How Advances in Genetics / Genomics are leading to Novel Treatments for Rare Diseases

An Honors Thesis for the Department of Center of Interdisciplinary Studies.

Dafni FT Frohman

Tufts University, 2019.

GENE DISCOVERY 1

The Human Genome Project 1
Sequencing Techniques 2
Bioinformatics 6
Medical Care and Follow-Up 8

Application of the Human Genome Project 10
International HapMap Consortium 10
Genome-Wide Association Studies 11
Animal Studies 14
Orphan Diseases 15

Precision Medicine 17

PHARMACOLOGY DEVELOPMENT 21

Animal Model to Human Model 21
Toxicology 22

Orphan Disease Legislation 24

Treatments 24
Pharmaceuticals 24

Noted Cases 26
Targeting Diseases 26
Family-Driven 28
Other Precision Medicine Successes 29

PAIN 31

Neuropathic Pain 31

Pain Generation 32

Channels 33

Sample Conditions 35
Erythromelalgia 35
Congenital Insensitivity to Pain (CIP) 35
Measurements of Pain 36
Quantitative Sensory Testing 37

PROTOCOL 41

Methods 41
Premise 41
Recruitment 41
Quantitative Sensory Testing Measures 42
Questionnaires 45

Results **45**
Recruitment Efforts 45
Enrollment 46
Patient Phenotypes 46

Conclusion **51**

CITATIONS 52

Gene Discovery

The Human Genome Project

In 1990, the National Institutes of Health (NIH) and the Department of Energy began a project known as the *Human Genome Project* with the goal of sequencing and mapping all of the genes of the human body ("NIH Fact Sheets - Human Genome Project,"). With the addition of this knowledge, the genetic factors underlying diseases could be approached from a new angle, potentially allowing for solutions to a number of incurable illnesses. In 2003, two years ahead of schedule, this project was completed.

One of the fundamental notions stressed during the Human Genome Project was the importance of free access to data. In 1996, the *Bermuda Principles* were created, emphasizing the need to publicize all genetic information within 24 hours of being generated, including prepublication data release ("Genome: Unlocking Life's Code,"). This accentuated the collaborative basis of the project, accelerating the pace of data collection and the number of researchers involved. As stated by Dr. Francis Collins, the director of the National Human Genome Research Institute and one of the two men who oversaw the Human Genome Project, this mindset highlighted that "it's up to the world to do this [project], we shouldn't get special treatment to get more recognition" (*The Human Genome: A Decade of Discovery, Creating a Healthy Future*, 2010). Dr. Collins, with public funding, and Dr. Craig Venter, with private funding, organized the sequencing of the human genome cooperatively (Shampo & Kyle, 2011). In the span of 14 years, the price to decode

an entire genome decreased from $2.7 billion in 2003 to approximately $1000 in 2017 due to improved sequencing techniques such as Next-Generation sequencing (Fig. 1) ("The Cost of Sequencing a Human Genome," 2018). The time to sequence a genome decreased tremendously, from 14 years (the Human Genome Project) to about 1 hour using Illumina, a well-established DNA sequencing company (Buhr, 2017).

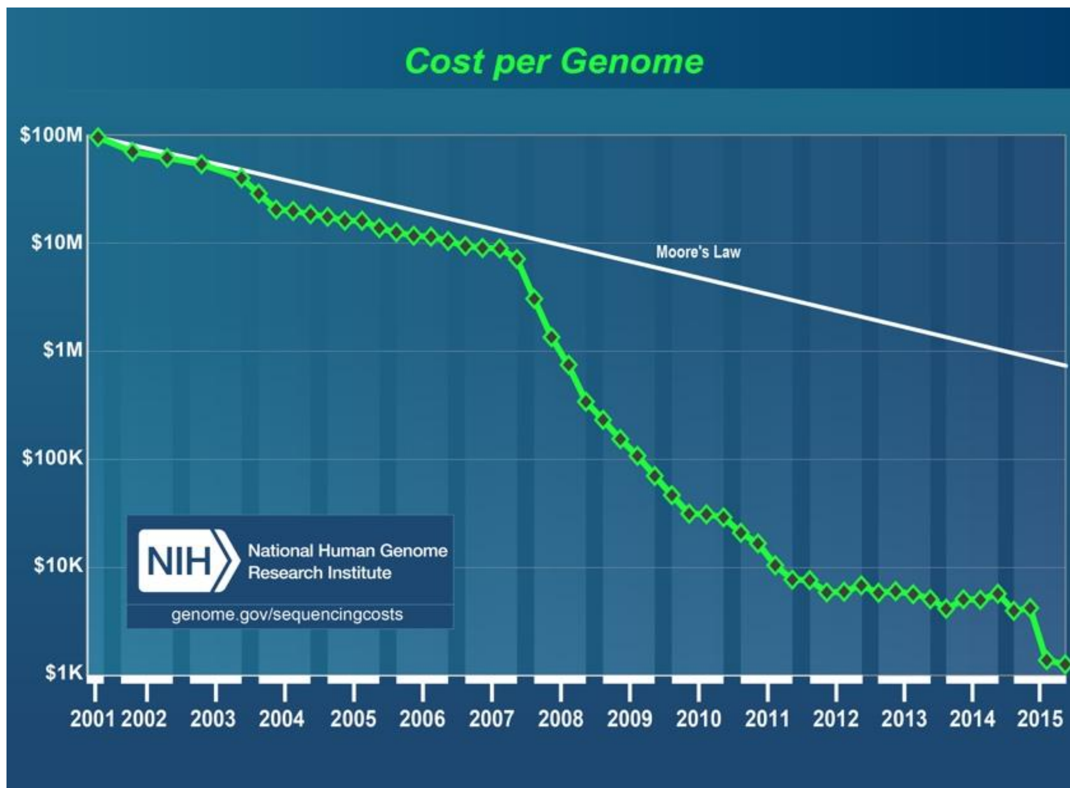

Fig. 1. Decreases in cost of genome sequencing from 2001 to 2015. From "The Cost of Sequencing a Human Genome," 2016, NHGRI, https://www.genome.gov/27565109/the-cost-of-sequencing-a-human-genome/

Sequencing Techniques

Sanger-Based Sequencing

Part of the tremendous decrease in price to sequence a genome was due to the advances in genetic sequencing methods. The Human Genome Project was completed using Sanger-based sequencing technologies ("DNA Sequencing Costs:

Data," 2018). Sanger sequencing, otherwise known as the chain termination method, was developed by Nobel laureate Frederick Sanger and his colleagues in 1977 and requires three main steps to determine the nucleotide sequence.

First, DNA fragments of various lengths are generated, each terminating with a labeled nucleotide. This terminating, labeled nucleotide is a dideoxynucleotide, as it can attach from its 5' end to the previously formed strand but is missing the hydroxyl group on its 3' end, preventing another nucleotide from attaching. Once strands of varying lengths have been formed, they are sorted by length via denaturing (capillary bed) electrophoresis. Here, the short strands travel faster, reaching the end of the capillary bed first. The final step involves using a laser to excite the labeled dideoxynucleotide, emitting a light that can be associated with the correct base. Put together, this generates a chromatogram in the correct order due to the prior electrophoresis ("Sanger Sequencing Steps & Method,").

Next-Generation Sequencing

In 2008, there was a shift in techniques used to sequence genomes as researchers began to focus on next-generation sequencing (NGS). NGS made it economically feasible for the average researcher to sequence a whole genome, exponentially increasing the number of labs able to collect data ("Next-Generation Sequencing (NGS) | Explore the technology,"). What makes next-generation sequencing less costly is its ability to process millions of DNA strands in parallel rather than the 96 at a time via Sanger sequencing. Cloning bias, or the tendency of certain regions of the genome to be represented less due to factors such as AT-richness, may be prevented via next-generation sequencing as well due to not performing conventional vector-based cloning (Mardis, 2008) (Clark, 2012).

Next-generation sequencing relies on a mixture of template preparation, sequencing and imaging, and genetic alignment and assembly methods. The genomic DNA is typically broken into smaller fragments to prepare the templates. Adaptors, or short DNA sequences, are added to these smaller fragments. These pieces are then denatured to form single-strands and are subsequently immobilized to a solid surface, allowing a multitude of sequencing reactions to occur simultaneously. Solid-phase amplification and emulsion PCR are used for amplification of the templates (Fig. 2) (Metzker, 2010) (Knief, 2014). Primers and fluorescently-labeled dideoxynucleotides are added to the mixture, which can then bind to the single-stranded DNA. Lasers are passed over the strands and their fluorescence is detected via a camera and recorded via a computer ("What is the Illumina method of DNA sequencing?," 2015). With this technique, genome sequencing can be done for approximately $1,000, making it a useful technique for health professions and medical care (Mardis, 2008).

At such an affordable cost, next-generation sequencing is being embraced in clinical practice and as an integral part of genetics research. For example, it has allowed researchers to categorize cancer as genomic as well as discover new cancer-related genes. It has also led to the opportunity for targeted treatments based

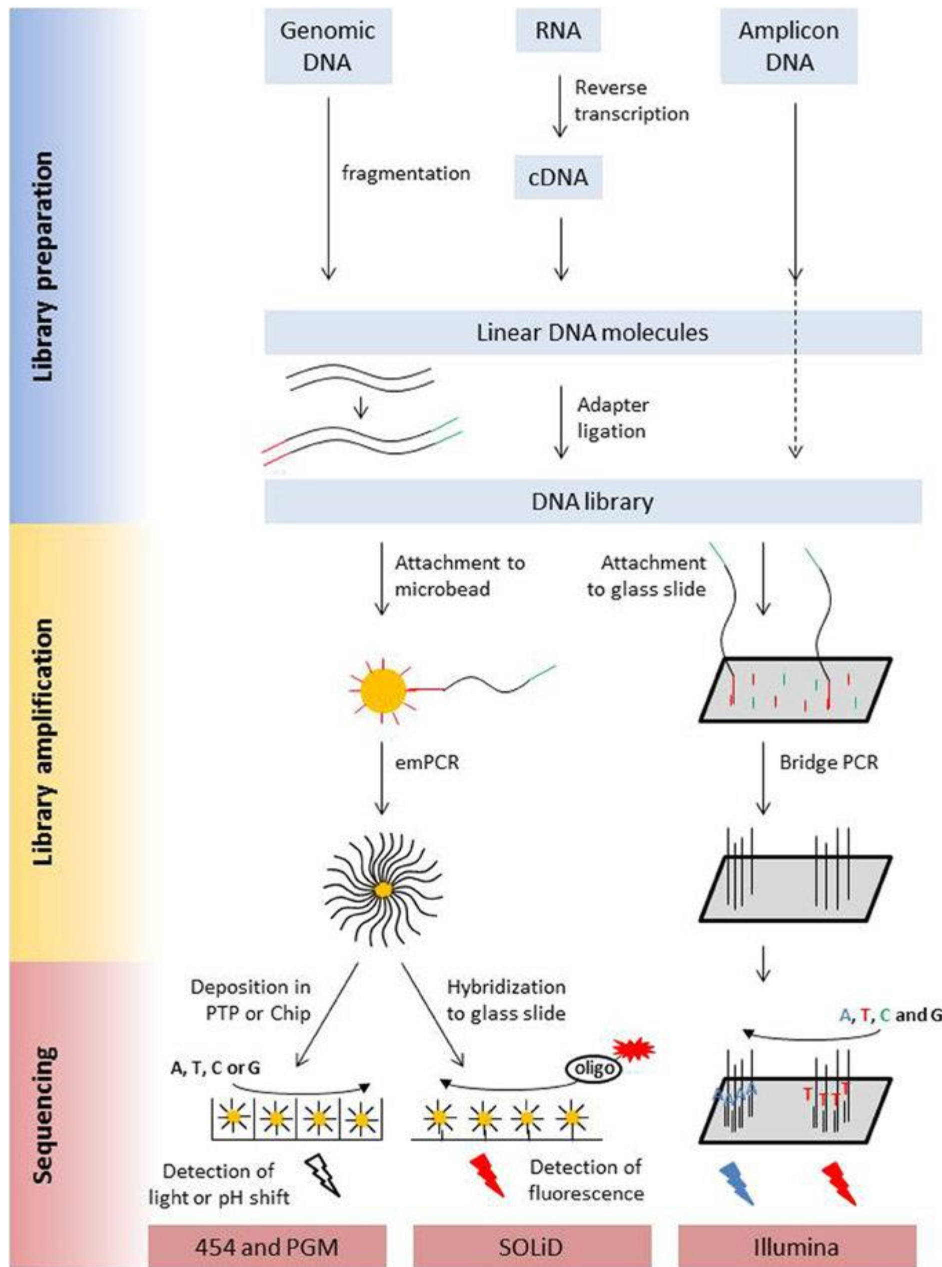

Fig. 2. Next generation sequencing process. From Knief, C, "Analysis of Plant Microbe Interactions in the Era of Next Generation Sequencing Technologies", 2014, Frontiers in Plant Science, 5:216.

on a specific genetic mutation. Clinically, the sequenced data can serve as a great diagnostic and prognostic instrument to help a healthcare provider specify a patient's characteristics (Zhao, 2014).

