to successfully leverage studies in PKU mice to translational studies in human PKU. Federal funding for PKU research is severely limited and funding from NPKUA is essential to improve the management of PKU leading to improved health and reduced health care costs.

Research Publications Directly Related to NPKUA Grants to Denise M. Ney

Solverson, PM, Murali, SG, Brinkman, AS, Nelson, DW, Clayton, MK, Yen, E and **Ney, DM**. Glycomacropeptide, a low-phenylalanine protein isolated from cheese whey, supports growth and attenuates metabolic stress in the murine model of phenylketonuria. Am. J. Physiol. (Endoc & Metab) 302:E885-E895, 2012.

Solverson, P, Murali, SG, Litscher, SJ, Blank, RD and **Ney, DM**. Low bone strength is a manifestation of phenylketonuria and is attenuated by a glycomacropeptide diet. PLoS ONE 7(9): e45165, 2012. DOI:10.1371/journal.pone.0045165

Hansen, KE and **Ney D**. A systematic review of bone mineral density and fractures in phenylketonuria. J Inherit Metab Dis. 37:875-880, 2014.

Dr. Donna Santillan
University of Iowa

The goal of our lab is to develop a long-term non-dietary therapy that efficiently reduces Phenylalanine and reverses or prevents adverse physiologic conditions associated with PKU. Directly because of our funding from the NPKUA, we were able to make significant strides in our research to test the capabilities of our artificial liver system to reduce Phe and to correct metabolic changes that occur under high Phe in the PKU model mice. In humans these measurements are very difficult to make and would require very large populations of participants with PKU and without PKU for comparison. However, because we can use an animal model that recapitulates what has been observed in humans, we can more easily gain a preliminary determination of whether our therapy is effective.

In addition in our NPKUA funded research, we found adverse changes under high Phe in the immune system of PKU mice that we believe explains why people with PKU are more prone to infections and have a more difficult time clearing the infection. his is very preliminary data and we are currently applying for funding to pursue this finding in more depth.

We have presented our results at several conferences including a poster presentation at the University of Iowa Center for Immunology Conference in September 2014 and at the Society for Maternal Fetal Medicine 33rd annual meeting in February 2013. We were honored with the Best Poster Award for our poster session at the Society for Maternal Medicine Meeting.

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In addition to submitting abstracts and internal and external grant proposals, our lab is currently preparing 2 publications based on our projects that were funded by the National PKU Alliance. These publications will be very important to our success in obtaining grants.

Funding is very difficult in these times, especially to study rare disorders. In PKU this challenge is also great because many people do not fully understand the shortcomings of the PKU diet. Without funding for preliminary data, it becomes practically impossible to receive large extramural funding. Even without doing any experiments, the costs of maintaining a PKU mouse colony are very prohibitive for many researchers. Funding from the National PKU Alliance has been instrumental in allowing us to generate important preliminary data that increases our knowledge, advances our knowledge of the effects of PKU, and builds the foundation of our research program.

Dr. Kristen Skvorak
University of Pittsburgh School of Medicine

The goal of my research interests is to successfully shift stem cell transplantation for the treatment of metabolic disease, including phenylketonuria (PKU), from the laboratory into the clinic. Though the first cell transplant to treat a metabolic disease patient using human hepatocytes was successfully done 16 years ago, and a clinical trial is currently underway at the UPMC Children's Hospital in Pittsburgh, PA, this therapy is still considered experimental. The use of human hepatocytes has many of the same issues and concerns as a liver transplant, which is slowing and ultimately may stop progress if the problems cannot be solved. Excitingly, stem cells derived from human placenta may be able to circumvent many of these issues, and funding from the National PKU Alliance has been instrumental in much of the progress made thus far.

Investigation of cell transplantation to treat metabolic disease began for me with a mouse model of maple syrup urine disease, and research would have also ended with MSUD if not for a postdoctoral fellowship from the NPKUA. With this support, significant strides have been made towards our goal. Cell

transplantation, both with mouse hepatocytes and human placental stem cells, have produced marked improvements in blood and brain Phe levels in a mouse model of PKU. We have also seen improvements in other important brain chemicals, including dopamine metabolites, and growth was more normalized. No negative side effects have been noted in any of the transplanted animals. The stem cells themselves have also been optimized for greater yield from a human placenta and best viability out of cryogenic storage prior to transplant, which is of the upmost importance for clinical use. We are currently applying for additional funding to now optimize donor cell engraftment and function using drugs approved and commonly used in patients.

