The NIH Undiagnosed Diseases Program

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In the United States, a rare disease defined by the 1983 Orphan Drug Act, is a disease that affects fewer than 200,000 people [1]. Unfortunately, for these patients, receiving treatment, let alone a diagnosis provides to be a challenge. The drug development pipeline for relatively common disorders such as hypercholesterolemia is incredibly expensive and time consuming. However, due to these disorders being common, sales of drugs that treat these disorders prove to be hugely profitable for pharmaceutical companies with sales of, for example, atorvastatin generating more than $125 billion in sales over it's 14.5 year lifetime. The fact of the matter is that diseases which affect more people get more attention, and diseases which affect less are neglected. Therefore, the traditional research and development pipeline for these disorders needs to be reimagined. Before that can happen, a more fundamental understanding behind the pathogenesis of these disorders needs to be appreciated. Understanding the pathophysiology behind rare diseases not only offers hope to patients, but also furthers our understanding of normal human physiology. Quoting Dr. William Gahl at the National Human Genome Research Institute,

> *Evolution requires imperfect fidelity of replication, that is mutations, and these mistakes ultimately reveal the exquisite functionality of Nature. The rare diseases that populate our species represent the manifestations of Nature's errors. Rare though they are, their study has revealed important insights about normal physiology that in turn have provided a better understanding of common disorders, universal mechanisms, critical pathways, and therapies that are useful for treating more than one disease. [1]*

A classic example of translation from rare disease research to the clinic is illustrated by the role of low–density lipoprotein receptor (LDL–r) in cholesterol homeostasis [2]. Early investigations into familial hypercholesterolemia, an extremely rare genetic defect caused by mutations in LDL–r elucidated the mechanism in which LDL–r regulates cholesterol biosynthesis. Not only did it provide an understanding on the basic science level, but it also paved the way into the importance for cholesterol in the development of atherosclerosis and eventually to the use of statins (such as the atorvastatin example illustrated above) to lower cholesterol levels and decrease the risk of coronary artery disease.

A more enigmatic example of how rare disease can provide insight into normal human physiology involves the study of Hutchinson–Gilford Progeria Syndrome which is a rare, sporadic, autosomal dominant disease characterized by a remarkable display of accelerated aging ultimately leading to premature death at approximately 13 years of age due to myocardial infarction or stroke [3]. This results from a mutation in the

LMNA gene which leads to the production of a mutant protein termed *Progerin*. This leads to nuclear membrane instability, the perturbation of global gene expression, and the early senescence of cells. Interestingly, it was found that the pathological process implicated in Progeria also exists in normal human cells, albeit on a much smaller scale. Therefore, Progeria can serve as a model for normal human aging and treatments targeted towards Progeria can perhaps also be used to slow down the normal process of aging.

Realizing the importance of rare disease research, the Office of Rare Disease of the National Institutes of Health launched the Undiagnosed Disease Program (UDP) in 2008 with an initial seed grant of $280,000 [4, 5]. A primary motivation factor for initiating this program was from data that indicated that it took one to five years to reach a proper diagnosis for 33% of patients with rare disease, and more than 5 years for 15% of these patients [6]. The second critical motivation factor, as illustrated above, was the importance of extracting medically and biologically relevant insights into human physiology from the study of rare diseases. After the program launched in 2008, the popularity of the program grew immensely with funding increased to $1.9 million in FY 2009, $3.5 million annually for FY 2010 through 2012 [4], and most recently, more than $15 million for FY 2013 [7].

Overall Methodology

The research pipeline of the UDP starts with a summary letter from a referring clinician and complete medical records including imaging and histologic slides of biopsy material. The case files are reviewed by a team of one to five physicians representing 25 different specialities. In the first 32 months since the inception of the UDP, 4700 inquires were received, along with the medical records of more than 1700 individuals. Approximately 1000 cases were rejected (usually due to poorly defined symptoms, subjective complaints, and more far–fetched inquiries[1]), 400 were accepted, and with the rest under consideration.

Accepted patients are scheduled for a week–long inpatient visit to the NIH Clinical center and diagnostic investigations including specialized tests and genomic studies (i.e. SNP arrays and whole–exome sequencing) are performed. For both the applicant and accepted patients pool, several key demographics surfaced. Nearly half of the patients had a chief complaint related to a neurological problem. Other systems involved include gastrointestinal disease, fibromyalgia, chronic fatigue syndrome, immune and autoimmune disorders, psychiatry, pain, dermatology, and cardiovascular disease[2]. Approximately 40% were children and 60% were female. For those admitted

[1] Anecdotal: An inquiry was submitted to the UDP with a chief complaint of "excessive masturbation".