Bioinformatics

With all the new information generated via next-generation sequencing comes the necessity for the field of bioinformatics. This discipline uses intensive computational tools and modeling to interpret massive amounts of genetic data. Sequencing information can therefore be converted from signals to data to interpretable information, which can finally be used in the discovery of genomic knowledge (Oliver, Hart, & Klee, 2015).

While the cost and time of DNA sequencing have decreased tremendously, the cost of processing the information (the bioinformatics), has not seen such a drop and is not assumed to follow this trend anytime soon (Christensen, Dukhovny, Siebert, & Green, 2015). Once the DNA is sequenced, it is sent to laboratories to align reads, determine sequencing depth, and is converted into the appropriate files for bioinformatics analysis. Variants are filtered via computer algorithms based on what is available in public databases, the frequency within a respective family, and functional predictions. These results are then manually curated to ensure correct classifications of variants. Allele frequency clinical databases have arisen to help with this endeavor, showing relationships between medically significant variants and phenotypes (Landrum et al., 2014). These include both public databases such as ExAC and private databases such as TriNetX. Though access to allele frequency clinical databases reduces the quantity of variants needed to be manually reviewed, the novel variants that will arise as well as the time taken to assess and reassess the variants and their pathological associations creates a limitation on the ability to decrease genome interpretation costs (Christensen et al., 2015).

The Bermuda principles have held strong over the years, allowing computational biologists to take full advantage of many datasets shared in repositories. Nevertheless, this vast amount of data poses an issue in terms of the time necessary for its download and the computational power needed for storage and analysis. For reference, one byte contains about four bases and the basic output of sequencing results in about 600 billion nucleotides. Compression-based methods have been created to minimize these costs by ignoring the sample 'noise,' but the improvements have yet to parallel those seen in sequencing. In fact, one could argue that it is more cost-effective to re-sequence a sample than it is to store one. However, logistically this is not an option as we cannot retrieve organ tissue and antibody samples regularly (Sboner, Mu, Greenbaum, Auerbach, & Gerstein, 2011).

Even after the data has been roughly managed, the results need to be interpreted. This requires a multidisciplinary team that can link the unbiased data to phenotypes while not overestimating each variant's significance. As this activity requires a lot of expertise and work, it cannot be automated, resulting in a process that may often take up to months to complete thoroughly and precisely (Fig. 3) (Sboner et al., 2011).

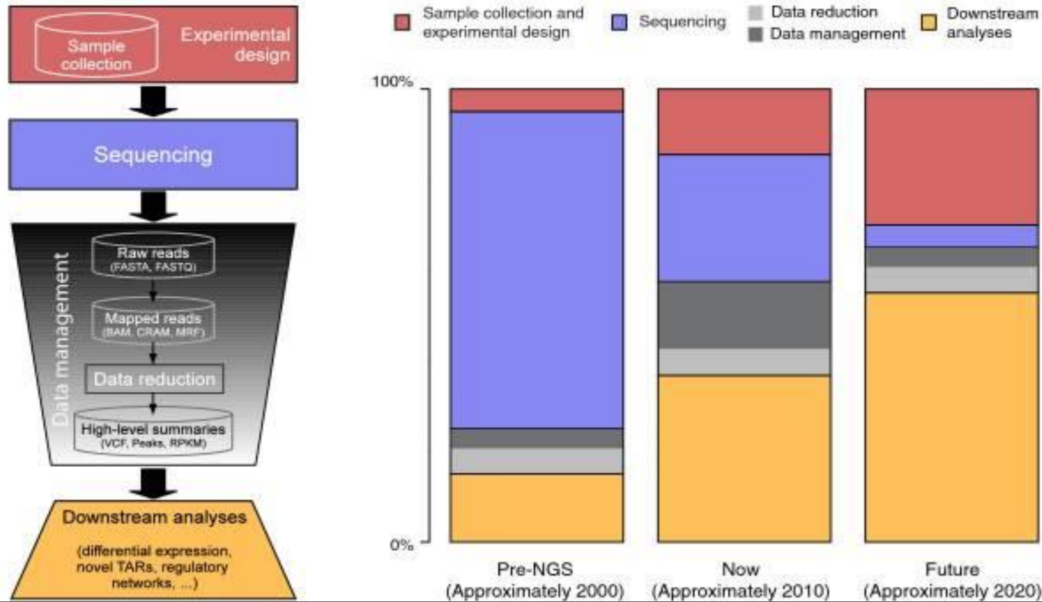

Figure 3. The relative expenses for experimental design, sequencing, data management and downstream analyses over time. From Sboner, A. et al, "The real cost of sequencing: higher than you think!", 2011, Genome Biology, 12(8): 125.

Medical Care and Follow-Up

Following the recommendations of the American College of Medical Genetics (ACMG) in 2013, many laboratories began to proactively screen for genetic variants that have the potential to carry actionable disease risk (Green et al., 2013). The selection for how many genes they screen for and which variants are analyzed is dependent on the context and risk of the screening. These types of diagnostic screenings have shown notable successes in pediatric cases. An observational study at Baylor College of Medicine found that sequencing resulted in diagnosis for approximately 25% of the cases., 57% of which were previously unreported. Patients with rare diseases have also benefitted greatly from sequencing (Christensen et al., 2015). However, the results of these studies and its interpretation in the clinical context may also pose a burden to clinicians and patients that would likely result in additional procedures and otherwise unnecessary

follow-ups that may drastically increase the price of a clinic visit. They also have the potential to lead to overdiagnosis and overtreatment, increasing the risk of harm (Christensen et al., 2015). It is important that healthcare providers, when given the bioinformatic interpretation of genetic results, are prepared to use the genomic information effectively rather than just ordering an additional number of diagnostic tests (Vassy et al., 2014).

The *MedSeq* Project is trying to integrate whole-genome sequencing (WGS) and clinical medicine. To do this, they seek to address how genomic results should be processed and reported by laboratories in a manner that brings to light the results while not oversimplifying the data and still emphasizing the uncertainty of its significance. They also are looking into how physicians should discuss and manage this information with their patients and how the delivery of the material will impact the further healthcare-related proceedings (Vassy et al., 2014). In 2015, a preliminary one-page summary report of sequencing results was created to demonstrate how this information could be conveyed to physicians and patients (Vassy et al., 2015). While improvements are still being made, this report provides one step toward integrating genomics into the clinical practice.

Similarly, families at Boston Children's Hospital and Brigham and Women's Hospital are enrolling babies in a similar study known as the *BabySeq* Project. This study is looking at newborns who are either sick or healthy, and for each population group, half get their genomes sequenced and reported to the clinician and family. However, they are not only looking at the best method to discuss the genomic reports, but also at how the data influences the medical

management of the infants and the medical, behavioral, and economic impacts of introducing this new set of data in infant care (Holm et al., 2018).

Application of the Human Genome Project

International HapMap Consortium

Once completed, the Human Genome Project began to be used for comparisons of genotypic differences between those with and without various diseases. The International HapMap Consortium - one way of approaching this - was created in 2005. This haplotype map provides a reference for all the single nucleotide polymorphisms (SNPs), or the sites in which DNA bases vary frequently, across many human genomes. SNPs grouped together are inherited as a block of DNA known as a haplotype ("International HapMap Project,"). New haplotypes may be formed via mutations (which occur at a very low rate) or via genetic recombination during meiosis. As the likelihood of SNP alleles being inherited together corresponds with their proximity on the genome, associations can be made with various alleles in a population. This is known as linkage disequilibrium ("The International HapMap Project," 2003). The HapMap project allows for a smaller quantity of variants to be analyzed by forming these haplotypes, reducing the cost and time of sequencing (Eisenstadt, 2017). With collaboration between six countries, the common genetic variants across the world can be analyzed ("What is the International HapMap Project?," 2019).

The HapMap project therefore notes different variants, their frequencies, and any correlations between them and a phenotype. By documenting this worldwide, indirect associations can be made between SNPs and risk factors for

diseases as well as correlating specific variants to certain populations ("The International HapMap Project," 2003).

Genome-Wide Association Studies

The Human Genome Project has also been used to perform genome-wide association studies (GWAS). These studies work by comparing the genomes of various populations and searching for similarities (*The Human Genome: A Decade of Discovery, Creating a Healthy Future*, 2010). Typically, a GWAS collects data to look for common variants within a specific population using SNP arrays, relying on the "common disease, common variant" hypothesis (Pearson & Manolio, 2008). Variants associated with the respective phenotype may appear at a higher frequency when compared to the SNPs of a control population. That variant can then be analyzed to determine its statistical likelihood of being associated with the given trait ("What are genome wide association studies (GWAS)?,"). According to the NIH, GWAS are studies of common genetic variations "across the entire human genome designed to identify genetic associations with observable traits" (Pearson & Manolio, 2008).

Typically, GWA studies have four parts: first, a large number of individuals with a trait of interest are selected along with an appropriate control group. Next, the participants are genotyped to a high-quality level to maximize accuracy. Then, statistical analysis is performed to look for associations between SNPs within and between the population groups. Finally, GWAS are replicated in independent populations to examine the reproducibility of those results (Pearson & Manolio, 2008).

GWAS rely on and take advantage of linkage disequilibrium (LD). Through this, GWAS can detect associations between genetic variants and traits, taking into account variant frequency and the number of loci between the respective variants (Visscher et al., 2017). They have had immense success in identifying genetic variations that contribute to the risk of various diseases, uncovering the underlying biological basis. For example, in 2005, three separate studies found a correlation between blindness and a variant in the gene for complement factor H (which is involved in inflammatory regulation). This mutation is now referred to as age-related macular degeneration. Similar correlations have also been found for type 2 diabetes, Parkinson's disease, heart disorders, obesity, and some cancers ("Genome-Wide Association Studies Fact Sheet," 2015).

GWAS do not guarantee an outcome, but they do inform about an increased risk for an individual. As said by the Broad Institute, GWAS can be “most accurately viewed as a ‘hypothesis-generating’ endeavor” (Eisenstadt, 2017). Direct to consumer genetic companies such as 23andMe and Knome use this strategy to provide customers with their genome-based risks for various common conditions (Norrgard, 2008).

The GWAS approach permits comparisons of a multitude of unrelated individuals, unconstrained by preconceived hypotheses. However, it also creates a large potential for false-positive outcomes, affecting the reasonable cutoffs for what is considered to have statistical significance (Pearson & Manolio, 2008). In 2011, 96% of GWAS data subjects were noted to be of European ancestry (Bustamante, Vega, & Burchard, 2011). Due to this lack of diversity, those of non-European

descent have a higher likelihood of receiving false positive genetic results (Popejoy et al., 2018). It also results in an overestimate of relative risks for common diseases and may be difficult with old-age onset disorders. Finally, it is highly vulnerable to genotyping errors (Pearson & Manolio, 2008).

Using a GWA study, variants in a DNA sequence are identified. This may be done using a SNP chip or a microarray with software that can perform heavy computation to identify the different variants (Leek). Each SNP chip contains a particular set of bases that are complementary to the SNPs of interest. The selected SNPs are chosen to effectively capture the genetic variations of a genome (LaFramboise, 2009).

Next, confounders are taken into account. One of the most common confounders is population stratification, or the variants and diseases that are common within a population. This can be addressed by software such as Eigensoft (Leek). Additionally, as there are so many comparisons being made, Bonferroni adjustments are made to reduce the Type I errors that may occur (Johnson et al., 2010).

