CLOVES Syndrome Gap and Landscape Analysis

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Prepared for:
CLOVES Syndrome Community

Scope of Work: The goal of this document is to provide actionable recommendations to the CLOVES Syndrome Community about how best to direct their research efforts for CLOVES syndrome. To this end, we have reviewed published material and public databases, reviewed foundation provided materials, and integrated learnings from other related fields and disorders to inform our recommendations. The activities we are suggesting should be considered alongside the organizational priorities, available funding, and bandwidth or resources needed to pursue these activities. CLOVES is a multifaceted disease that can affect multiple biological systems at once. Priorities may shift over time as scientific understanding of the disease progresses and as therapeutic technologies mature. We recommend revisiting strategic plans and community priorities yearly to ensure your foundation's goals are aligned with your research activities. Due to the length of the document several sections repeat content since we assume the reader might skip around and we do not want key information to be missed. The intended audience for this document is the CLOVES Syndrome Community Board Members and CSC constituents. The writing style aims to be accessible to patients and families that are familiar with CLOVES syndrome.

Priority Recommendations

1. Community Organization: We suggest undertaking efforts that formalize diagnostic practices in an effort to increase awareness and identify patients earlier. Data collection is always important in the rare disease community. In the case of CLOVES where repurposing cancer treatments is a common practice, data collection would be particularly beneficial in accelerating understanding of outcomes.
    - Development of diagnostic criteria as a collaboration between the Foundation, clinicians, specialists, and researchers. This should be published, and peer reviewed and distributed internationally to raise awareness and facilitate communications about needs in the CLOVES community.
    - Part of the diagnostic process exercise should be a focus on neonatal identification of CLOVES risk factors and guidance on how and when to apply appropriate interventions.
    - Development of patient registries and support of natural history studies. The Foundation should take a leadership stake in a registry effort and support clinical centers in natural history study development and execution.

2. Therapeutic Research: While repurposing efforts have been effective in identifying CLOVES treatments, there are challenges specific to the unique genetic nature of CLOVES that should be considered when designing the next generation of treatments for CLOVES. Priority areas of research to enable therapeutic development include:
    - Allele specific targeting mechanisms
    - Localized delivery and tissue targeting
    - Targeted protein degradation
    - Therapeutics targeting the interaction of p110 with p85 or expression of either subunit

3. Investment recommendations: research tools and disease mechanism understanding
    - Animal models that recapitulate mosaic expression of CLOVES relevant mutations
    - Cell-based endpoints
    - Biological understanding: how do different patient mutations increase catalytic activity? Is this often the same mechanism or is there a diverse array of effects?

CLOVES Syndrome Overview

CLOVES Syndrome (Congenital Lipomatous Overgrowth, Vascular malformations, Epidermal nevi and Scoliosis/skeletal/spinal anomalies) is a rare, nonheritable disorder (meaning neither parent is a carrier for the disorder) characterized by tissue overgrowth and complex vascular anomalies. This is a spectrum disorder, meaning there is a broad range of symptoms and severity and not all patients will have the same phenotype. The most life-threatening and debilitating symptoms of this disorder include vascular malformations, lipomatous masses, and overgrowth. Generally, the first diagnosable sign of CLOVES identified in patients is a fatty mass present at birth. Unfortunately, it is often hard to progress from identification of the first symptom to diagnosis, therefore age of diagnosis can range dramatically due to severity of symptoms, familiarity of physicians with rare overgrowth disorders, and availability of genetic testing.

CLOVES is caused by somatic (mutation occurs after conception), mosaic (meaning the mutation is not found in all cells), activating mutations in the PIK3CA gene (Kurek et al., 2012). The PIK3CA gene encodes the catalytic, or active, subunit of the phosphoinositide 3-kinase (PI3K). A kinase is a type of protein which can regulate the biological activity of other proteins. Understanding how a genetic mutation occurs, what protein it disrupts, and how it disrupts function is very important for identification of treatments. The main function of PI3K is activation of the PI3K/AKT pathway. The genetic mutations found in CLOVES patients increase PI3K activity. Therefore, these activating mutations in the PI3K protein cause abnormally high PI3K/AKT pathway activation, (see section *PIK3CA Biology and the PI3K/AKT Pathway*) which can lead to a number of downstream effects including increased cell growth, increased protein synthesis, and decreased cell death.

The mosaic nature of mutations in PIK3CA means that each patient will have mutated protein in different cells or cell populations. Because the mutations arise after conception, the precise timing of when the mutation arises during development and in which cells it occurs in will determine the symptoms of each individual patient and may lead to different diagnoses. While mosaic, activating mutations in PIK3CA can lead to different clinical diagnoses (see section on *Learning from PROS and other related syndromes*) we will be focusing on CLOVES syndrome specifically.