[2] For a detailed table of primary phenotypes of UDP applicants and accepted patients, refer to Table 1 in [5].

to the UDP program (n=272 as of 2011), 20%–25% received a diagnosis on clinical, biochemical, pathologic, or molecular grounds [4].

Standard protocol [5] (in addition to specialized tests) that all UDP patients undergo include the collection of DNA samples and subsequent genomic analysis. For SNP genotyping, peripheral whole blood DNA is analyzed using the Illumina Bead Array Platform which can identify regions of homozygosity [8], copy number variation [9], chromosomal mosaicism [10], and uniparental disomy. The most powerful analytic tool is arguably elucidating the regions of homozygosity [5]. Multiple small regions of contiguous homozygosity (RCH) are expected in any human genome, however, large RCHs are potential candidates for disease causing genes and form the basis for homozygosity mapping. It is in these regions in which one would expect to find the causative gene for an autosomal recessive disorder. More exotic disease mechanisms require a more thorough searching of the genome including whole genome and exome sequencing.

One would expect to generate an extremely long list of variants and potential disease causing genes. Various filters such as dbSNP, the 1000 genomes project, Mendelian consistency, and pathogenicity correlation using CDPred [11] were used to generate a set of highly polymorphic genes that could be involved in the disease pathology. If multiple families exhibit the same disease phenotype, genomic data can be compared across families to further narrow down the candidate gene list. This narrowed down list (usually by 1–2 orders of magnitude) is then Sanger Validated in an order that ranks the likelihood of that gene to participate in a specific disease process (*i.e.* ECM remodeling, cell signaling, etc...)[3]. Finally, when a gene is identified, the disease process is reconstituted in a cell–based and often animal model to validate the disease pathogenesis. After understanding the molecular characteristics of the disease, one can then look to potential treatments, often through drug repurposing and off–label use.

Cases where a diagnosis were reached consisted mostly of known conditions. The fact that these cases were previously undiagnosed can be attributed to lack of consideration due to the rarity of the disease, misleading laboratory data, and the emergence of newer tests and refined disease definitions [5]. **Table 1** summarizes some key diagnoses that the UDP has made.

[3] This part, arguably, is the most tedious as it takes a lot of educated guesswork and trial and error. There have been cases where Mendelian filtering has narrowed the candidate gene list down to <10 genes, and in other cases, more than 100. However, this is usually better than the original list (usually in the 10,000–100,000) of genes generated by homozygosity mapping and/or whole–exome sequencing. Table 5 in [5] gives a detailed summary of how filtering is accomplished with successive quantitative reduction in candidate variants.

FREQUENCY	DIAGNOSIS	COMMENTS
New Diseases	Arterial Calcification due to CD73 Deficiency	Mutation in *NT5E*
	Congenital neutropenia due to *VPS45* mutations	Mutation in *VPS45*
	Familial distal myopathy	*HINT3* mutation
<60 cases reported	Leukodystrophy with axonal spheroids	
	Spinocerebellar ataxia and hereditary spastic paraplegia	Only case associated with biallelic *AFG3L2* mutations
	Pitt–Hopkins Syndrome	*TCF4* Mutation
	Hereditary benign intraepithelial dyskeratosis	4q35.2 duplication
	Congenital disorder of glycosylation IIb	*LMNB1* Mutation
	Autosomal dominant cerebellar ataxia	Glucosidase I deficiency
	Aceruloplasminemia	*Cp* mutations with neurological involvement
<1 in 100,000	Facial dysautonomia	
	Hereditary spastic paraplegia	*SPG4* Mutations
	Smith–Magenis Syndrome	*RAI1* Mutation
	CSF tetrahydro–biopterin deficiency	
	Immune–mediated cerebellar degeneration	
	GM1 gangliosidosis	
	Amyloid myopathy	
	Amyotrophic lateral sclerosis	*SOD1* mutation
	Progressive spastic paraparesis	*SPG7* mutation
	Call–Fleming Syndrome	
1–10 in 100,000	Primary progressive multiple sclerosis	
	Neuromyelitis optica	
	Progressive spranuclear palsy and corticobasal ganglia degeneration	
	Corticobasal ganglionic degeneration	
Common	Fibromyalgia	
	Psychogenic tic cough	

FREQUENCY	DIAGNOSIS	COMMENTS
	Somatization	
	Morgellon disease	
	Multiple myeloma	
	Functional Gait Disorder	
	Psychogenic movement disorder	

Table 1: Key diagnoses made by the NIH UDP [5, 12, 13]