Following this, statistical tests are performed, variant by variant, to determine the association with a particular outcome. A p-value can be established for every SNP and Manhattan plots can be created. Manhattan plots are set up with the y-axis depicting the $-\log_{10}$[p-value]. This results in lower p-values being higher on the plot (Leek). Each dot in the plot represents one haplotype, with vertical lines arising from the same genetic location (Fig. 4). The p-value that determines the height of the data point is related to the relative frequency of the haplotype in that

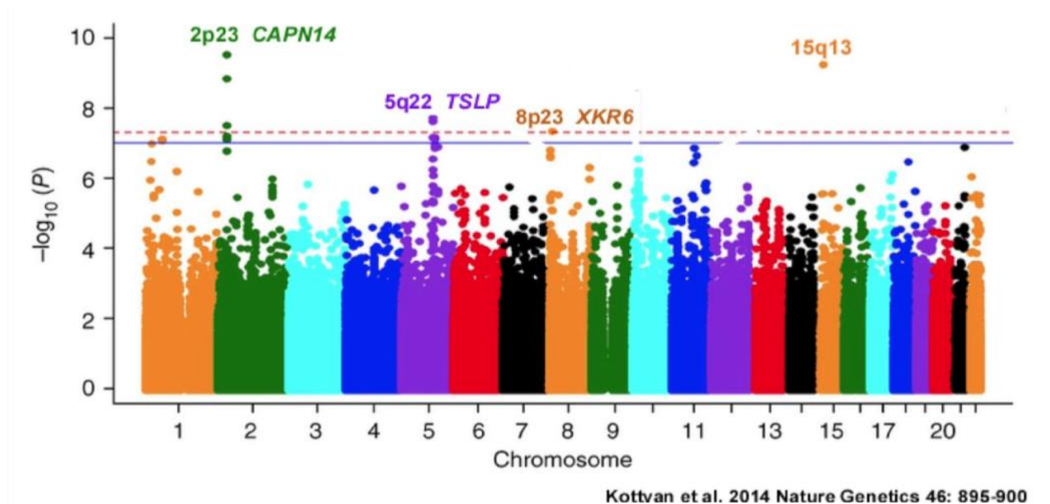

Figure 4. Output from Manhattan Plots. From Meneely et al, "Understanding Manhattan Plots and Genome-wide Association Studies," 2017, Oxford Academic, https://www.youtube.com/watch?v=Pdic7p_dk0I

region in individuals that are affected vs. the control sample ("Understanding Manhattan Plots and Genome-wide Association Studies - YouTube," 2017). GWAS use a p-value of about 10^{-8} to securely identify a genotype-phenotype association (Johnson et al., 2010).

Once a variant is visualized on these plots, it can be analyzed to note if it is a casual variant for the particular trait. Software such as PLINK can help identify if SNPs are associated with any specific exons and could be relevant factors in the respective phenotype (Leek). The development of sequence-based -omic analyses have led to the association of variants with potential mechanistic interferences. Expression quantitative trait locus (eQTL) studies focus on the correlations of sequences and complex traits (Visscher et al., 2017).

Animal Studies

The Knock-Out Mouse Project (KOMP) was started in 2003 ("Knockout Mouse Project,"). This project strove to create a mouse in which one gene is knocked out for every protein-coding region in the mouse genome. This strategy

has helped in studying the effects of loss-of-function alleles. Similarly, knock-in mice could be used to study gain-of-function and dominant-negative alleles (Boycott, Vanstone, Bulman, & MacKenzie, 2013).

Another technique used in animal models to look at gene function is via CRISPR-Cas9 or Clustered Regularly Interspaced Short Palindromic Repeats. Similar to CRIPSR's intrinsic function in bacteria, researchers create small RNA sequences that bind to a specific target of DNA. This RNA sequence also binds to the Cas9 enzyme, which then cuts the DNA at a specified location. Once cut, researchers may add or delete pieces of genetic material via the cell's own repair system ("What are genome editing and CRISPR-Cas9?," 2019).

As many species such as rats, mice, and monkeys have similar genomes to humans, we frequently extrapolate findings from animal study research to our species. However, this translation is not exact, resulting in issues of toxicity or unexpected side effects of potential treatments. In fact, John P. A. Ioannidis in his paper *Extrapolating from Animals to Humans* noted "limited concordance exists between treatment effects in preclinical animal experiments and clinical trials in human subjects" (Ioannidis, 2012).

Orphan Diseases

Another manner in which the genetics from the Human Genome Project has been used is to study orphan diseases. An orphan disease can be defined as a condition that affects under 200,000 people in the United States. Termed in 1983 by Congress, rare diseases became otherwise known as orphan diseases due to the lack of interest and adoption from pharmaceutical companies. From their perspective, these uncommon diseases are not worth the investment due to the

minimal profit a theoretical treatment would generate. Additionally, were they to investigate a rare disease, it would be a challenge to successfully perform a traditional clinical trial due to the low prevalence within the population. That being said, there may be as many as 7,000 rare diseases affecting approximately 25-30 million citizens of the United States ("FAQs About Rare Diseases | Genetic and Rare Diseases Information Center (GARD) an NCATS Program,"). These diseases are typically chronic, progressive, disabling, and potentially life-threatening (Wastfelt, Fadeel, & Henter, 2006). They are also often of genetic origin, so exploration into the diseases may assist in providing more knowledge about the biological processes of the human body.

Using next-generation sequencing, rare-genetic-disease research has identified many causative genes (Boycott et al., 2013). Most rare diseases can be traced to mutations in a single gene, making them monogenic ("FAQs About Rare Diseases | Genetic and Rare Diseases Information Center (GARD) an NCATS Program,"). By studying these monogenic diseases, insight can also be made for polygenic forms of more common diseases (Chial, 2008). Long-term complications of rare disease symptoms may also be lessened if diagnosed and treated earlier (Boycott et al., 2017).

As of 2013, whole-exome sequencing is the preferred method for the discovery of rare-disease-causing genes. Comparing sequences to public databases reduces the approximate 20,000 variants typically found in whole-exome sequencing to under 500 variations for rare-disease analysis. When the disease is known to be hereditary, the likelihood that its phenotype-causing mutation is

monogenic increases (Boycott et al., 2013). Rare diseases may be phenotypically presented as mosaic mutations (present in only a portion of the body cells), gain-of-function mutations, etc. In general, genes causing recessive and *de novo* dominant disorders are easier to identify than other mutations, as shown by the diseases discovered to date (Boycott et al., 2013).

Many organizations have been created to facilitate the progress of rare-disease research, such as the International Rare Diseases Research Consortium (IRDiRC). This association unites international governmental and non-profit funding bodies, companies, umbrella patient advocacy organizations, and researchers to create a space for collaboration across various continents ("About Us," 2019). Sites, such as Orphanet, have created a searchable database of centers, organizations, known drugs, and physicians that are relevant for each rare disease. Thus, finding information about an orphan disease has become much easier and connecting those with the condition worldwide is more manageable ("The portal for rare diseases and orphan drugs," 2019).

Precision Medicine

With these genetic discoveries came the field of precision medicine. This approach "takes into account individual variability in genes, environment, and lifestyle for each person," providing a more specific treatment instead of a one-size-fits-all solution ("What is precision medicine?," 2019). This specificity may include taking into account family history, newborn disease screenings, or tailoring preventions to one's genetics ("Precision Medicine: What Does it Mean for Your Health? | Features | CDC," 2018). It allows for a shift from organ-based medicine

to a more realistic all-inclusive assessment of health (Beckmann & Lew, 2016). While this concept has been considered for centuries such as in blood donations and other medical treatments, the field grew with the expansion of knowledge that came from the Human Genome Project.

One way in which precision medicine is applied to healthcare is through pharmacogenetics. This can be seen via the analysis of drug metabolism or the development of an ideal drug in terms of efficacy and safety based on an individual's genome (Hodson, 2016). Inherent in the strategy of using precision medicine comes the maximization of clinical outcomes and minimization of side effects for the respected individual (Jameson & Longo, 2015). Through sequencing, one can become aware of the mutation of a specific inhibitor or enhancer that needs to be targeted to increase the likelihood of a successful treatment.

For instance, codeine is a drug that becomes converted into its active form, morphine, by the polymorphic cytochrome P450 CYP2D6 enzyme system within our bodies. The metabolism of codeine varies within the human population based on the number and efficiency of one's gene alleles. Those with over 2 functional CYP2D6 genes may be at risk for serious adverse events due to their quick metabolism. While these fast metabolizers make up 1-2% of the general population, they make up about 30% in certain areas of the world. St. Jude's Research Hospital approached this issue by genotyping 230 pharmacogenes and integrating the test results into clinical settings. This has allowed healthcare professionals to determine drug dosages based on individualized genetic information (Hudak, 2016).

Personalized drug development has unfortunately been shown to be extremely complex, including innumerable genetic alterations and heterogeneity not only between, but also within, individuals. Examples of this have been seen in cancers. The heterogeneity both leads to drug resistance and the need for multiple angles of treatment (Hoelder, Clarke, & Workman, 2012). Another challenge of pharmacogenetics is known as the 'druggability gap,' in which the drug targets have shown to be beneficial if successfully reached, but the ability to get to them via a small molecule is extremely difficult (Hoelder et al., 2012).

Nevertheless, with the fall in cost of genetic sequencing, electronic medical records may be prepopulated by genetic data, supplying healthcare providers with applicable information regarding which patient may benefit from a certain treatment vs. another and how the individual's body might process the drug (Jameson & Longo, 2015). As a matter of fact, drug targets influenced by genetics have a higher likelihood of making it to a phase III clinical trial and to the market. This suggests a potential cost-efficient solution for pharmaceutical companies (Visscher et al., 2017).

Another significant possibility brought forth by precision medicine is drug repositioning. This involves repurposing existing drugs to tailor a treatment for individual patients. This idea is based on the premise that an existing drug has already passed clinical trials and will be less of a risk in terms of toxicity than a newly developed drug, resulting in faster drug approval (Li & Jones, 2012).

While precision medicine focuses on the individual by collecting a multitude of data on each person, it is limited by its specificity. With millions of

data points per person, each case may be so unique that the population of interest would be limited to an n = 1 scenario. If so, it may be hard to determine the efficacy of a drug due to statistic power limitations and may restrict the ability to compare the results to a control group (Beckmann & Lew, 2016).

Pharmacology Development

Animal Model to Human Model

Once a molecule has been found that acts on a target of interest, it has to be tested for efficacy in computerized models, cells, and animals. This drug must express high specificity, potency, and selectivity, and show safety for the individual (Brodniewicz & Grynkiewicz, 2010). If successfully tested *in vitro* in assays, it is then further analyzed in pre-clinical testing, or the discovery phase. The discovery phase is carried out on animals in multiple dosage groups to determine the efficacy of the drug *in vivo* (Brodniewicz & Grynkiewicz, 2010). If the preclinical testing shows promising results, it can be transitioned into clinical trials. Clinical trials occur in three phases: safety, efficacy in the desired population group, and efficacy relative to the current treatment, respectively.

However, as mentioned before by John Ioannidis, there are still difficulties correlating treatment effects in animal trials with human clinical trials (Ioannidis, 2012). This may be in part to animal models not capturing the human physiology and disease to an appropriate extent. Additionally, bias within an animal study design may also contribute to the lack of correlation. Nonblinding and nonrandomization in animal studies have a 3.2- and 3.4-fold higher likelihood of claiming statistical significance in their animal model studies compared to those that were randomized and blinded (Ioannidis, 2012). Furthermore, there is a risk of selective outcome and analysis reporting. In other words, if desirable results are obtained, the studies will be published even if, in reality, the treatment is not effective (Ioannidis, 2012). Even in journals such as Nature and Cell, only 37% of the most-cited animal studies were replicated in subsequent human randomized

trials and 18% were contradicted in clinical trials (Scarborough, 2017). One famous instance of poor translation from animal models to the human population was with thalidomide, a drug put on the market to treat morning sickness for pregnant women. While proven to be safe in mice and rats, it caused "an international epidemic of birth defects" in the middle of the 20th century, causing limb malformations and other deformities (Scarborough, 2017). Due to this incident, we now test in pregnant and infant animal models, looking at age-specific risks. We are also able to take human liver cells to analyze drug metabolism and functionality.

While it is precarious to rely on solely animal data to predict the success of an intervention in the human population, challenges arise when testing for diseases that are not ethically testable in clinical trials (Ioannidis, 2012). In these cases, the Food and Drug Administration (FDA) can grant approval of a treatment based on adequate and well-obtained data from animal studies. These studies are required to have strong reasoning that the treatment would prove clinically beneficial for humans ("Product Development Under the Animal Rule Guidance for Industry," 2015).

Toxicology

Most drugs shown as safe in animals fail in clinical trials because of safety and efficacy. In fact, "the typical compound entering a Phase I clinical trial has been through roughly a decade of rigorous preclinical testing, but still only has an 8% chance of reaching the market" (Shanks, Greek, & Greek, 2009). Animal models provide information about how a drug is absorbed and how it interacts with surrounding tissues as well as how it is eliminated (Scarborough, 2017). However, animal models are likely to absorb and process a drug differently than the human

species. If this difference is large enough, it may result in detrimental effects in one species and not the other. Thalidomide, for example, breaks down much faster in rats and mice compared to humans. Additionally, the embryos of rats and mice also have increased antioxidant defenses, preventing the birth defects often seen in human embryos (Scarborough, 2017).