For those patients diagnosed with CLOVES syndrome, there is a broad spectrum of symptoms and severity that at this time remain frustratingly hard to predict. While there are several main symptoms, not all patients present with these symptoms and some patients present with symptoms not on the 'common to CLOVES' list. Main symptoms include (Martinez-Lopez et al., 2017):

- Low and high flow vascular malformations
- Thoracic lipomatous hyperplasia (fatty overgrowth affecting one or both sides of the body)

- Skin anomalies (including birthmarks, prominent veins, epidermis nevus)
- Asymmetric growth (including abnormal extremities and scoliosis)
- Visceral disorders (including kidney anomalies)
- Neurological disorders (including arteriovenous malformations in and around the spinal cord)

The diversity of these symptoms often leads patients to visit multiple specialists and receive treatments on a symptom-by-symptom basis, rather than assessing the disorder overall. Additionally, there are other less common symptoms that affect patients with CLOVES syndrome such as kidney abnormalities and Wilms tumors, which patients should be regularly screened for. The life expectancy of patients with CLOVES syndrome is unknown since it is a relatively newly described condition (first reported in 2007). Life expectancy differs for patients based on severity of disease and given the rarity of CLOVES, this is still an area that will benefit from more research and more data collection. Natural history studies and patient registries would help patients and clinicians better understand the trajectory of the syndrome, average age of diagnosis, treatment outcomes, and life expectancy.

In the case of CLOVES syndrome, therapeutic design and discovery is more complicated than similar monogenic disorders:

- The multi-system nature of the disease combined with the mosaic presentation of healthy tissue alongside 'affected' tissue makes it harder to specifically and effectively target the pathogenic event with drugs.
- The ubiquity of the PI3K pathway and its importance in numerous basic cellular functions means that treatments for CLOVES would ideally modulate function rather than totally block or inhibit activity. This can be more difficult to balance from a chemical design perspective.
- Heterogeneity of symptoms in a disease can increase the complexity of properly treating patients and designing therapeutics that improve the quality of life for a large portion of the population.
- The use of surgery in CLOVES allows for effective targeting of the affected tissue, but its utility is limited due the regrowth of tissue which is common. This suggests that combination treatments may be needed to more effectively treat CLOVES long-term.

CLOVES Diagnosis

Early diagnosis is critical for early intervention and provides patients and their families with a better understanding of their prognosis, as well as access to the CLOVES community. Clinical diagnosis has historically been complicated, and many cases are confused with other overgrowth syndromes (Martinez-Lopez et al., 2017). Genetic tests can rule out syndromes

caused by mutations in other genes and symptom analysis can rule out other PI3K-associated disorders.

There are many barriers to early diagnosis including clinician lack of awareness, availability or accessibility of genetic testing, and misdiagnosis. However, in retrospective analyses, physical manifestation of CLOVES is often present at birth. Initial symptoms can range from mild to severe and may affect soft tissue, blood vessels, bones, and/or internal organs (Mahajan et al., 2019). Because other syndromes, especially Proteus syndrome, have similar characteristics, confirmation of diagnosis requires molecular genetic testing for mutations in PIK3CA in the affected tissues (Keppler-Noreuil et al., 2015). Additionally, the manifestations present at birth generally increase with age, but are not considered rapidly progressive (Mahajan et al., 2019). Currently, it is estimated that there are around 2,000 patients with CLOVES around the world (Kristen Davis, Executive Director CLOVES Syndrome Community), making it difficult for patients and their families to receive a diagnosis since few physicians are familiar with CLOVES syndrome or have seen more than one case in their career. *Despite multiple obstacles to diagnosis, a focus on increased clinician awareness programs and genetic testing would likely have a significant impact on diagnostic rate and overall numbers.*

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Another obstacle to diagnosis is the mosaic nature of CLOVES. Routine prenatal genetic tests, including blood tests and amniocentesis, are unlikely to pick up mutations in PIK3CA since this mutation is found in only a subset of the fetus' cells. The exception being if the mutation is present in the placenta, then a chorionic villus sample or a sample of cell-free fetal DNA from the maternal plasma may be able to detect a mutation. However, this could lead to a false negative diagnosis or identify a mutation which is found only in the placenta and may not impose a risk on the fetus. More invasive procedures such as sampling directly from a vascular malformation in the fetus are possible, however it is likely that the risks outweigh the benefits of such procedures (Emrick et al., 2014).

Prenatal ultrasounds can sometimes identify lymphovascular malformations, segmental overgrowth and skeletal defects which should raise suspicion for a possible CLOVES diagnosis (Emrick et al., 2014); however, this remains rare and often CLOVES is not suspected until after birth. *Increasing awareness of the risk factors with prenatal radiology specialists and physicians may help with early identification just after birth when samples can be taken with the least amount of risk to the mother and fetus.* In cases where the location of the overgrowth prenatally may put the fetus at early risk, additional interventions should be considered prenatally. Since symptoms of CLOVES syndrome present prenatally, the community should consider engaging key clinicians to design diagnostic and early intervention guidance for publication and dissemination to the medical community. These types of educational programs

have had a significant impact on increasing diagnoses or improving outcomes in other rare diseases.

Because abnormal growth is sometimes identifiable by ultrasound prior to birth, there should be a clear, standard set of tests done after birth on infants that present with potential CLOVES manifestations to determine the exact cause. Mutations in several different genes can lead to overgrowth, so it would be advantageous to test for mutations in these genes early to identify the cause of symptoms and understand the likely trajectory of symptoms for the individual. Additionally, the better clinicians are at diagnosing CLOVES, the more likely we are to know the true prevalence of the disorder which in turn can increase awareness and provide additional pressure to develop better treatments.

A key recommendation for improving early diagnosis is the development of educational programs and materials, focused on the early differential diagnosis of CLOVES and related disorders.