Unsolved cases that are still under investigation include (1) two women with increased circulating vascular endothelial growth factor and either thrombotic microangiopathy or hepatic and bone hemangiomas; (2) a man with renal stones and elevated vitamin D levels; (3) a young woman with fibro-inflammatory tumors of her lungs, liver, and pterygomaxillary region; (4) two women with decreased cerebrospinal fluid tetrahydrobiopterin and neurotransmitter levels who responded to sapropterin supplementation; (5) a child with developmental delays and copper storage in Zone 3 of the liver; (6) a child with idiopathic renal tubular Fanconi syndrome and hearing loss; (7) a woman with lung nodules and thick pulmonary mucus; (8) a woman with a possible pathogenic mutation in platelet-derived growth factor-α; (9) a woman with follicular keratosis producing painful spikes of keratin protruding from her skin and scalp; and (10) a woman with autoimmune-mediated cerebellar degeneration [5]. The breadth and diversity of cases presented to the UDP show incredible potential for understanding a wide range of cellular processes. The following two case studies will illustrate how the study of a previously undiagnosed disease shed light on to a undiscovered molecular process elucidated only after investigation into what happens when that process goes wrong.

Case Study — Arterial Calcifications due to CD73 Deficiency (ACDC)

Vascular calcification resulting from a hardening of the tunica intima or media of vessels is a disease process associated with an increased risk of cardiovascular events [14]. Three families present to the NIH UDP with radiographic findings of intense arterial calcification [12]. The patient from family 1 (VI.1) was a 54–year old female with a 20–year history of intermittent claudication of the calves, thighs, and buttocks and chronic ischemic paint in her feet at rest. Her parents are third cousins. On examination, her ankle–brachial blood pressure index values were markedly reduced, but levels of serum calcium, phosphate, vitamin D, Alk–Phos, creatinine, cholesterol, and other lab values were normal. Contrast enhanced magnetic resonance angiography revealed extensive occlusion of the iliac, femoropopliteal, and tibial arteries. Plain radiographs of the lower extremities reveal extensive calcification and arteriomegaly. Calcification was also found in the juxta–articular joint–capsules of the fingers, wrists, ankles, and feet. Interestingly, no calcifications were found above the

diaphragm. The patient along with all four of her siblings has disabling intermittent claudication (inability to talk more than 6 blocks) and hemodynamically significant lower–extremity obstructive peripheral artery disease.

The proband in family 2 (II.4) is a 68–year old northern Italian woman who may or may not be a part of a consanguineous conception (mother's surname was the same as that of some of her father's relative four generations ago). This patient presented with intense joint paint in her hands which was unresponsive to glucocorticoids administered from 14 to 27 years of age. Similar to patient VI.4, radiograph revealed extensive arterial calcifications and normal blood laboratory values. Two sisters of II.4 also had lower–extremity pain and vascular calcifications.

The proband in family 3 (II.1) is a 44–year old woman and at age 42, presented with mild paresthesias in the lower limbs. Extensive calcifications were revealed in the distal arteries. Laboratory values were in the normal range; however, concern about ischemia in the right leg prompted a femoral–popliteal bypass at the age of 43.

None of the affected patients nor their parents or children have abnormal bone morphology, type 2 diabetes, or decreased kidney function.

Upon admission to the NIH UDP, fibroblast cultures were prepared from a skin punch–biopsy. Genomic DNA was extracted from peripheral leukocytes and genotyped on the Illumina Human 1M Duo platform. Regions of homozygosity were analyzed and a LOD score was established with the use of parametric multipoint linkage analysis [15]. The consanguineous relationship in family 1, with disease confined to one generation, suggested autosomal recessive inheritance. Therefore, candidate genes should appear in homozygous regions common to all five affected siblings. Upon RCH analysis, one region of the genome was discovered: a 22.4 Mb region on 6q14 containing 7977 genotyped SNPs and 92 genes. Of these 92 genes in this region, three were evaluated based on their involvement[4] in degenerated cellular processes that could lead to calcification — *ATG5* (E3 ubiquitin ligase necessary for autophagy), *CASP8AP2* (implicated in apoptosis), and *NT5E*. *NT5E* was an interesting candidate because its enzyme substrate is the product of *ENPP1* which is the only other gene implicated in a single mendelian disorder of isolated vascular calcification [16].