While toxicology has advanced greatly since the era of thalidomide for traditional small molecules directed at protein targets, etc., a new field of toxicology has been created for drugs targeting transcription, translation, and gene insertions. To convert dosages from animal models to humans, careful consideration of body surface area, pharmacokinetics, and physiological time is necessary. Although commonly perceived to be the case, dosage conversions are not based on body weight alone. (Nair & Jacob, 2016) In the dose by factor method, one approach for assessing the initial dose, selection is based on the minimization of dose toxicity in the preclinical toxicological studies to establish the human equivalent dose (HED) (Nair & Jacob, 2016). This level of dosage is termed the No Observed Adverse Effects Level (NOAEL) in the animal species. Once the NOAEL is selected, it is converted to a HED based on the body surface area correction factor between the specific animal model and humans. The HED is then divided by a factor of 10, increasing the safety of the first respective clinical trial and accounting for any physiological and metabolically distinct differences between species (Nair & Jacob, 2016).

More so, acute toxicology, or the effect of a single dose on an animal species, is now recommended to be tested out on at least two different species, one

being a rodent, one being a nonrodent. The results from different doses of the drug of interest are observed over the course of a set period of time, with any significant outcomes noted (Parasuraman, 2011).

Orphan Disease Legislation

In the 1960s, amendments were made to federal laws in the United States, mandating that all drugs must be proven to be safe and effective using "adequate and well-controlled studies" before they can be put on the market (Wastfelt et al., 2006). This undoubtedly raised the price of drug development, diminishing the effort pharmaceutical companies spent on orphan diseases. The Orphan Drug Act of 1983 initiated financial incentives for pharmaceutical companies to do research on these diseases ("FAQs About Rare Diseases | Genetic and Rare Diseases Information Center (GARD) an NCATS Program,"). The Rare Diseases Act of 2002 established the Office of Rare Diseases within the NIH, creating a Rare Diseases Clinical Research Network. This network facilitates collaboration between various groups on their respective rare diseases, allowing for multi-site clinical trials and sharing of knowledge (Wastfelt et al., 2006).

Treatments

Pharmaceuticals

Gene therapy, or "the delivery of genetic material to supply gene products that will permanently restore missing function or bring in target tissue / cells a therapeutic gene," has been used clinically to target diseases that have yet to be solved by pharmacology. In particular, neurodegenerative diseases can be targeted

in this manner (Piguet, Alves, & Cartier, 2017). Gene therapy may work to replace a mutated gene that causes a disease, to knockout a mutated gene, or introduce a new gene to help fight a disease ("What is gene therapy?,").

Type of Gene Therapy	Technique	Examples
Viral Vectors: Adeno-associated vectors (AAVs)	These vectors are ssDNA parvoviruses that are engineered via recombination to enter the cell membrane via receptors and deliver the DNA to the nucleus of the cell (Piguet et al., 2017). They do not integrate into host genomes and therefore eventually get diluted as the cell replicates. They are very effective in getting the ssDNA into the cell as well (Naso, Tomkowicz, Perry, & Strohl, 2017).	SMA
Viral Vectors: Lentiviral vectors (LVs)	These are ssRNA viruses that are also highly permeable in cell membranes via receptors. Using reverse transcriptase, the RNA can be used to create DNA to be incorporated into the host's genome. They can be used to specifically target cells or tumors (McIntosh, 2012).	HIV
Antisense oligonucleotides (ASOs)	ASOs knockout mutant genes (Piguet et al., 2017). This occurs by creating the complementary strand to a specific mRNA to inhibit its expression and prevent it from forming RNA (Dias & Stein, 2002).	SMAs; Spinraza
CAR-T cell therapy	This involves genetically engineering T-cells (immune cells) to express artificial proteins (chimeric antigen receptors) which navigate immune cells to surface tumor antigens. This technique is used often in cancer treatment (Mollanoori, Shahraki, Rahmati, & Teimourian, 2018).	Leukemia
CRISPR/cas9 system	The Cas9 enzyme works to cut DNA at a specific locus so DNA can be added or removed. Once cut, the cell's natural DNA repair system comes in to fix the damage, repairing the mutation that is also present ("What is CRISPR-Cas9?,").	Potential for Sickle Cell disease

Noted Cases

Targeting Diseases

Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) attacks the body's immune system by targeting CD4 cells. CD4 cells are T cells that are important for our ability to fight infections. Untreated, HIV reduces the amount of CD4 cells, weakening the body's defense capabilities ("About HIV/AIDS | HIV Basics | HIV/AIDS | CDC,").

A genetic mutation *CCR5-delta 32* results in HIV resistance, impeding the virus's ability to enter those respective CD4 cells. With this mutation, the CCR5 receptor that normally allows for HIV to enter the cell is unusually small and cannot sit on the extracellular cell membrane. About 1% of people are homozygous for this mutation, implying they are completely resistant to HIV. Another 10-15% have one copy of the mutation, reducing (although not eliminating) their susceptibility to the virus (Paoli, 2013). This mutation's phenotypic outcome could be theoretically replicated in a drug to lessen the danger of HIV.

Sickle Cell

Sickle cell anemia is a disease in which there is an insufficient amount of normal red blood cells in the body to carry enough oxygen to tissues and organs. The red blood cells, which are normally round and flexible, take on a crescent moon shape due to the disease. The abnormal shape gets stuck in smaller blood vessels, which slows or stops blood and oxygen from getting to various areas of the body ("Sickle cell anemia - Symptoms and causes,").

At Boston Children's Hospital in combination with Dana Farber, 21-year-old Manny Johnson presented with a form of sickle cell disease that was so severe

that he needed monthly blood transfusions (Fliesler, 2018). While the disease affected the conformation of his adult hemoglobin, the researchers looking at his genotype noticed his fetal hemoglobin genes were not affected. Because the production of fetal hemoglobin is inhibited by the gene *BCL11A,* the researchers decided to silence it. This allowed Manny to produce fetal hemoglobin once again, minimizing (and potentially even reversing) the symptoms of sickle-cell disease (Fliesler, 2018).

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is a disease characterized by weakening and wasting due to a loss of motor neurons. It is typically worse in the proximal areas of the body relative to one's extremities and worsens with age ("Spinal muscular atrophy,"). SMA has been discovered to be caused by the lack of *SMN* protein, formed mostly by *SMN1*, but also by *SMN2*. Those without a functional *SMN1* will show symptoms of SMA, regardless of their copies of *SMN2* (Ellis et al., 2018).

Spinraza is an antisense oligonucleotide (ASO) approved in 2016 by the FDA to treat SMA. Spinraza works to target *SMN2* and increase the amount of *SMN* protein it creates, mitigating the effects of SMA (Ellis et al., 2018). It modulates alternative splicing by binding to pre-mRNA and disrupting the binding of splicing factors in specific spots (Shen & Corey, 2018). Specifically, it binds to *SM2* to enable the production of a full-length protein that can work to make up for the lack of *SMN* protein from the non-functional *SMN1* gene ("European public assessment report (EPAR) for Spinraza," 2017).

Family-Driven

Mila Makovec

Until about age 2.5, Mila Makovec was a very active and accelerated child. By the age of 3, she began to show some abnormal symptoms; her feet turning inward and frequent clumsiness. At age six, she had gone completely blind. She was soon diagnosed with Batten disease, a rare neurodegenerative and fatal recessive genetic disorder. Within a year of diagnosis, a Spinraza-like drug was designed for Mila's exact mutation (Keshavan, 2018).

Batten disease is a lysosomal storage disorder, resulting in a toxic buildup of proteins and lipids in the brain. While various forms of the disease exist and research is being conducted by the FDA for therapeutics, nothing was available for Mila's specific mutation, *CLN7*. Mila's mother Julia Vitarello began a foundation, *Mila's Miracle Foundation to Stop Batten*, to raise awareness and funding for the disease. With the help of social media, Julia connected with Dr. Timothy Yu of Boston Children's Hospital, who both sequenced Mila's genome and proposed a solution. Mila's variant involved a retrotransposon, a 2,000-letter stretch of code, that had been mistakenly inserted into an intron of her *CLN7* gene. This insertion caused exon trapping, inactivating the gene (Yu et al., 2019). While that resulting *CLN7* protein was ineffective, the necessary DNA coding sequence was still present (Keshavan, 2018).

Dr. Yu realized it was possible to use the mechanism of action used in *Spinraza* to undo this exon trap. The ASO specific to Mila was eventually created and tested in her cell cultures. Next, many of the departments at Boston Children's

Hospital worked together to progress the manufacturing, toxicology, and testing stages of the drug (Keshavan, 2018).

The drug *Milasen* was administered to Mila, who had been experiencing worsening symptoms by the end of that year. Since that point, her seizures have decreased, and she has become more alert than she previously was. While it is unlikely the treatment can be used on other patients with Batten disease, it has shown optimistic results for Mila (Keshavan, 2018).

Other Precision Medicine Successes

The Mights

Bertrand, born from Matt and Christina Might, began to worry his parents shortly after being conceived. He was described as "jiggly" and in constant distress. The following months of Bertrand's life were full of misdiagnoses, announced then eliminated shortly after via various screenings. In the meantime, he became wheelchair-bound and hospitalized. As with many other genetic disorders, Bertrand had various facial abnormalities and did not produce any tears. To prevent damage from the latter characteristic, Matt and Christina would treat their son with eyedrops every few hours. Bertrand also rarely seemed to get sick (Mnookin, 2014).

In 2010, by the time Bertrand was three-years-old, the family was contacted by Duke University researchers looking at genetic sequences to diagnose unknown conditions. The researchers assembled a dozen participants with various undiagnosed disorders and sequenced their exomes with their parents' exomes. This comparison created a baseline of about 15,000 variations, most of which could be eliminated due to their association to already known conditions or their frequent variance in the general population. Each patient was left with about 12 variant

genes. Some participants were found to have rare symptoms of a known disease, some had *de novo* mutations on the same gene, some had mutations that accounted for a subset of the participant's phenotypes. Bertrand was found to have a mutation on both of his *NGLY1* genes, resulting in a new glycosylation disorder (Mnookin, 2014).

With this conclusion, the Duke researchers admitted they could not progress in any drug development steps, especially without other patients. However, they did mention the likelihood that ten to fifty other citizens of the United States may have this condition. With this and Matt's previously established internet presence, the Mights wrote a blogpost in May 2012 called *Hunting Down My Son's Killer* and it slowly was spread across the world (Mnookin, 2014).

By June 2013, nine other people with two NGLY1 abnormalities were identified and connected. With acquisition of money and research, the symptoms were dissected, and the disorder understood (Mnookin, 2014). The kids went through a series of procedures for deep phenotyping, revealing multiple biomarkers of the disease. In a conference in June 2015, Matt Might revealed that while they initially thought the symptoms arose from the build-up of a glycoprotein, it turned out many of the disease characteristics arose from the monosaccharide component that normally allows the protein to aggregate but was in fact deficit in those with the disorder. When Matt gave Bertrand this monosaccharide, he cried his first tear and his seizures ceased. Soon after, the family went to the state senate and passed the Right to Try bill, giving "any Utah resident with a fatal disease the right to try anything passed Phase 1 safety testing with the FDA" (Might, 2015).

Pain

Neuropathic Pain

Unlike the rare diseases mentioned, neuropathic pain is not generated by a single mechanism, but rather by multiple mechanisms operating together, resulting in phenotypic heterogeneity (Smith et al., 2017). Defined as "pain caused by a lesion or disease of the somatosensory nervous system," neuropathic pain affects as many as 7-8% of adults (Murnion, 2018). Within a diagnostic group, different patterns of sensory abnormalities emerge, hinting at many influences from different underlying pain mechanisms. Likewise, across diagnostic groups, similar patterns of sensory abnormalities may be seen (Cruz-Almeida & Fillingim, 2014). Currently, clinical diagnoses do not focus on pathways and mechanisms underlying pain, making the associated treatments less effective.

Many effective drugs developed from preclinical animal model studies are proven to be ineffective in humans as the animal studies tend to focus on evoked, rather than spontaneous, pain, and do not account for any emotional pain factors (Cohen & Mao, 2014). Pain medications such as opioids and non-steroidal anti-inflammatory drugs (NSAIDS), which target nociceptive pain well, tend to only moderately succeed with neuropathic pain due to the difference between the two and the imprecision within the underlying biological mechanisms of neuropathic pain. In fact, these treatments tend to temporarily reduce the sensation of pain, but lack the ability to target the underlying somatosensory mechanisms (Guedon et al., 2015).