- These materials should focus on early detection and awareness of the disorder
- Diagnostic criteria and best practices- this should vary for different age groups and potentially for different symptomatic presentations
- Guidance should be drafted as a collaboration between CLOVES clinical experts, perinatal specialists, and the CLOVES community

Areas of further refinement:

- Prenatal presentation of CLOVES: retrospective analysis of outcomes, possible interventions, and associated risks
- Prenatal diagnostic decision tree: when to intervene, when to use invasive testing procedures, and postnatal diagnostic practice and care

Patient Data

Patients, families, and clinical researchers should be engaged to collect longitudinal data bout CLOVES to better characterize CLOVES Syndrome, identify important risk factors for the most severe outcomes, and to recontact patients to participate in further research opportunities or clinical trials. There are two primary tools to capture patient data in a meaningful way: formal natural history studies and patient registries.

Natural history studies follow patients over a period of time to measure progression and better characterize the disease. Natural history studies are often initiated and managed by clinical researchers and require substantial financial support due to the cost of clinical visits and the data capture mechanisms to support such data. A foundation can participate in this process even when responsibility lies with a university or other entity. *Foundations should request collaboration on protocol design, consent language, research questions, data sharing, and use of data policies.*

Patient registries, nowadays, are usually computer-based questionnaires that are distributed directly to the patient or family member, or both, and can range in complexity from a contact registry solely to disease characterization and progression tracking. Patient registries are often managed by a foundation, a company, or clinical researchers and are usually much cheaper on a per research participant consideration compared to a natural history study. Although registries collect less formal data, they have the advantage of incorporating feedback from far more participants, who are usually more diverse in their geographical and financial situations as well as race, age, sex, and stage of disease.

Areas to consider tracking information through direct to patient and family registries:

- Symptoms
- Medications taken
- Surgical intervention
- Quality of Life (patient and family)
- Opt-in to future research and/or clinical trials
- Health economics considerations
- Specialists seen

Genetic Panels

The Genetic Testing Registry (GTR) lists 43 clinical genetic tests for CLOVES syndrome, and 228 tests include screening the PIK3CA gene as it relates to different disorders. Collectively, panels screen for a range of genetic mutation types including deletions, duplications, mutation scanning of the entire coding region, mutation scanning of select exons, targeted variant analysis, methylation, and sequence analysis of the entire coding region. Outside of the USA, at least 6 additional countries have genetic screening panels for CLOVES. The following countries have registered CLOVES genetic panels in the Genetic Testing Registry:

- Canada
- Netherlands
- Germany
- Portugal
- Spain
- Turkey

Below is a list of the labs involved with testing in each country and the link to this information in the Genetic Testing Registry is included here:
https://www.ncbi.nlm.nih.gov/gtr/all/labs/?term=C2752042&LHistId=MCID_6201714fbd3ab22aeb46ade5&qkey=2

Table 1. Information on genetic testing relevant to cloves in countries outside the USA.

Location	Country
Laboratory of genome diagnostics, Academic Medical Center, University of Amsterdam	Netherlands
CeGaT GmbH	Germany
Centogene AG - the Rare Disease Company	Germany
CGC Genetics	Portugal
Genologica Medica	Spain
Intergen Genetic Diagnosis and Research Centre, Intergen Genetic Diagnosis and Research Centre	Turkey
LifeLabs Genetics	Canada
MVZ Dr. Eberhard & Partner Dortmund	Germany
Institute for Human Genetics, University Clinic Freiburg	Germany

The following link provides further information on each lab and testing panel being used. Not all of the testing panels that include testing for PIK3CA mutations are intended for CLOVES syndrome diagnosis. Keep in mind that the practice of returning a disease diagnosis for a disease that was not being tested for will vary between countries and labs. Not all countries or labs will disclose an incidental finding (i.e. finding a mutation that indicates a disease that was not being tested for originally), therefore, the presence of a panel that tests for PIK3CA mutations in a country does not mean the testing lab will return a CLOVES diagnosis to the patient if one is identified.

https://www.ncbi.nlm.nih.gov/gtr/all/tests/?term=C2752042&filter=testtype:clinical;location:124__276__528__620__724__792

PIK3CA Biology and the PI3K/AKT Pathway

To identify the spectrum of therapeutic options for CLOVES, it is important to first understand the protein and biological pathways that are disrupted by mutations to PIK3CA. As previously described, CLOVES syndrome is caused by mosaic, activating mutations in the PIK3CA gene. The PIK3CA gene encodes a 110-kD catalytic subunit of PI3K known as p110α, which is considered a subunit of class 1 PI3Ks. Proteins such as PI3K are made up of more than one subunit, which also means that more than one gene is responsible for the proper creation of the entire PI3K protein complex. A catalytic subunit is the piece of a functional protein complex which is responsible for the activity of the protein complex. Therefore, this catalytic subunit is often the subunit responsible for the primary function of a protein complex. A regulatory subunit of a protein serves to facilitate or reduce the activity of a protein without having any direct functional activity itself. It can do this through several mechanisms including: targeting the protein to the proper place in a cell, creating a change in the protein's shape which can act like an on/off switch for function, binding to the protein's target to keep it close to the catalytic subunit, etc. One catalytic and one regulatory subunit combine to make up PI3K for a total of three classes of PI3Ks (Table 1). For the purpose of this report, we will focus solely on class 1 PI3K subunits. Within class 1 PI3Ks, there are several genes that encode the catalytic and