Our discoveries have been presented at many national and international scientific conferences, all of which were accepted for oral presentation. We were also honored with several awards for our submitted abstracts, including the Cell Transplant Society Young Investigator Travel Grant in 2013 to attend and speak at their conference in Milan, Italy, the European Society of Organ Transplantation Award for Excellence in 2012 in Vienna, Austria, and the Transplant Society Mentee-Mentor Travel Award and President's Choice Award in 2012 for their conference in Berlin, Germany. We also have a publication in preparation, which is very important to the success of obtaining future grants as well as the clinical advancement of placental stem cells. I also had the pleasure of addressing the Tennessee PKU Foundation (2012) and PKU Organization of Illinois (2014) as keynote speaker at their annual meetings to share my work and progress.

Funding is extremely difficult, especially when the main focus of your work is in rare diseases. Unfortunately many misunderstand the special diet to be a cure. Alternative therapies are needed, but without generating strong preclinical data there would be no hope of advancing from bench to bedside. Importantly, this therapy may be a viable option for many types of metabolic disease, not just PKU. Funding awarded by the NPKUA allowed me to complete a study demonstrating the positive effects of cell transplant in a PKU mouse model. This has led to greater knowledge of the benefits and safety associated with placental stem cells, a greater understanding of how transplanted cells in the liver can improve the brain, and improved techniques for cell isolation, storage, and transplant, all of which are vital information for clinical implementation.

Dr. Eddy van der Zee
University of Groningen

Notwithstanding the successes that have been made by introduction of neonatal screening and the phenylalanine (Phe)-restricted diet, outcome of phenylketonuria (PKU) patients still remains suboptimal, especially in adult patients who no longer manage to keep up with the burdensome dietary treatment. Therefore, our research group aims to develop new treatment strategies directly targeting the brain to improve neurocognitive and neuropsychological outcome and possibly liberalize the dietary restrictions for PKU patients. Using the PKU mouse model, we aim to further investigate the mechanisms underlying brain dysfunction in PKU, and to develop new treatments directly targeting at these mechanisms.

The association between elevated blood Phe levels and cognitive dysfunction in untreated PKU is well-known. However, the underlying mechanisms by which increased blood Phe levels lead to cognitive dysfunction have not been fully elucidated yet. Off course, you may ask whether that is of major interest, as the present treatment, decreasing brain Phe by decreasing blood Phe levels, is effective. Indeed, the diet has changed the outcome of PKU patients from severe mental retardation to near normal, but still outcome is not optimal, and the diet is very demanding. Thus, new treatments are required, and for this, we need to understand the mechanisms.

Coming back to the mechanisms, probably, the increased transport of Phe from blood to brain and the consequentially decreased transport of other large neutral amino acids (LNAA) plays a central role. As a possible new treatment for PKU, supplementation of LNAA other than Phe aims to restore the disturbed transport from blood to brain. This could be accomplished by inhibiting the transport of Phe, while increasing the transport of other LNAA. This idea has already been considered 40 years ago, and investigated in some small studies, but not in full detail. If effective, however, in the future, such LNAA

supplementation may improve outcome and liberalize the dietary restrictions for PKU patients.

The NPKUA funding for our research has been of critical importance to further develop our research line on this possible new treatment strategy for PKU. Before, we had performed some pilot studies showing promising effects of LNAA treatment in both young and adult PKU mice. However, we lacked the resources to take this research to a next level. The NPKUA funding has enabled us to perform such a study comparing different LNAA treatment regimens at the same time instead of investigating these regimens one by one. This comparison of different LNAA treatment regimens will be of utmost importance to our ultimate goal: to develop optimal LNAA treatment to improve outcome of PKU patients.

Therefore, we, as a research group from The Netherlands, feel very honored having received a grant from the USA PKU community as one of the first from Europe. We are very grateful for having received funding from the NPKUA to be able to investigate this idea in much more detail and to see whether this really is the way to go for our patients both in USA and Europe. Although many differences in care for PKU patients exist between Europe and USA, the disease is the same, and treatment should be further optimized for all patients.

To develop innovative and alternative treatment strategies for PKU, solid pre-clinical studies in models like the PKU mouse are urgently required. We are fortunate to have this PKU mouse model, and we very much appreciate the fact that the NPKUA acknowledges the value of such pre-clinical studies on possible new treatments in the PKU mouse model.

Currently, we are writing two papers on our pilot experiments on LNAA treatment in the PKU mouse. As our funded research was granted only 9 months ago and includes such a broad study, we are still working on the project and strongly await the first results. We sincerely believe, however, that our study as granted by the NPKUA will result in some additional posters, abstracts, and a paper, and will be of much help in receiving additional funding from other parties. We also applied for a second year of funding to enable to further work towards an optimal LNAA treatment regimen for PKU patients. With the answers of both studies we hope we can bring the PKU community the start of a new treatment which has already been on the table for so many years, but has never been investigated well enough.