Furthermore, there are little new medications being approved by the FDA that relate to neuropathic pain. From 2005 to 2009, of the approximate 100 new

drugs approved by the FDA, only a few targeted chronic pain (Institute of Medicine (US) Committee on Advancing Pain Research, 2011). Most recently approved drugs are variations of or involve the repackaging of existing molecules.

Pain Generation

The generation of pain in response to injury occurs in four main steps. First is transduction, in which nociceptors, or receptors for painful stimuli, convert a noxious stimulation to a signal. Transmission occurs next, sending the signal along nerve fibers to the Central Nervous System (CNS). Transformation or plasticity subsequently occurs as the signal travels up to the CNS, inhibiting or enhancing the signal at synaptic sites. Finally, perception is achieved by integrating cognitive and emotional significance to the noxious signal (Cohen & Mao, 2014).

Great for evolutionary protective adaptions, this process is very precise in sensing small noxious stimulations, as are other sensory receptors, but the human body is not as adept in dealing with chronic pain such as surgery and disease. Additionally, the divisions of nociceptive and neuropathic pain have different underlying mechanisms, adding to the complexity of finding an effective treatment. While nociceptive pain is localized and typically more somatic, neuropathic pain directly affects the somatic system, bypassing transduction (Cohen & Mao, 2014). Further, neuropathic pain and nociceptive pain both have many subcategories.

The transition from acute to chronic pain is a plasticity-driven event that affects the responsivity to pain. Differences in phenotype may be a result of neuronal, immune, or other cell alterations (National Academies of Sciences, Division, Policy, & Disorders, 2018).

Channels

Action potentials in nerve fibers are generated by ionic currents. With a resting state of about -70 mV, the cell membrane depolarizes if an impulse results in excitation. To depolarize, the impulse membrane has to reach the threshold of about -55 mV. Following this is the rising phase until the membrane reaches its peak of about +40 mV, and then begins to once again repolarize. Repolarization leads to hyperpolarization, or a voltage below -70 mV until it finally returns to the resting potential voltage ("The generation of action potential in nerves," 2014).

Nerve fibers may spontaneously fire for a multitude of reasons, one being the overexpression of sodium (Na) channels in the dorsal root ganglia and around the area of injured axons (Cohen & Mao, 2014). Sodium channels have been proven to be involved in the sensation of pain, some increasing expression more than others in the presence of nerve injury. The proliferation of some sodium channels such as $Na_v1.7$ and $Na_v1.8$ lowers the stimulation threshold needed for a noxious stimulus to reach the CNS, resulting in a higher likelihood of spontaneous pain. Na channels also stimulate central sensitization, or increased responsiveness of the CNS to normal sensory stimulation. This results in sensations that typically are not perceived as noxious to be sensed as so, which is known as allodynia (Cohen & Mao, 2014). Drugs that have tried to target and inhibit sodium channels have been minimal in their efficacy due to their lack of specificity to the subtypes of the channels and often come with a variety of side effects (Cohen & Mao, 2014).

All of the voltage-gated sodium channels (Na_v channels) have four homologous domains, each with six transmembrane helices. During an action potential, the Na_v channels switch from closed to open to inactive. Activation is

mediated by the fourth transmembrane helix and inactivation is mediated by an intracellular section of the channel between domain three and four known as the P-loop (Ekberg & Adams, 2006).

Of the nine types of Na_V channels found in mammals, six are expressed at high concentrations in sensory neurons. $Na_V1.1$, $Na_V1.2$, and $Na_V1.6$ channels are expressed in large dorsal root ganglion (DRG) neurons, exhibiting fast activating and inactivating sodium currents. $Na_V1.8$ and $Na_V1.9$ channels generate slow-inactivating and persistent sodium currents and are expressed in small DRG neurons. $Na_V1.7$ channels exist in DRG neurons of all sizes (Ekberg & Adams, 2006).

Calcium and potassium channels have also been noted to be related to neuropathic pain and drug development has had more success in targeting these voltage-gated channels. This can be seen with treatments such as gabapentinoids (Cohen & Mao, 2014).

Nociceptors can also be broken down into A-delta fibers and C fibers. C fibers are typically small in diameter and unmyelinated, with slow-conducting axons. A-delta fibers are relatively larger and lightly myelinated, resulting in a faster signal conduction. One type of C fiber responds to all pain modalities (mechanical, chemical, and thermal). A-delta fibers, on the other hand, are activated by high-intensity stimuli (Caterina & Julius, 2001).

Sample Conditions

Erythromelalgia

As pain mechanisms are multifaceted and symptoms may be experienced to differing degrees, one approach to obtaining more information on origin and properties of neuropathic pain is to study the extremes of hyper- and hyposensitivity. Familial erythromelalgia is widely recognized as a genetic model of human pain, and in some cases, it is clearly associated with a mutation that causes gain-of-function changes of sodium channels $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$, activating mutations in gene *SCN9A* and triggering pain attacks (McDonnell et al., 2016). Many with the autosomal dominant mutation, it is characterized by intense, burning pain, severe redness, and increased skin temperature (Mark Denis P. Davis, 2018).

Familial erythromelalgia is often shown to have onset within the first 10 years of life (Tang, Chen, Tang, & Jiang, 2015). It is usually diagnosed by screenings for the *SCN9A* mutation and treatment is typically unsatisfactory. Concentrated in the extremities, aggravations such as warm temperatures, exercise, and gravity and can be soothed with cooling and elevation. Another method of treatment is through sodium channel blockers such as lidocaine (Tang et al., 2015).

Erythromelalgia may also develop later in life as a form known as secondary erythromelalgia. This often presents as a secondary symptom to diseases most often myeloproliferative in nature (Effraim, Waxman, & Greene, 2007).

Congenital Insensitivity to Pain (CIP)

On the other side of the spectrum is pain hyposensitivity, a recessive mutation. When *SCN9A* is inactivated due to mutations on both alleles, the $Na_v1.7$

channel is no longer transcribed. This results in a loss-of-function phenotype known as Congenital Insensitivity to Pain (CIP). These patients do not perceive any painful sensation in the presence of a noxious stimulation (McDermott et al., 2019).

A critical barrier to drug development for this population group is the lack of understanding of the mechanisms underlying CIP. As we do not currently understand the locus of action, it is unclear whether the $Na_V1.7$ blockers need to cross the blood-brain barrier and whether other developmental side effects are associated with any replication (McDermott et al., 2019).

Pakistani Family

One case of CIP was found in a group of related families in Pakistan, who were found to have a novel mutation in their *SCN9A* gene. It was first noticed in one of the children doing street performances such as walking on coals and stabbing his extremities with knives. Then, his relatives were found to all have bruises and other injuries (Hopkin, 2006).

Without the sodium channel generated by a functional *SCN9A* gene, pain signals do not reach the CNS. This family was autosomal recessive and felt no pain to various typically noxious stimuli including burns, fractures, and lip bites. Other sensory modalities, including touch, tickle and pressure, warm and cold temperatures, and proprioception showed to be within normal ranges (Kurban, Wajid, Shimomura, & Christiano, 2010).

Measurements of Pain

Most methods of measuring pain involve structured self-reporting or behavioral observations of the patients. Subjective reports are the most common type of data collection in regard to pain measurement. This may be done via verbal

communication, rating scales, or questionnaires. Rating scales may be portrayed as numerical (pain from 0-100, etc.), depicted by photos of faces, or by descriptions of varying intensity. For example, Melzack and Torgerson introduced a scale in 1971 which instructed participants to select the word that felt most similar to their pain intensity. The options were *mild, discomforting, distressing, horrible, excruciating*. One disadvantage of this system is the tendency for people to choose values in the middle of the scale (Institute of Medicine (US) Committee on Pain, Osterweis, Kleinman, & Mechanic, 1987).

More complex versions of the subjective rating method involve questionnaires such as the McGill Pain Questionnaire (MPQ). This questionnaire provides many vocabulary words typically used to describe pain. Instead of marking one instance on a unidimensional scale, patients can select all the appropriate words (Institute of Medicine (US) Committee on Pain et al., 1987).

Similarly, in a clinical context, a physician may use the patient's observable behaviors to note his or her level of pain. This might involve a patient's capability to do certain tasks. This method has been criticized for the enormous amount of bias that may be present in a healthcare provider's judgement and the variability of behaviors that correlate to a specific level of pain (Institute of Medicine (US) Committee on Pain et al., 1987).

Quantitative Sensory Testing

Quantitative Sensory Testing (QST) was developed as a way to focus on the mechanisms underlying pain. This testing involves 13 non-invasive procedures that provide insight about myelinated Aδ fibers and unmyelinated C fibers, the main pain-conducting nerve fibers, and their respective pathways (Beissner et al., 2010).

While these procedures do not provide the exact source of dysfunction, they do provide a more specific sensory profile and, therefore, assist in providing the patient with a better chance of improvement. These exams are also useful for both loss- and gain-of-function assessments, providing quantifiable measurements, and allowing comparison between patients of similar age and gender (Cruz-Almeida & Fillingim, 2014). Therefore, patients can be compared to their healthy equivalents. As QST is so specific to each patient that it has been referred to as "personalized pain medicine" (Smith et al., 2017).

QST uses a method of limits to determine a patient's sensory profile. In this method, the stimulus is slowly increased or decreased until the participant reports its sensation. The stimuli include non-painful and painful sensations. The non-painful sensations include detection thresholds, used to assess loss of sensation. The painful thresholds consist of pain thresholds and pain tolerances. Limitations of QST include reaction time, a concern in older patients and specific patient populations, and the time it takes to perform a QST – it takes approximately 30 minutes for each complete profile (Rolke et al., 2006). Regardless, QST is known to have sufficient test reliability (Cruz-Almeida & Fillingim, 2014).

Efforts have been made to create a multicenter database of comparing individuals with chronic pain conditions to a normative range. As of 2017, this adult database comprised of over 300 healthy human subjects' sensory profiles and over 200 subjects with various neuropathic pain conditions. In this database, the subjects are classified according to loss or gain of function of their afferent fibers (Smith et al., 2017).

Genetics / Genomics Discovery in Pain Research at BCH

In the Chronic Pain Clinic at Boston Children's Hospital, there are frequent referrals of children with either increased spontaneous pain due to conditions such as erythromelalgia as well as children with reduced sensitivity to pain.

We have been collaborating with a neuroscientist, Michael Costigan, and geneticists, Catherine Brownstein and Casie Genetti, affiliated with *the Manton Center for Orphan Disease Research Gene Discovery Core* at BCH to tie phenotypic information with the genotype of the patients. Many children with hyper- and hyposensitivity and no previous molecular diagnosis have been genotyped through this process. Some have additionally provided potential new leads for variants of interest. Dr. Costigan has been able to use the genotypic information to create mouse models that has some phenotypic similarities to the patients' pain responses.

After a series of novel gene variants were identified, a team at Amgen, Inc., a biopharmaceutical company in Cambridge, MA, became interested in this line of research. As discussed above, there have been few truly novel analgesics developed in many years. The team at Amgen was willing to support high risk, high reward research on characterizing the molecular bases of novel gene variants in patients with these rare disorders of pain sensitivity, as a path to drug discovery. Amgen has a subsidiary in Iceland, deCODE Genetics, that is one of the world's leading gene sequencing and discovery research teams. Through this collaboration, and through the Manton Center protocols, there is an ongoing program of whole genome sequencing and advanced informatics. While this work is very preliminary, these researchers have already identified some potentially interesting targets.

Phenotyping and Variability in Pain Responsiveness

As previously described, QST is a useful approach to phenotyping increased or decreased pain sensitivity. In applying QST to children, it was first necessary to study normal reference ranges in typically developing children, and to ascertain feasibility and tolerability. Our group reported on QST using the Medoc apparatus in the hands and feet in 101 healthy children aged 6-17 years (Meier, Berde, DiCanzio, Zurakowski, & Sethna, 2001). Our lab has been working on creating a juvenile and adolescent database of subjects' sensory profiles. In one study, we measured 60 children aged 7-17 years that were either healthy controls or had juvenile idiopathic arthritis (JIA). QST was performed at their affected joint and the contralateral thenar eminence (Cornelissen et al., 2014). QST data was also collected in 42 pediatric patients with unilateral lower extremity complex regional pain syndromes (CRPS), comparing the data to those of sex- and age-matched healthy controls (Sethna, Meier, Zurakowski, & Berde, 2007). QST can be used to assess reduced pain sensitivity in patients with rare disorders and in patients receiving local anesthetics in clinical use and clinical trials. For example, our lab successfully used QST to evaluate a novel local anesthetic, neosaxitoxin, in a Phase 1 trial of healthy adult males (Lobo et al., 2015). Based on this previous experience, a protocol was adapted for evaluating altered cutaneous sensitivity in children with rare variants in pain responses, as detailed below.