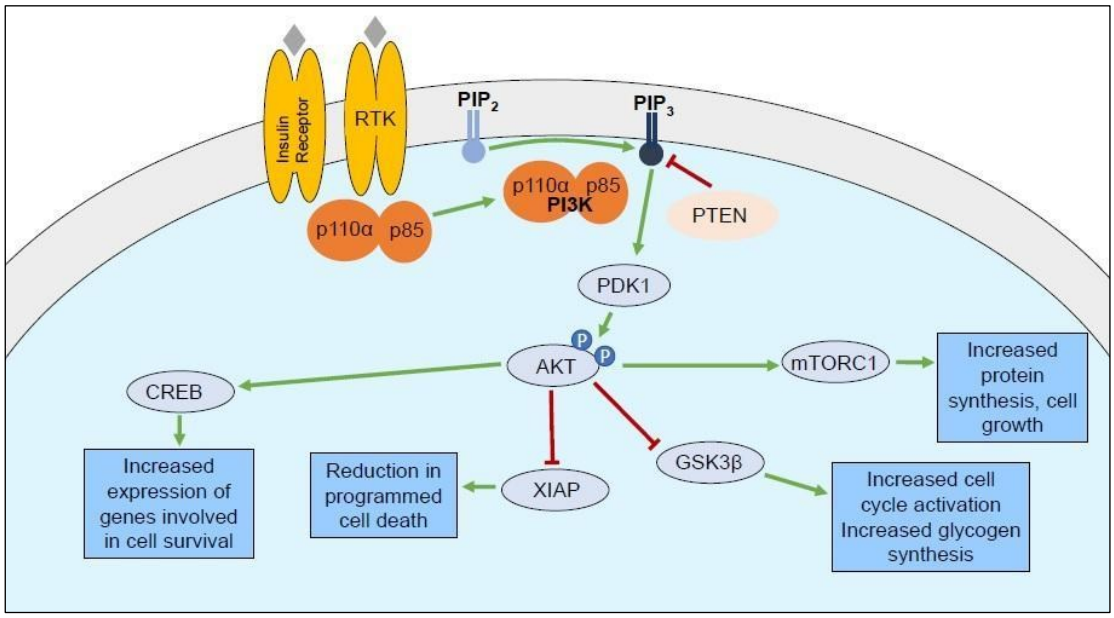
regulatory subunits, including PIK3CA, PI3KCB, PI3KCG, PI3KCD (catalytic subunits) and PIK3R1, PIK3R2, PIK3R3, PIK3R5, PIK3R6 (regulatory subunits). Class 1 PI3Ks are a ubiquitously expressed (expressed in all cells) kinase involved in cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking (Fruman et al., 2017).

Table 2. Classes and subunits of PI3K (Jean and Kiger, 2014)

	Genes encoding catalytic subunits	**Genes encoding regulatory subunits**
Class 1 PI3K	***PIK3CA***, PI3KCB, PI3KCG, PI3KCD	PIK3R1, PIK3R2, PIK3R3, PIK3R5, PIK3R6
Class 2 PI3K	PIK3C2A, PIK3C2B, PIK3C2G	No known
Class 3 PI3K	PIK3C3	PIK3R4

For PI3K to be functional it must form a dimer (an interaction of two proteins) between a catalytic subunit, such as p110α, and a regulatory subunit which serves to both modulate function and correctly localize PI3K within a cell. Once a functional PI3K is formed its main function in the cell is to increase the presence of the signaling molecule, PIP_3. PIP_3 is an important upstream regulator of the Akt pathway which is involved in promoting cell growth and survival. The increase in Akt phosphorylation following increased PIP_3 leads to up or down regulation of many proteins which in turn have major effects on cell health. Please see Figure 1 for a summary on some of the proteins involved in this pathway.

Figure 1. PI3K/AKT Pathway



In addition to understanding the protein's functions, knowing how a protein is regulated often facilitates discovery of potential therapeutics. In the case of CLOVES syndrome, it would be helpful to know how to downregulate overactive PI3K. In other words, how to decrease the activity level of PI3K without completely silencing it. This is an important distinction from completely turning off all PI3K function since normally functioning cells need some active PI3K. The function of PI3K is regulated in several different ways: upstream by receptor tyrosine kinases, G-proteins, and growth factor receptors such as the insulin receptor (Fruman et al., 2017). It is also self-regulated by interaction with its regulatory subunit. Knowing these mechanisms provides insight into potential ways to dampen the activity of an overactive PI3K. Advice on follow-up research to assist in drug design and/or selection can be found in the *Potential Future Therapeutic Targets* section.

Therapeutic Considerations for the Next Generation of CLOVES treatments

Developing therapeutic approaches that treat CLOVES syndrome is difficult since the clinical manifestations of this disorder can range drastically between individuals. There has been recent progress in the testing and use of Piqray (Venot et al., 2018) for treatment of CLOVES, but there may be additional treatments not yet identified which could also benefit the CLOVES community.