Direct Sanger Sequencing of these candidates revealed a homozygous nonsense mutation (c.662C>A, p.S221X) in exon 3 of *NT5E* in all five siblings in family 1, and the same heterozygous mutation in both parents. qRT–PCR analysis demonstrated

[4] They certainly got *very* lucky in their initial picks since one of the very first genes they decided to sequence was the causative gene. Imagine if they had to run through all 92! Unfortunately, for many of the cases in the UDP backlog, there *is not a* logical order to picking genes for sequencing since there is a lack of understanding of the genes involved or insufficient biological knowledge to draw inferences from. For these cases, it's essentially finding a needle in a haystack. Hopefully more refined computational methods can streamline this process and allow for more logical picks.

decreased expression of *NT5E* in VI.1 and VI.4 in family 1. Affected members of family 2 were homozygous for a missense mutation, c.1073G>A (p.C358Y) in exon 5 of *NT5E*. The proband in family 3 was a compound heterozygote for c.662C>A (nonsense) and c.1609dupA (p.V537fsX7), both leading to premature stop codon in *NT5E*.

NT5E is a gene located on 6q14–q21 which encodes CD73, a membrane bound ecto–5'–nucleotidase involved in extracellular ATP metabolism. This enzyme binds adenosine monophosphate and converts it into adenosine and inorganic phosphate *[17]*. Western blot analysis of fibroblast extracts from patients VI.1 and VI.4 of Family 1 indicated significant reduction in expression of CD73 protein as compared to normal controls. This was corroborated with an enzyme activity study which showed nearly absent activity. CD73 function for the patient's parents showed an activity of 72% as compared to a control. To further validate that CD73 is the causative defect, lentiviral vector rescue of wild type NT5E produced normal CD73 levels and AMP–dependent inorganic phosphate production. Additional testing in HEK293 cells (which has low endogenous CD73 activity) showed that transfection with wild type *NT5E* cDNA resulted in high CD73 activity, whereas transfection with mutated forms of *NT5E* corresponding with each patient's mutation failed to restore CD73 activity.

CD73 and it's role in arterial calcification lies in the fact that calcification is dependent on tissue–nonspecific alkaline phosphatase (TNAP) activity *[18]*, an enzyme that converts pyrophosphate (PPi) to inorganic phosphate (Pi). Calcific stimulation of *ACDC* fibroblasts increased staining for TNAP, whereas treatment with adenosine reduced the amount of TNAP staining. TNAP staining was also reduced when *ACDC* cells were transfected with wild type *NT5E*. Calcific stimulation results in abundant calcium phosphate crystal formation in ACDC fibroblasts but not in normal fibroblasts. Adenosine treatment largely obliterated the calcification process and the noncompetitive TNAP inhibitor levamisole also prevented calcification in ACDC cells.

These set of findings is incredibly interesting from a basic science perspective because it reveals a novel physiological role of adenosine as an indirect negative regulator of arterial calcifications. CD73 participates in an extracellular pathway that converts ATP to adenosine on the surface of cells. First, ENPP1 catalyzes the conversion of ATP to AMP and PPi which is then coupled to the production of adenosine and Pi by CD73. Cellular calcification depends on the level of pyrophosphate, a strong inhibitor of calcification, and TNAP, which degrades pyrophosphate *[18]*. To connect these dots, we look to similar conditions involving this pathway. In patients with generalized arterial calcification of infancy, ENPP1 deficiency leads directly to decreased PPi levels *[16]*. In the case of ACDC, decreased CD73 activity may not lead *directly* to decreased PPi levels, but the consequent reduction of adenosine can increase TNAP activity which was demonstrated by the reversal of TNAP by adenosine rescue. A hypothesized mechanism is that decreased CD73 leads to decreased adenosine which leads to increased TNAP activity which reduces PPi levels leading to calcification. This is again

supported by the notion that inhibition of TNAP by levamisole ameliorates the calcific process. The exact process of how adenosine interacts with TNAP (possibly through a GPCR signaling cascade) is unclear.

With the knowledge of the molecular mechanism of the defect in ACDC patients, treatment options can be considered. The overall strategy is to either provide adenosine rescue or to supply a TNAP antagonist. Dipyridamole, an antithrombotic drug used successfully for aneurysmal vascular remodeling inhibits cellular reuptake of adenosine and can increased the amount of extracellular adenosine available to the adenosine receptor *[19]*. Adenosine–receptor agonists *[20]* or TNAP inhibitors such as lansoprazole *[21]* can also show efficacy. Drugs repurposed from similar calcification disorders such as bisphosphonates for ENPP1 deficiency can also prove useful *[22]*.

As one of the first success stories from the NIH UDP, this gives a first glimpse into the power of genomics to provided answers that have eluded diagnostic methods. Not only does it provide some closure to the patients involved, it also revealed a novel pathway for arterial calcifications and a molecular understanding of the disease.