Protocol

Methods

Premise

We performed a cross-sectional, exploratory study. For individuals with suspected hyposensitivity or erythromelalgia, we determined their sensory profiles using QST measurements. We collected additional phenotypic data from patient medical records as well as QST measurements from immediate family members. When possible, we also complemented this information with the genetic profiles of patients enrolled in *The Manton Center for Orphan Disease Research Gene Discovery Core* (IRB: 10-02-0053).

Recruitment

Patients were recruited from the Pain Treatment Service (PTS), Neurology, and Rheumatology at Boston Children's Hospital (BCH). Additionally, outside erythromelalgia patients that had previously been seen were contacted and asked to come in. Potential participants included those suspected to have hyposensitivity or erythromelalgia that were above the age of 6.

The weekly schedules of the Pain Treatment Service (PTS), Neurology, and Rheumatology clinics were reviewed to screen for possible study participants. An introductory letter was sent beforehand to the families of these patients. This letter introduced the study and let the families know that a member of the research team would approach them at the clinic visit. The letter also gave the families the opportunity to opt out of the study and to not be approached further or the opportunity to contact us prior to the appointment to schedule a convenient time to perform the QST.

If the family had expressed interest in participating in the study, we scheduled a separate visit to perform the QST measures on the patient and any interested immediate family members. The QST took approximately 1 hour per subject. All testing was performed after informed consent had been obtained for the patient and family members.

Quantitative Sensory Testing Measures

QST was performed in two areas for each subject, once in the thenar eminence and once on the big toe. Patients were set comfortably in a chair/examination table that provided adequate support for the arms and legs. The chair/examination table allowed easy access to the examined body sites. The testing facility was set at a comfortable room temperature and provided a quiet and neutral environment with no distraction. The patient was also instructed to keep their eyes closed for the duration of the data collection. Instructions were provided clearly and consistently. The measures tested included mechanical detection threshold (MDT), mechanical pain threshold (MPT), temporal summation (wind-up ratio), dynamic mechanical allodynia, cold detection threshold (CDT), warm detection threshold (WDT), cold pain threshold (CPT), hot pain threshold (HPT), paradoxical heat sensation, vibratory detection threshold (VDT), and pressure pain threshold (PPT).

MDT was measured using Von Frey hairs. These are made of nylon monofilaments that exert a precise force when pressed downward. The discrete amount of force applied may range from 0.01 to 300 grams. First, the lightest filament tip was applied to the test area perpendicularly and pressed downwards until the filament bent. Once bent, it was held there for at least two seconds. The subject then indicated whether the filament caused a sensation. If no sensation was

felt, the next filament was used. The filaments were applied in ascending order until the subject answered yes. When so, the same filament was then used twice more. If two of the three sensations were felt, the filament weight was recorded as the subject's value. This entire process was repeated three times to determine MDT.

MPT was measured using both Von Frey hairs and Pin Prick stimulators. Pin Prick stimulators are made of weighted filaments that are attached to a freely moveable weight inside a tube. The weights may range from 8 mN to 512 mN (0.82 gram-force to 52 gram-force). Both instruments were used following the same procedure as MDT. However, instead of instructing the patient to note when they sensed the tool, the patient was instructed to note when the sensation felt *sharp*.

Temporal summation (wind-up ratio) was obtained by taking the heaviest Pin Prick simulator (512 mN) and performing the motion 10 times in a row. A pain rating of 1-10 was recorded after the first sensation and after the tenth sensation. There was a 3-second delay between each occurrence.

Dynamic mechanical allodynia was performed using a SENSELab Brush-05. The brush was swept over the testing area and the participant was instructed to note whether it felt painful. This was performed three times.

CDT, ***WDT***, ***CPT***, ***HPT***, and ***paradoxical heat sensation*** were obtained using the thermal sensory analyzer (TSA) II. This machine delivers heat stimuli to the skin using a 16x16 cm thermode. A central unit generates and transmits the signal to the thermode and a computer unit controls the thermode temperature. Stimuli were delivered with inter-stimulus intervals of 30 s and the baseline thermode temperature was set at 32 ^{o}C. The maximum and minimum temperatures

the thermode could reach were 50.5 ^{o}C and 0 ^{o}C, respectively. For CDT and WDT, the thermode temperature changed at 1 ^{o}C/s. The first four stimuli increased in temperature (WDT). The last four stimuli decreased in temperature (CDT). The subject had a hand-held control to mark when they felt a change in temperature. After each stimulus, the participant was instructed to note whether it felt *warm* or *cold* in order to test for paradoxical heat sensation. For CPT and WPT, the temperature changed at 1.5 ^{o}C/s and returned to baseline at 10 ^{o}C/s. The first three stimuli increased in temperature and the following three decreased in temperature. The participants were instructed to note when the temperature became uncomfortable by clicking the hand-held control.

VDT was obtained using the Vibratory Sensory Analyzer (VSA) module of the TSA II. This module delivers vibratory stimuli to the skin using a flat, round, and plastic 1 cm vibrometer. The subject's hand was placed on a flat area and the vibrometer perpendicularly over the skin. Following the pre-programmed test algorithm, the vibrometer started increasing in frequency until the participant noted a change in sensation via the hand-held control. Eight trials were collected in total, with 30 seconds in between each.

PPT was collected via a pressure algometer. This is a hand-held device that has a small flat disk with a surface are of 1.1 cm^2. The pressure was applied at a rate of 1 N/sec until the subject noted a painful / uncomfortable sensation. The pressure was applied on the thumb nailbed and the procedure repeated three times.

Questionnaires

Questionnaires were provided to all participants, immediate family members, and extended family members. The questionnaires were based on the Pain Sensitivity Questionnaire and the Pediatric Pain Questionnaire and were used to compare the information of those who were able to do QST with those who were not.

Results

Recruitment Efforts

As mentioned above, there were significant efforts directed toward identifying and enrolling patients with a variety of pain related disorders in order to learn more about potential underlying genetic mechanisms. The diagnoses of interest included the following:

- Markedly diminished pain sensitivity
- Erythromelalgia
- Paroxysmal extreme pain disorder
- Hereditary sensory and autoimmune neuropathy
- Insensitivity to local anesthetics

Patients were identified at the Pain Treatment Service at BCH and through referring physicians in the United States and around the world. In addition, a patient-facing ad was posted on the Erythromelalgia Foundation group, Facebook groups, and on the Channelopathy Congenital Insensitivity to Pain group to further boost patient enrollment in the study.

Participant enrollment entailed a conversation around Informed Consent, obtaining informed consent, and DNA sample collection. QST was performed on

patients, when possible, either clinically or via a standardized protocol. Enrollment is ongoing at this time.

Enrollment

Overall, we have collected DNA samples from 23 probands (total number of samples including parents and siblings = 70) with an additional 10 families in the pipeline for recruitment. It is important to note that due to the enormous time required from patients and families for conducting QST testing, recruitment on this front has been relatively slow.

For patients that are already enrolled in this research, efforts are being made to contact family members outside the immediate family if they have had symptoms similar to the diagnoses of interest. We are mindful that genetic research poses complex ethical issues with regard to disclosure and informed consent and hence the study team has taken extra steps to ensure patient confidentiality and maintaining consent as an ongoing process.

Patient Phenotypes

A few example summaries of patient phenotypes are presented below:

Example 1: Eleven-year old proband presents with severe burning bilateral foot pains, moderately relieved by cold temperatures, suggesting a diagnosis of erythromelalgia. Similar symptoms were noted in proband's mom and one of the siblings, although presenting with milder symptoms.

Example 2: Eight-year old proband presents with severe spontaneous pain and itching, relieved by cold temperatures. The proband's parents and siblings are noted to be asymptomatic.

All QST measures were collected in one to two locations for probands and family members. Figures 5, 6, and 7 depict some of the QST values from the thenar eminence of the participants.

Figure 5 depicts the Mechanical Pain Threshold (MPT) for both example families. From the data, it appears that relative to parents, proband from example family 1 and the affected sibling are more sensitive in MPT. This is in accordance with the clinic notes, which suggest that the sibling and proband show signs of hypersensitivity. Interestingly, the unaffected sibling shows the most sensitivity relative to the family in regard to MPT. The second example family demonstrates a hyposensitive proband in comparison to the mother.

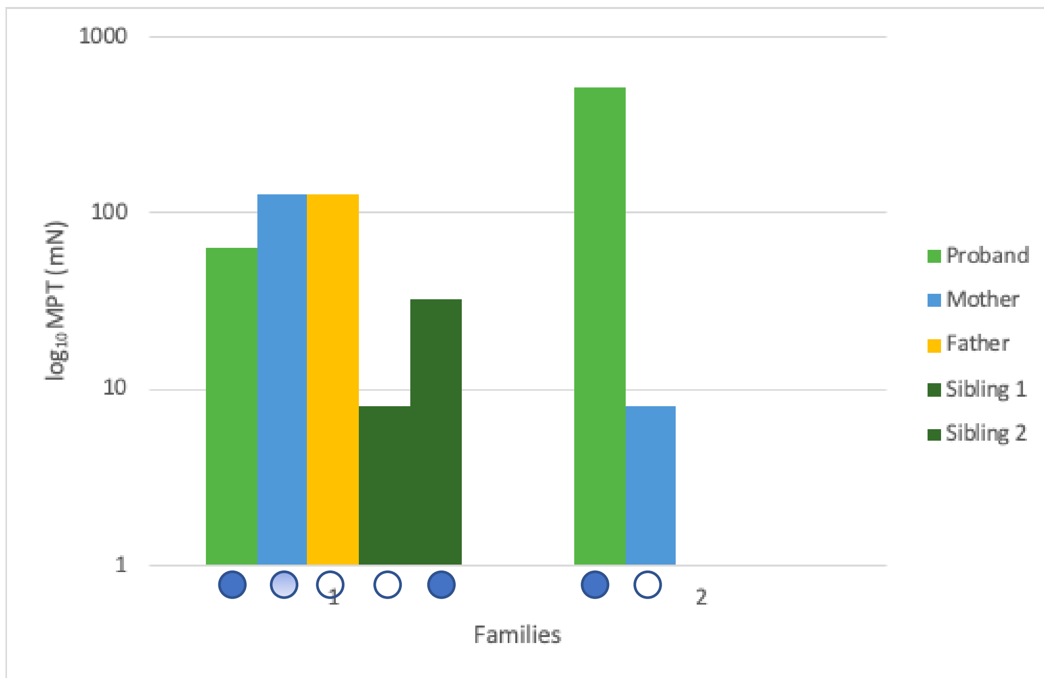

Figure 5. Mechanical Pain Threshold. The average of the closest two values are recorded for each subject. MPT was obtained using the Pin Prick stimulators. The closed circles below the bars represent those presenting with symptoms. The shadow circle represents partial phenotypic expression. The open circles represent asymptomatic participants.

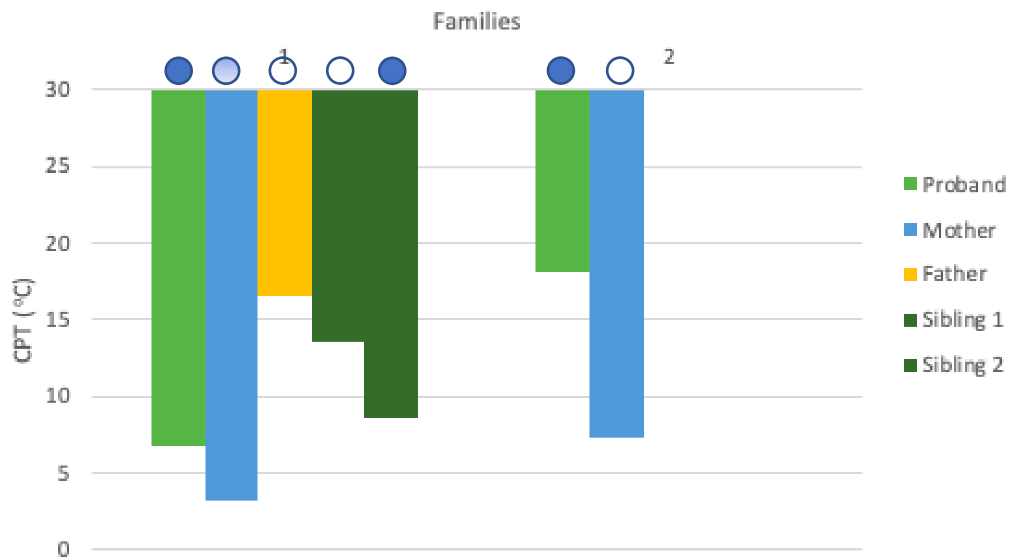

Figure 6. Cold Pain Threshold. The average of the closest two values are recorded for each subject. CPT was obtained using the TSA II and a 16x16 thermode. The closed circles above the bars represent those presenting with symptoms. The shadow circle represents partial phenotypic expression. The open circles represent asymptomatic participants.