Below is a general list of different therapeutic modalities or approaches to drug development:

1. Small molecule screening: when screening compounds already approved by the FDA this is called drug repurposing. Examples include Tylenol and Zyrtec. While cost effective and sometimes the most efficient path to find a drug, this approach is what we call a "fishing expedition." By the nature of the term screening usually large libraries of chemical compounds are tested to see if they influence a disease-relevant outcome in cells lines. For example, a screen might test a library of a thousand or tens of thousands of different compounds to see if the compounds affect a DNA repair mechanism when exposed to a DNA damaging toxin. Those compounds that protect the cell against damage would be studied further. While this can be a cost-effective approach when an effective compound is identified, there isn't always an effective compound identified or the outcome is not truly disease specific. Also, if there is a potential drug identified, it isn't always clear what the chemical or molecular mechanism of action is. And small molecules usually don't target the root cause of the disease.

2. Biologics (antibodies, soluble proteins, hormones): These are large protein-based molecules that are usually injected into the patient and target circulating proteins. Because of their size they do not usually penetrate cells easily or cross certain barriers in the body and therefore they must exert their effects outside of the cell. This limits their utility to certain tissues or localized use.

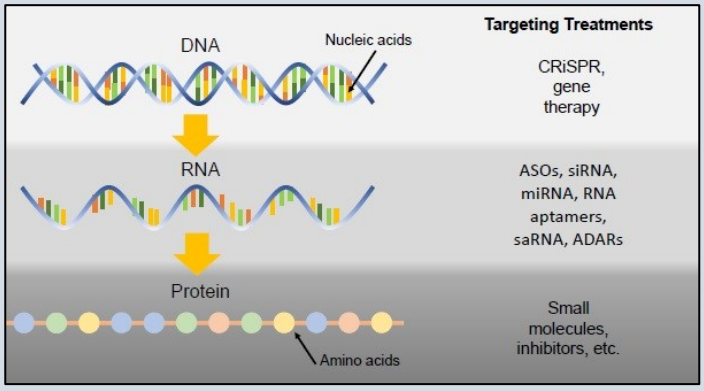
3. Cell therapies (bone marrow transplants, or iPSC delivery): The injection into or replacement of cells in the body is called cell therapy. Sometimes a functional cell is put into the body when the previous cells die off or are dysfunctional, and sometimes cells are taken from the body and treated with a gene therapy and then put back into the body.

4. Gene therapies and genome editing (AAV, lentivirus, CRiSPR): These approaches deliver genetic material (such as a functional gene) or directly edit the mutation in a genome.

5. RNA therapeutics (siRNAs, ASOs, aptamers, etc): Targeting RNA is sometimes easier than delivering DNA, especially when the aim is to decrease the expression or function of a protein. RNA targeting therapeutics utilize a diverse and growing number of approaches that can exert different effects on the RNA itself including upregulation, downregulation, and allele-specific targeting to name a few.

Inside of a cell, DNA is transcribed into RNA before being translated into protein (Figure 2). Therefore, a mutation in DNA can result in the mutation being carried into the RNA transcript. This mutation in turn may result in a change in amino acid in the protein which could affect function of the protein. Because only one copy of the RNA transcript for the mutated version of the protein is different from the wildtype (non-mutated) RNA transcript, it is possible to target this RNA specifically. This opens the door for RNA therapeutics. Additional methodologies can target DNA or protein.

Figure 2. Dogma of DNA



Recommendations for Research

From our initial assessment, these are the recommend priority research areas that hold the most potential for treatment of CLOVES:

- Small molecule screen
  - The most cost-effective place to begin therapeutic discovery is often with a repurposing screen. This can be done in an unbiased fashion, meaning all compounds are treated equally, or it's possible to prioritize testing certain classes of compounds that are suggested to work based on the biology of CLOVES, such as focusing on known PI3K inhibitors not yet tested for CLOVES syndrome (See section on *Current Treatment* for list of PI3K inhibitors).
  - Caveats and considerations: screening for small molecules requires development of a biologically relevant assay. In *Resources and Tools*, we discuss the utility of

developing better animal models and considering cell lines to support drug development. In the absence of these tools initially, researchers can focus on outcomes that relate to alterations of the PI3K-AKT pathway, such as measuring the phosphorylation of Akt which has increased phosphorylation due to increased PI3K activity.

- RNA therapeutics
    - RNA therapeutics include a broad range of technologies that target the gene transcript to: increase expression, decrease expression, target specific alleles, or alter the sequence of the eventual protein (i.e. exon skipping, etc.). Current approaches include antisense oligonucelotides (ASOs), siRNA, miRNA, RNA aptamers, short activating RNA (saRNA), and RNA editing with CRiSPR/Cas9 systems or Adenosine deaminases acting on RNA (ADARs).
    - Caveats and considerations: Although RNA therapeutics are generally thought to have a better safety profile compared to DNA targeting therapeutics, there are still barriers to effective delivery in certain organs and tissue types that need to be overcome. While this can be limiting for systemic treatment, there may be unique advantages to localized delivery (i.e. topical or injections, etc.) since therapeutics for CLOVES may aim to modulate the mutation-bearing cells only. This would need to undergo rigorous safety and pharmacodynamic testing to ensure the therapeutic does in fact penetrate the relevant tissue.

- Targeted development of drugs based on underlying biology.
    - Focusing research on targeting the underlying genetic cause of the disease is a more direct approach with fewer unknowns. Unfortunately, these approaches are sometimes much more costly to fund. This type of screen includes developing novel PI3K inhibitors as well as therapeutics which impact the PI3K pathway through partial modulation without direct PI3K inhibition. Based on the current knowledge of PI3K pathway biology, we have several recommendations which are explored in the *Potential Future Therapeutic Targets* section.