A New Model for Genomic Medicine

The translational research paradigm has mostly concerned itself with brining innovations from the bench to the bedside; however, there is enormous potential for the bedside observations to drive basic science research. Since it's inception, the NIH UDP has provided a transformative model for how scientists and physicians can work together to open a two–way avenue between the two. The experience of the UDP has also provided insight into how we view disease pathologies and progression.

First, the disproportionate amount of neurological cases being referred to the UDP reflects not only the current lack of understanding of the brain, but also suggests that the brain is the next big frontier for medicine. It is apparent that both clinicians and patients are currently unsatisfied with the diagnostic tools available today and affected patients often get a myriad of disparate diagnoses among various specialists. Neurologists are often restricted to symptomatic management and therapeutic maneuvers as a means of diagnosis — i.e. using intravenous immune globulin or steroids for suspected inflammatory CNS disease or the use of L–DOPA for Parkinson's [4].

Second, systemic problems in healthcare often impede access to patients with chronic, illusive, and multi–systemic disorders. UDP patients recall that a single week of tests at the NIH Clinical Center would take more than a year to obtain under the approval process of various insurance plans. Unscrupulous diagnoses are often made either to placate nervous patients or for billing purposes. Fragmentation and lack of care coordination in the healthcare system is also a huge driver of multiple inchoate diagnoses. It is often difficult for a single physician to manage a disorder which

manifests itself as multiple systems — therefore, many of these patients hop from specialist to specialist — with each specialist diagnosing "what they want to see".

Third, genomic studies can prove to be a valuable screening tool in families with pedigrees that suggest a certain degree of consanguinity. SNP arrays can identify regions of homozygosity that can contain candidates for single gene recessive disorders. Whole–exome sequencing can not only identify a large amount of genes with potentially deleterious variants but also can provide deep insight into large groups of gens known to cause certain *groups* of disorders. As we approach the sub $1,000 genome, there will most certainly be an overwhelming deluge of clinical data and more patients will be brining these issues up with their physicians. As medicine becomes more data–driven and the full potential of the genomic revolution is realized, physicians will need to be sufficiently trained in interpreting such data and applying it in clinical practice.

Fourth, the study of rare disease can not only lead to new discoveries about pathophysiology, but can reveal previously unknown normal physiology. As illustrated in the ACDC example above, a previously unrecognized role of adenosine in inhibiting vascular cell calcification was elucidated. More recently, *VPS45* was implicated in congenital neutropenia with bone marrow fibrosis, nephromegaly, migration defects, and severe bacterial and fungal infections [13]. The fact that *VPS45* plays an important role in vesicular trafficking can suggest that other immunodeficiency disorders such as Hermansky–Pudlak Syndrome Subtype 2 and Griscelli Syndrome may share a common mechanism.

Fifth, genomic medicine can refine what a diagnosis is. Traditionally, a diagnosis could refer to a simple histological description, a collection of clinical symptoms and signs, or a gene mutation. However, the most informative definition of a diagnosis is one that provides a "grand unified theory" for a specific disease which includes a description of the disease pathogenesis along with genetic and clinical findings that ultimately inform prognosis and treatment. By understanding the specific molecular pathology behind a patient's disease, medicine has the potential to become incredibly specific, targeted, and personalized.

Sixth, patients with rare disease would rather know if they have a serious disease rather than continuing with no diagnosis. Patients relate feelings of isolation when branded with a condition without a name, and how physicians — perhaps with feelings of inadequacy — become distant or even hostile. Surprisingly, these patients, despite knowing the high failure rate of the UDP, are generally appreciative not because of the possibility of a diagnosis, but rather because of the personalized attention they receive. The latter point is especially important as the care of patients

with these mysterious conditions offers a caricature of the human condition[5] and the idealized patient–physician relationship. It also teaches physicians to realize the limitations of medicine and many of the clinicians involved in the UDP know and accept that their efforts will most likely fail. This is in stark contrast to the archetype of the commanding physician that is used to successfully diagnosing, treating, and understanding a disease.

And lastly, as with any new medical innovation comes with a complex set of ethical and social considerations. Cases of non–paternity is a frequent issue that arises in genetic testing. Access to care, and more importantly, patient education in what genetic testing will mean to the patient will be an ongoing challenge. As genomic expands around the globe, cross–cultural differences and socioeconomic context will also need to be addressed [23]. However, this new paradigm in diagnosis and it's effects on prognosis and treatment is significant. It reflects the best that both science and medicine has to offer. The study of rare disease not only offers a glimpse into nature's molecular imperfections, but also offers an introspective window in the human condition.

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[5] Anecdote: A patient actually *thanked* the UDP team after we gave the person a terminal diagnosis. It shows that living with uncertainty is actually harder to bear than accepting what is reality.

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