Figure 6 depicts the Cold Pain Threshold (CPT) for both example families. The proband in example family 1 appeared to withstand a relatively cold temperature when compared to asymptomatic family members. Proband's symptomatic mother and sibling showed similar results. This would suggest that the mother and her two symptomatic children can tolerate lower temperatures, which might reflect the affinity of patients with erythromelalgia for cold temperatures. The data from example family 2 shows the proband was more sensitive to cold than the mother.

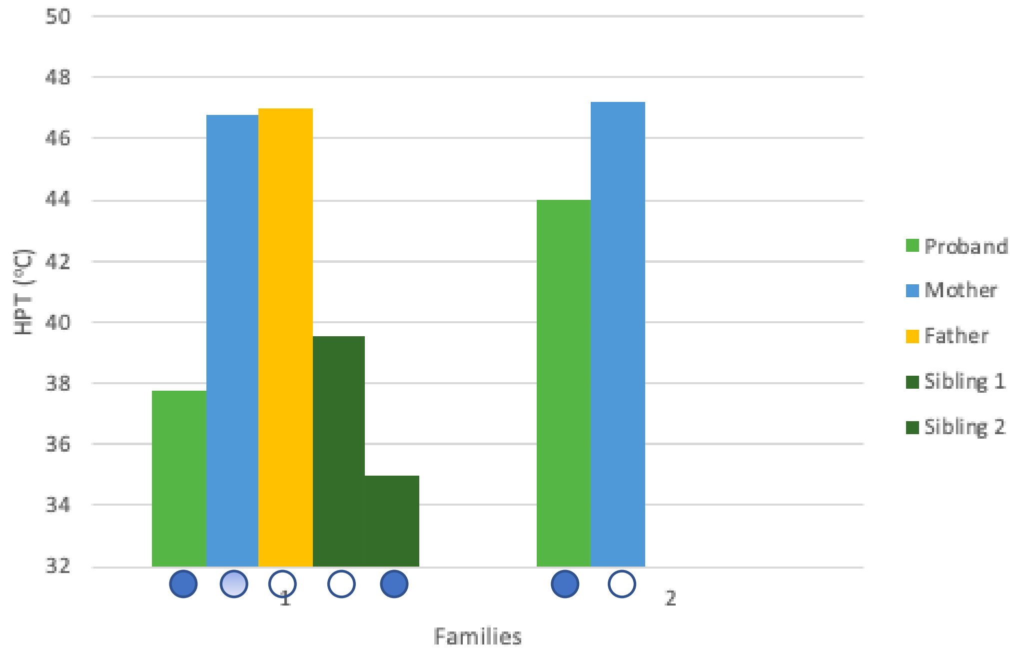

Figure 7. Hot Pain Threshold. The average of the closest two values are recorded for each subject. HPT was obtained using the TSA II and a 16x16 thermode. The closed circles below the bars represent those presenting with symptoms. The shadow circle represents partial phenotypic expression. The open circles represent asymptomatic participants.

Looking at the HPT values in in Figure 7, the proband and affected sibling in example family 1 were much less able to tolerate heat relative to the other sibling and parents. This is in accordance with the phenotypic information from the clinical notes. Interestingly, the mother had approximately the same HPT as the asymptomatic father and was more tolerant of heat than her asymptomatic child, which can reflect a milder symptomatology. In example family 2, the proband was relatively more hypersensitive to heat pain than the mother.

The families discussed above are just two examples from our sample of enrolled patients. Given that the diagnoses of interest are extremely rare, and the vast amount of data and time required from the patients, recruitment was initially relatively slow. In the past 6 weeks, with permission to post on social media, many participants are now in the pipeline for recruitment. Although this work is very

preliminary, the research is evolving, and our group has already identified potentially promising gene targets.

Specifically related to QST, as it is difficult to compare data of family members of differing ages and genders, Z-scores are typically used to normalize the data. Research is ongoing and this will be possible as additional data is collected. Finally, as recruitment increases, it will be possible to perform between-family comparisons and strengthen our findings.

Conclusion

Genetics is a rapidly evolving and highly promising field. With the ability to analyze the human genome, we can now complete population studies, create animal models, and build up variant consortiums that are publicly available to address the many diseases present in human populations, which can be used in clinical care settings, personalizing medicine to a greater extent than we have been able to previously.

The advancements in genetic research have also allowed for drug development to be significantly more effective. New techniques such as CRISPR-Cas9 and ASOs have been established to target diseases from different angles. Legislation has additionally resulted in increased attention to orphan diseases, which, due to the small financial revenue that finding a treatment would generate, are not often adopted by pharmaceutical companies.

Boston Children's Hospital has a unique collaboration with Amgen, Inc. to study patients presenting with a variety of pain disorders to understand the molecular bases of novel gene variants in these patients, as a path to gene discovery including whole genome sequencing and advanced bioinformatics. The results presented here are preliminary, however provide an overview of the ongoing research, which may potentially lead to the identification of promising gene targets.

Citations

About HIV/AIDS | HIV Basics | HIV/AIDS | CDC. (2019-02-28T10:12:13Z). Retrieved from https://www.cdc.gov/hiv/basics/whatishiv.html

About Us. (2019). Retrieved from http://www.irdirc.org/about-us/

Beckmann, J. S., & Lew, D. (2016). Reconciling evidence-based medicine and precision medicine in the era of big data: challenges and opportunities. In *Genome Med* (Vol. 8).

Beissner, F., Brandau, A., Henke, C., Felden, L., Baumgartner, U., Treede, R. D., . . . Lotsch, J. (2010). Quick discrimination of A(delta) and C fiber mediated pain based on three verbal descriptors. *PLOS ONE, 5*(9), e12944. doi:10.1371/journal.pone.0012944

Boycott, K. M., Rath, A., Chong, J. X., Hartley, T., Alkuraya, F. S., Baynam, G., . . . Lochmuller, H. (2017). International Cooperation to Enable the Diagnosis of All Rare Genetic Diseases. *Am J Hum Genet, 100*(5), 695-705. doi:10.1016/j.ajhg.2017.04.003

Boycott, K. M., Vanstone, M. R., Bulman, D. E., & MacKenzie, A. E. (2013). Rare-disease genetics in the era of next-generation sequencing: discovery to translation. *Nat Rev Genet, 14*(10), 681-691. doi:10.1038/nrg3555

Brodniewicz, T., & Grynkiewicz, G. (2010). Preclinical drug development. *Acta Pol Pharm, 67*(6), 578-585.

Buhr, S. (2017). Illumina wants to sequence your whole genome for $100.

Bustamante, C. D., Vega, F. M. D. L., & Burchard, E. G. (2011). Genomics for the world. *Nature, 475*(7355), 163. doi:doi:10.1038/475163a

Caterina, M. J., & Julius, D. (2001). The vanilloid receptor: A molecular gateway to the pain pathway - ProQuest. *Annual Review of Neuroscience, 24*, 487-517.

Chial, H. (2008). Rare Genetic Disorders: Learning About Genetic Disease Through Gene Mapping, SNPs, and Microarray Data. *Nature Education*.

Christensen, K. D., Dukhovny, D., Siebert, U., & Green, R. C. (2015). Assessing the Costs and Cost-Effectiveness of Genomic Sequencing. *J Pers Med, 5*(4), 470-486. doi:10.3390/jpm5040470

Clark, D. (2012). What's the meaning of "Cloning Bias"? Retrieved from https://www.researchgate.net/post/Whats_the_meaning_of_Cloning_Bias

Cohen, S. P., & Mao, J. (2014). Neuropathic pain: mechanisms and their clinical implications. doi:10.1136/bmj.f7656

Cornelissen, L., Donado, C., Kim, J., Chiel, L., Zurakowski, D., Logan, D. E., . . . Berde, C. B. (2014). Pain hypersensitivity in juvenile idiopathic arthritis: a quantitative sensory testing study. *Pediatr Rheumatol Online J, 12*, 39. doi:10.1186/1546-0096-12-39

Cruz-Almeida, Y., & Fillingim, R. B. (2014). Can quantitative sensory testing move us closer to mechanism-based pain management? *Pain Med, 15*(1), 61-72. doi:10.1111/pme.12230

Dias, N., & Stein, C. A. (2002). Antisense Oligonucleotides: Basic Concepts and Mechanisms. Retrieved from http://mct.aacrjournals.org/content/1/5/347

DNA Sequencing Costs: Data. (2018). Retrieved from https://www.genome.gov/27541954/dna-sequencing-costs-data/

Effraim, P. R., Waxman, S. G., & Greene, J. G. (2007). Erythromelalgia. Retrieved from http://www.medlink.com/article/erythromelalgia

Eisenstadt, L. (2017). After a decade of genome-wide association studies, a new phase of discovery pushes on.

Ekberg, J., & Adams, D. J. (2006). Neuronal voltage-gated sodium channel subtypes: key roles in inflammatory and neuropathic pain. *Int J Biochem Cell Biol, 38*(12), 2005-2010. doi:10.1016/j.biocel.2006.06.008

Ellis, A. G., Mickle, K., Herron-Smith, S., Kumar, V., Cianciolo, L., Rind, D., & Pearson, S. D. (2018). *Spinraza® and Zolgensma® for Spinal Muscular Atrophy: Effectiveness and Value*. Retrieved from

European public assessment report (EPAR) for Spinraza. (2017). In *European Medicines Agency*.

FAQs About Rare Diseases | Genetic and Rare Diseases Information Center (GARD) an NCATS Program. Retrieved from https://rarediseases.info.nih.gov/diseases/pages/31/faqs-about-rare-diseases

Fliesler, N. (2018). Reviving fetal hemoglobin in sickle cell disease: First patient is symptom-free.

Genome-Wide Association Studies Fact Sheet. (2015). Retrieved from https://www.genome.gov/20019523/genomewide-association-studies-fact-sheet/

Genome: Unlocking Life's Code. Retrieved from https://unlockinglifescode.org/timeline

Green, R. C., Berg, J. S., Grody, W. W., Kalia, S. S., Korf, B. R., Martin, C. L., . . . Biesecker, L. G. (2013). ACMG Recommendations for Reporting of Incidental Findings in Clinical Exome and Genome Sequencing. *Genet Med, 15*(7), 565-574. doi:10.1038/gim.2013.73

Guedon, J. M. G., Wu, S., Zheng, X., Churchill, C. C., Glorioso, J. C., Liu, C. H., . . . Hao, S. (2015). Current gene therapy using viral vectors for chronic pain. In *Mol Pain* (Vol. 11).

Hodson, R. (2016). Precision medicine. *Nature, 537*(7619). doi:doi:10.1038/537S49a

Hoelder, S., Clarke, P., & Workman, P. (2012). Discovery of small molecule cancer drugs: Successes, challenges and opportunities. *Molecular Oncology, 6*(2), 155-176. doi:https://doi.org/10.1016/j.molonc.2012.02.004

Holm, I. A., Agrawal, P. B., Ceyhan-Birsoy, O., Christensen, K. D., Fayer, S., Frankel, L. A., . . . Beggs, A. H. (2018). The BabySeq project: implementing genomic sequencing in newborns. In *BMC Pediatr* (Vol. 18).

Hopkin, M. (2006). The mutation that takes away pain. *Nature News*. doi:doi:10.1038/news061211-11

Hudak, M. L. (2016). Codeine Pharmacogenetics as a Proof of Concept for Pediatric Precision Medicine. doi:10.1542/peds.2016-1359

Institute of Medicine (US) Committee on Advancing Pain Research, C., andEducation. (2011). Research Challenges. doi:https://www.ncbi.nlm.nih.gov/books/NBK92512/

Institute of Medicine (US) Committee on Pain, D., and Chronic Illness Behavior, Osterweis, M., Kleinman, A., & Mechanic, D. (1987). Measuring Pain And Dysfunction. doi:https://www.ncbi.nlm.nih.gov/books/NBK219245/

International HapMap Project. Retrieved from https://www.genome.gov/10001688/international-hapmap-project/

Ioannidis, J. (2012). Extrapolating from Animals to Humans. *Science Translational Medicine, 4*(151), 151.