At this time, a number of therapeutic modalities (primarily cell therapy, gene therapy, and biologics) are not likely to be effective for the treatment of CLOVES:

- Biologics are a lower priority recommendation. They mainly exert effects extracellularly (outside of cells) so their utility in impacting the intracellular PI3K/AKT pathway is limited. However, if a biologic was identified which could impact a target upstream of the PI3K/AKT pathway, for example insulin receptors, it could be useful if targeted locally to mutated cells.

- Cell therapies are unlikely to treat the activating PIK3CA mutation, because the mutated PIK3CA containing cells often 'out-compete' the wildtype PIK3CA cells so this is not expected to have a lasting effect.
- Gene replacement therapy is currently the most common type of gene therapy and includes the delivery of a non-mutated form of the gene to certain cells in the body. This is not expected to attenuate the expression of the mutated PIK3CAs since there is no conclusive evidence suggesting increasing the amount of wildtype PIK3CA in cells that express mutated PIK3CA would diminish the effects of the mutated PIK3CA. This may be an area worth exploring further, although research would need to assess the effects of increasing the expression of wildtype PIK3CA in cells that already express wildtype PIK3CA. That being said, there are alternative therapeutic approaches that would be recommended for increasing wildtype expression of PIK3CA before the use of gene therapy, such as allele specific RNA targeting technologies, genome editing, and potentially even drug screening approaches discussed previously. Additionally, gene therapy delivery is most effective when targeting non-dividing cells. Gene therapy is lost over successive cell divisions and therefore not ideal for many dividing cell types. For this reason, direct editing would hold more potential to intervening on the effects of the mutated PIK3CA directly.

Primary Obstacles and Considerations for Therapeutic Development

While there is a broad spectrum of symptoms, the most critical unmet need is reduction of vascular malformations and lipomatous masses. There has been recent advancement in the use of PI3K targeted therapeutics, originally developed for cancer treatment, being used to treat CLOVES and other PROS. But there is still a need to refine therapeutic approaches to meet the specific needs of the CLOVES community. CLOVES syndrome presents unique therapeutic challenges that are necessary to overcome in order to develop effective treatments. Therefore, it is important to understand the biology underlying CLOVES to identify treatments and overcome obstacles.

1. Targeting PIK3CA mutation-bearing cells specifically: PIK3CA and its protein PI3K are expressed ubiquitously. In CLOVES syndrome, a subset of these cells express a mutated form of PIK3CA which leads to aberrant increased activity of PI3K only within this subset of mutation expressing cells. A treatment which ubiquitously decreases PI3K activity would be greatly beneficial in mutated cells but may be detrimental to the normal functioning of non-mutated cells. This is not necessarily an insurmountable problem, but it is one that requires creativity and further understanding.

"While there is a broad spectrum of symptoms, the most critical unmet need is reduction of vascular malformations and lipomatous masses."

    - This obstacle highlights the need to disrupt mutated p110α ONLY.

- Using targeted therapy, either by targeted development of treatment or development of treatments that selectively target only certain cell types/organs, is one way to overcome this obstacle.
- Continued research into ways to target just the mutated p110α would also lead to a treatment option which treats the mutated proteins but leaves the healthy, non-mutated p110α alone.

2. Lack of understanding of mutational effects on increased PI3K activity: Knowing that mutations increase activity is very important. However, it is only part of the story. There are a number of mechanisms that result in increased protein activity, and research focusing on elucidating these mechanisms in CLOVES would aid therapeutic development. Different mechanisms that can result in increased protein activity include: conformational (protein shape) changes that alter protein-protein interactions, resistance to normal degradation pathways, increased expression of the protein due to increased transcript stability, to name a few.

   Genetic mutations can cause changes in protein shape when the mutation affects the amino acid sequence at a particular location. Different amino acids have different properties and by changing even one it is possible that it could cause the protein to dramatically change its shape. This can change how a protein interacts with other proteins. In the case of an activating mutation this can increase a protein's localization to the subcellular compartment where it is active or it can increase a protein's affinity for its substrate (i.e. make it more likely for the protein to be active).

   Additionally, normal degradation pathways exist within cells to regulate levels of proteins and to eliminate dysfunctional or damaged proteins. However, some proteins are able to evade these pathways and certain mutations may make a protein more likely to do so. If this is the case, increasing selective degradation of the protein of interest would reduce overall protein activity.

   In order to produce a protein, a gene must first be transcribed from DNA to RNA. This RNA transcript is the code that the cell uses to make proteins. A genetic mutation may occur which makes the RNA transcript stick around for longer in a cell than it normally would, causing an increase in the amount of associated protein made. This will increase the levels of a protein in a cell without changing the intrinsic level of activity of each individual protein. The different ways a mutation can increase protein function can be important for designing effective therapeutics. For CLOVES, because patients have different mutations, they may have different underlying biological causes for increased PI3K activity. Not only may this have an impact on symptoms and symptom severity, it may also have an impact on which therapeutics may work for each patient. Therefore, determining exactly what is causing the increase in activity has implications for treatment.

- This obstacle highlights the need for more research into the basic biology of the impact of mutations on PI3K function. This type o
- f research can be done in cell culture instead of in animal models, which means that it is often less expensive and time consuming than research in animal models. It is important to utilize multiple different patient mutations and biologically relevant tissues to better understand the sometimes-nuanced effects a specific mutation can have on regulation.
- Identifying the cause of the increase in activity could lead to novel therapeutics that target protein expression and/or protein degradation.

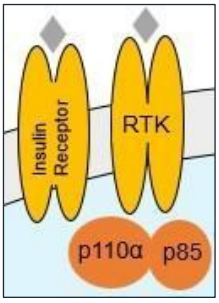
3. The PI3K/AKT pathway is delicate: Disrupting the homeostatic balance of such an integral cellular pathway as the PI3K/AKT pathway can, unfortunately, have unwanted side effects.
    - This obstacle highlights the need to find treatments which manipulate this pathway subtly, specifically, sufficiently, and without causing extreme side effects.
    - This is not an easy task and highlights the need for use of animal models before treatments in humans. While there are drugs that target this pathway that are successful in treating cancers, the longitudinal nature of CLOVES syndrome creates an additional consideration. Because CLOVES effects multiple organ systems and is a life-long disorder, short term treatments used in cancer need to be tested thoroughly for safety and side effects before treatment in CLOVES patients. While many of these treatments are promising, long-term use can increase off-target effects that may add to patient discomfort, suffering, or serious long-term risks.

Potential Future Therapeutic Targets

Continued research into the pathogenic mechanisms of disease is important for identifying the most effective treatments. Suggestions for focused investment in future CLOVES research include:

1. Regulation of the regulatory subunit p85: The catalytic activity of PI3K is contingent upon the interaction between p110α and its regulatory subunit p85. Therefore, inhibiting this interaction could reduce the activity of PI3K. This is recommended only in those cells expressing the PI3K mutation. There are two ways to impact this interaction (1) direct targeting of p85 and (2) targeting the region of p110α where the interaction takes place. Future research into this mechanism could elucidate new drug targets.

2. Upstream or downstream regulation of PI3K activity: Drugs that regulate p110α activity (i.e upstream through receptor tyrosine kinases such as insulin receptors or downstream via Akt) would reduce the detrimental increase in PI3K activity without directly impacting PI3K protein function.



3. Disruption of p110α and p85 binding: A more thorough understanding of exact binding mechanisms of the interaction between p110α and p85 would provide insight into the regions of both proteins that are responsible for this interaction. With this knowledge, it may be possible to design a therapeutic that inhibits or reduces the interactions between these subunits thus decreasing the activity of PI3K.

4. Alteration of the p110 subunit expression and downstream effects: The expression of many proteins is regulated intracellularly. Therefore, it is possible that increasing a different endogenous or exogenous p110 subunit may cause the cell to reduce the level of the mutated p110α. Research should focus on elucidating different p110 expression mechanisms.

5. Targeted protein degradation: Recently, a technology has been developed which is capable of targeting specific proteins for degradation, called Proteolysis targeting chimeric (PROTAC). This is a relatively new technology that uses what is known about the ubiquitin-mediated degradation pathway to selectively target a protein of interest (in this case p110α) for degradation. This technology is interesting because it could be used to degrade p110α selectively without effecting the degradation of other proteins in the cell. No drug using this technology has been approved for use yet, but there are several ongoing clinical trials using this technology to treat cancer (Qi et al., 2021). PROTAC technology continues to be improved upon but there are still some problems to overcome. Improvements currently focus on the creation of optimal linkers, increasing the limited number of E3 ubiquitin ligases that can be used, as well as improving protein binding dynamics. Overall, this has the potential to be a useful tool and developments in the field should be followed.

Learning from PROS and other related syndromes

Similarities between CLOVES syndrome, PROS, and other overgrowth disorders should be explored to determine if it is possible to gain insight into potential therapeutics. CLOVES syndrome is part of a family of disorders, PIK3CA-related overgrowth spectrum (PROS), which encompasses different syndromes with mutations in PIK3CA (Martinez-Lopez et al., 2017).

PROS include (Keppler-Noreuil et al., 2015):

- CLOVES syndrome
- CLAPO syndrome
- Fibroadipose hyperplasia
- Fibro-Adipose Vascular Anomaly (FAVA)
- Facial infiltrating lipomatosis (FIL)
- Fibroadipose hyperplasia (FAO)
- Hemihyperperplasia-multiple lipomatosis syndrome (HHML)
- Hemimegalencephaly (HME)
- Isolated Lymphatic Malformation (ILM)
- Klippel-Trenaunay Syndrome
- Macrodactyly
- Muscular Hemihyperplasia (MHH)
- Megalencephaly-capillary malformation syndrome (MCAP)

Because these disorders are caused by a similar biological mechanism, treatments for one disorder have the potential to benefit others. Additionally, clinicians who specialize in one of these disorders may also be a useful resource for other PROS.

Beyond PROS, there are multiple disorders which are caused by mutations in other genes which affect the same pathway as PIK3CA and a number of disorders which cause phenotypically similar disorders but whose biological cause is unrelated to the PI3K/AKT pathway. Understanding these disorders and the therapeutics used to treat them could help identify treatments already in existence which could be repurposed for CLOVES and/or provide further insight into therapeutic options.