Jameson, J. L., & Longo, D. L. (2015). Precision Medicine — Personalized, Problematic, and Promising. *http://dx.doi.org/10.1056/NEJMsb1503104*. doi:NJ201506043722313

Johnson, R. C., Nelson, G. W., Troyer, J. L., Lautenberger, J. A., Kessing, B. D., Winkler, C. A., & O'Brien, S. J. (2010). Accounting for multiple comparisons in a genome-wide association study (GWAS). In *BMC Genomics* (Vol. 11, pp. 724).

Keshavan, M. (2018). Saving Mila: How doctors raced to stop a young girl's rare disease.

Knief, C. (2014). Analysis of plant microbe interactions in the era of next generation sequencing technologies. *Front Plant Sci, 5*. doi:10.3389/fpls.2014.00216

Knockout Mouse Project. Retrieved from https://www.genome.gov/17515708

Kurban, M., Wajid, M., Shimomura, Y., & Christiano, A. M. (2010). A Nonsense Mutation in the SCN9A Gene in Congenital Insensitivity to Pain. In *Dermatology* (Vol. 221, pp. 179-183).

LaFramboise, T. (2009). Single nucleotide polymorphism arrays: a decade of biological, computational and technological advances. In *Nucleic Acids Res* (Vol. 37, pp. 4181-4193).

Landrum, M. J., Lee, J. M., Riley, G. R., Jang, W., Rubinstein, W. S., Church, D. M., & Maglott, D. R. (2014). ClinVar: public archive of relationships among sequence variation and human phenotype. In *Nucleic Acids Res* (Vol. 42, pp. D980-985).

Leek, J. (Producer). The Process for GWAS/WGS (6:12) - Module 4 | Coursera. Retrieved from https://www.coursera.org/lecture/statistical-genomics/the-process-for-gwas-wgs-6-12-KP30X

Li, Y. Y., & Jones, S. J. (2012). Drug repositioning for personalized medicine. In *Genome Med* (Vol. 4, pp. 27).

Lobo, K., Donado, C., Cornelissen, L., Kim, J., Ortiz, R., Peake, R. W., . . . Cravero, J. (2015). A Phase 1, Dose-escalation, Double-blind, Block-randomized, Controlled Trial of Safety and Efficacy of Neosaxitoxin Alone and in Combination with 0.2% Bupivacaine, with and without Epinephrine, for Cutaneous Anesthesia. *Anesthesiology, 123*(4), 873-885. doi:10.1097/ALN.0000000000000831

Mardis, E. R. (2008). The impact of next-generation sequencing technology on genetics. *Trends in Genetics, 24*(3), 133-141. doi:10.1016/j.tig.2007.12.007

Mark Denis P. Davis, M. (2018). Erythromelalgia. Retrieved from https://rarediseases.org/rare-diseases/erythromelalgia/

McDermott, L. A., Weir, G. A., Themistocleous, A. C., Segerdahl, A. R., Blesneac, I., Baskozos, G., . . . Bennett, D. L. (2019). Defining the Functional Role of NaV1.7 in Human Nociception. *Neuron*. doi:10.1016/j.neuron.2019.01.047

McDonnell, A., Schulman, B., Ali, Z., Dib-Hajj, S. D., Brock, F., Cobain, S., . . . Waxman, S. G. (2016). Inherited erythromelalgia due to mutations in SCN9A: natural history, clinical phenotype and somatosensory profile. *Brain, 139*(Pt 4), 1052-1065. doi:10.1093/brain/aww007

McIntosh, I. (2012). Lentiviral Vectors in Gene Therapy. Retrieved from https://microbewiki.kenyon.edu/index.php/Lentiviral_Vectors_in_Gene_Therapy

Meier, P. M., Berde, C. B., DiCanzio, J., Zurakowski, D., & Sethna, N. F. (2001). Quantitative assessment of cutaneous thermal and vibration sensation and thermal pain detection thresholds in healthy children and adolescents. *Muscle Nerve, 24*(10), 1339-1345.

Metzker, M. L. (2010). Sequencing technologies - the next generation. *Nat Rev Genet, 11*(1), 31-46. doi:10.1038/nrg2626

Might, M. (Producer). (2015). Matt Might on Patient-Driven Precision Medicine. Retrieved from https://www.youtube.com/watch?v=rx0kK6VnLSc

Mnookin, S. (2014). One of a Kind. Retrieved from https://www.newyorker.com/magazine/2014/07/21/one-of-a-kind-2

Mollanoori, H., Shahraki, H., Rahmati, Y., & Teimourian, S. (2018). CRISPR/Cas9 and CAR-T cell, collaboration of two revolutionary technologies in cancer immunotherapy, an instruction for successful cancer treatment. *Hum Immunol, 79*(12), 876-882. doi:10.1016/j.humimm.2018.09.007

Murnion, B. P. (2018). Neuropathic pain: current definition and review of drug treatment. In *Aust Prescr* (Vol. 41, pp. 60-63).

Nair, A. B., & Jacob, S. (2016). A simple practice guide for dose conversion between animals and human. In *J Basic Clin Pharm* (Vol. 7, pp. 27-31).

Naso, M. F., Tomkowicz, B., Perry, W. L., & Strohl, W. R. (2017). Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. In *BioDrugs* (Vol. 31, pp. 317-334).

National Academies of Sciences, E., and Medicine, Division, H. a. M., Policy, B. o. H. S., & Disorders, F. o. N. a. N. S. (2018). Clinical Development of Non-Addictive Pain Medications. doi:https://www.ncbi.nlm.nih.gov/books/NBK507100/

Next-Generation Sequencing (NGS) | Explore the technology. Retrieved from https://www.illumina.com/science/technology/next-generation-sequencing.html

NIH Fact Sheets - Human Genome Project. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/
Norrgard, K. (2008). Genetic Variation and Disease: GWAS. *Nature Education*.
Oliver, G. R., Hart, S. N., & Klee, E. W. (2015). Bioinformatics for clinical next generation sequencing. *Clin Chem, 61*(1), 124-135. doi:10.1373/clinchem.2014.224360
Paoli, J. (2013). HIV Resistant Mutation.
Parasuraman, S. (2011). Toxicological screening. In *J Pharmacol Pharmacother* (Vol. 2, pp. 74-79).
Pearson, T. A., & Manolio, T. A. (2008). How to Interpret a Genome-wide Association Study. *JAMA, 299*(11), 1335-1344. doi:10.1001/jama.299.11.1335
Piguet, F., Alves, S., & Cartier, N. (2017). Clinical Gene Therapy for Neurodegenerative Diseases: Past, Present, and Future. *Hum Gene Ther, 28*(11), 988-1003. doi:10.1089/hum.2017.160
Popejoy, A. B., Ritter, D. I., Crooks, K., Currey, E., Fullerton, S. M., Hindorff, L. A., . . . Bustamante, C. D. (2018). The clinical imperative for inclusivity: Race, ethnicity, and ancestry (REA) in genomics. *Hum Mutat, 39*(11), 1713-1720. doi:10.1002/humu.23644
Precision Medicine: What Does it Mean for Your Health? | Features | CDC. (2018). Retrieved from https://www.cdc.gov/features/precision-medicine/index.html
Product Development Under the Animal Rule Guidance for Industry. (2015). In. U.S. Department of Health and Human Services Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER).
Rolke, R., Magerl, W., Campbell, K. A., Schalber, C., Caspari, S., Birklein, F., & Treede, R. D. (2006). Quantitative sensory testing: a comprehensive protocol for clinical trials. *Eur J Pain, 10*(1), 77-88. doi:10.1016/j.ejpain.2005.02.003
Sanger Sequencing Steps & Method. Retrieved from https://www.sigmaaldrich.com/technical-documents/articles/biology/sanger-sequencing.html
Sboner, A., Mu, X. J., Greenbaum, D., Auerbach, R. K., & Gerstein, M. B. (2011). The real cost of sequencing: higher than you think! In *Genome Biol* (Vol. 12, pp. 125).
Scarborough, R. (2017). Of mice and men: why animal trial results don't always translate to humans.
Sethna, N. F., Meier, P. M., Zurakowski, D., & Berde, C. B. (2007). Cutaneous sensory abnormalities in children and adolescents with complex regional pain syndromes. *Pain, 131*(1-2), 153-161. doi:10.1016/j.pain.2006.12.028
Shampo, M. A., & Kyle, R. A. (2011). J. Craig Venter—The Human Genome Project. *Mayo Clin Proc, 86*(4), e26-27. doi:10.4065/mcp.2011.0160
Shanks, N., Greek, R., & Greek, J. (2009). Are animal models predictive for humans? *Philos Ethics Humanit Med, 4*, 2. doi:10.1186/1747-5341-4-2

Shen, X., & Corey, D. R. (2018). Chemistry, mechanism and clinical status of antisense oligonucleotides and duplex RNAs. *Nucleic Acids Res, 46*(4), 1584-1600. doi:10.1093/nar/gkx1239

Sickle cell anemia - Symptoms and causes.

Smith, S. M., Dworkin, R. H., Turk, D. C., Baron, R., Polydefkis, M., Tracey, I., . . . Witter, J. (2017). The Potential Role of Sensory Testing, Skin Biopsy, and Functional Brain Imaging as Biomarkers in Chronic Pain Clinical Trials: IMMPACT Considerations. *J Pain, 18*(7), 757-777. doi:10.1016/j.jpain.2017.02.429

Spinal muscular atrophy. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/

Tang, Z., Chen, Z., Tang, B., & Jiang, H. (2015). Primary erythromelalgia: a review. In *Orphanet J Rare Dis* (Vol. 10).

The Cost of Sequencing a Human Genome. (2018). Retrieved from https://www.genome.gov/images/content/costpergenome2015_4.jpg

The generation of action potential in nerves. (2014). Retrieved from http://www.animalresearch.info/en/medical-advances/nobel-prizes/the-generation-of-action-potential-nerves/

The Human Genome: A Decade of Discovery, Creating a Healthy Future. (2010). Paper presented at the The Human Genome: A Decade of Discovery, Creating a Healthy Future, Grand Hyatt Washington 1000 H Street NW Farragut Square and Lafayette Park Rooms Washington D.C., 20001.

The International HapMap Project. (2003). *Nature, 426*(6968), 789. doi:doi:10.1038/nature02168

The portal for rare diseases and orphan drugs. (2019). Retrieved from http://www.orpha.net/consor/www/cgi-bin/index.php?lng=EN

Understanding Manhattan Plots and Genome-wide Association Studies - YouTube. (2017). Retrieved from https://www.youtube.com/watch?v=Pdic7p_dk0I

Vassy, J. L., Lautenbach, D. M., McLaughlin, H. M., Kong, S. W., Christensen, K. D., Krier, J., . . . Green, R. C. (2014). The MedSeq Project: a randomized trial of integrating whole genome sequencing into clinical medicine. In *Trials* (Vol. 15, pp. 85).

Vassy, J. L., McLaughlin, H. M., MacRae, C. A., Seidman, C. E., Lautenbach, D., Krier, J. B., . . . Green, R. C. (2015). A One-Page Summary Report of Genome Sequencing for the Healthy Adult. *Public Health Genomics, 18*(2), 123-129. doi:10.1159/000370102

Visscher, P. M., Wray, N. R., Zhang, Q., Sklar, P., McCarthy, M. I., Brown, M. A., & Yang, J. (2017). 10 Years of GWAS Discovery: Biology, Function, and Translation. *Am J Hum Genet, 101*(1), 5-22. doi:10.1016/j.ajhg.2017.06.005

Wastfelt, M., Fadeel, B., & Henter, J. I. (2006). A journey of hope: lessons learned from studies on rare diseases and orphan drugs. *J Intern Med, 260*(1), 1-10. doi:10.1111/j.1365-2796.2006.01666.x

What are genome editing and CRISPR-Cas9? (2019). Retrieved from https://ghr.nlm.nih.gov/primer/genomicresearch/genomeediting

What are genome wide association studies (GWAS)?

What is CRISPR-Cas9? Retrieved from https://www.yourgenome.org/facts/what-is-crispr-cas9

What is gene therapy? Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/

What is precision medicine? (2019). Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/

What is the Illumina method of DNA sequencing? (2015).

What is the International HapMap Project? (2019). Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/

Yu, T. W., Hu, C., Kim, J., Larson, A., Lee, A., Black, L., . . . Berde, C. (2019). *PgmNr 3570: Patient-customized oligonucleotide therapy for an ultra-rare genetic disease.* EventPilot Web. Retrieved from https://eventpilot.us/web/page.php?page=IntHtml&project=ASHG18&id=180124037

Zhao, S. (2014). Clinical Applications of Next Generation Sequencing. *Journal of Health & Medical Informatics, 5*(5), 1-2. doi:10.4172/2157-7420.1000e129