The first step is to identify the disorders most similar to CLOVES. One disorder, Proteus syndrome, is sometimes clinically confused for CLOVES because the two disorders have overlapping clinical characteristics. Proteus syndrome is caused by a mutation in the AKT-1 gene. This gene acts in the same biological pathway as PIK3CA. Because this syndrome has a similar phenotype to CLOVES (including vascular malformations and scoliosis, to name a few) and is caused by an activating mutation in the same pathway, this could mean that a cross-over of treatments is possible. Another disorder, Klippel-Trenaunay syndrome (KTS) is a PROS disorder caused by mosaic, activating mutations in PIK3CA. This disorder differs from CLOVES clinically, but because it shares the same underlying biology, treatments for KTS may be effective for treating CLOVES, and vice-versa. The below table depicts the clinical similarities and differences between CLOVES syndrome, Proteus syndrome, and Klippel-Trenaunay syndrome. We consider these disorders most closely related to CLOVES and worth prioritizing for follow-up research and therapeutic identification.

Table 3. Comparison of the symptoms of CLOVES, Proteus and Klippel-Trenaunay syndromes identifies symptoms specific to each disorder as well as symptoms that are common across all three.

	Symptoms Specific to:			**Common to all**
	CLOVES	**Proteus**	**Klippel-Trenaunay**	
Mutation	Sporadic, mosaic activating mutation in **PIK3CA**	Mosaic activating mutation in **AKT-1**	Mosaic activating mutation in **PIK3CA**	
Vascular malformations	Spinal AVM	*See common to all*	*See common to all*	Capillary malformation, venous malformation, lymphatic malformation
Skeletal anomalies	Scoliosis, pectum excavatum	Scoliosis, soft and bone tissue hypertrophy	soft and bone tissue hypertrophy	Lower extremity asymmetry
Acral anomalies	Polydactyly, syndactyly, sandal gap	*See common to all*	Polydactyly, syndactyly	Macrodactyly
Neural anomalies	Syringomyelia, dysgenesis corpus callosum, seizures	Developmental delay	Seizures, developmental delay, spina bifida	Hemimegal-encephaly
Visceral anomalies	Renal agenesis/ hypoplasia, spleen lesions	Liver, spleen, thymus hypertrophy	Lymph node hyperplasia/hypoplasia	*None*
Additional Complications	Scoliosis, cardiac failure due to AVM	Developmental delay, lung restrictive syndrome	Developmental delay, Kassabach-Merrit syndrome, Disseminated Intravascular Coagulation	*None*

(Martinez-Lopez et al., 2017)

From this table, the main similarities of these three disorders are in their vascular malformations. Similar to CLOVES, the current vascular treatment recommendations for Klippel-Trenaunay are embolization, ligation and stripping, sclerotherapy, extraction surgery, laser therapy and endovenous thermal ablation. Currently, there are several ongoing clinical trials studying the safety and tolerability of the drug Miransertib, an Akt inhibitor administered orally, in Proteus and PROS syndromes (including CLOVES syndrome). Please find links to this clinical trial in the *Clinical Trials* section.

Learning about the treatment of additional overgrowth syndromes such as Beckwith-Wiedemann (the most frequent genetic overgrowth syndrome), Parkes-Weber, and PTEN hamartoma syndrome which includes Cowden and Bannayan-Riley-Ruvalcaba syndrome may provide important considerations and insight for CLOVES syndrome treatments. Importantly,

one recent clinical trial investigated the impact of a combination of chemotherapy and surgery in the treatment of Wilms tumors in Beckwith-Wiedemann syndrome (https://www.clinicaltrials.gov/ct2/show/results/NCT00945009). Wilms tumors are a type of rare, malignant childhood cancer originating in the kidneys. They are rare in CLOVES syndrome; however, they are more prevalent in the CLOVES syndrome community than in the general population (Peterman et al., 2017). Therefore, this could be useful for the subset of CLOVES patients who also suffer from Wilms tumors. Additionally, because most Wilms tumors are quite large before they are identified, it is prudent for patients diagnosed with CLOVES to be screened for this type of cancer. The CLOVES Syndrome Community recommends ultrasound screening every 3 months up to age 8 then once between 8 and 12 years old to rule out a late developing Wilms tumor.

Resources and Tools

Animal Models

Animal models provide a means to study the effect of genetic mutations on behavior and biology, as well as test therapeutics for efficacy, safety, and toxicity. The existence of an effective translational animal model enables disease communities to test therapeutics on relevant models prior to moving into human testing. *While this lowers risks that arise if a treatment is moved directly into human trials without animal testing, models can also have the added benefit of accelerating discovery of new therapeutics, decreasing wasted funding, and increasing pharma interest in a specific disease.* Animal models are validated based on certain criteria and can have predictive, face, and/or construct validity.

- Predictive validity: a measure of how well a model can be used to predict currently unknown aspects of human disease (e.g. correlation between the animal and human as it relates to therapeutic outcomes)
- Face validity: a measure of how well a model replicates the disease phenotype in humans (e.g. does the animal model exhibit all the symptoms and behaviors found in the human condition?)
- Construct validity: how well the mechanism of disease in the humans is matched in the animal model (e.g. does the underlying biology match between the human and the animal)

While it would be ideal to have an animal model that had predictive, face, and construct validity, this is rarely possible. Important findings can still be made using an animal model which does not perfectly recapitulate the human condition.

Animal Models for CLOVES

Main issues with current animal models for CLOVES syndrome:

- Many are not commercially available
- Homozygous animals are often embryonically lethal
- Lack of mosaic models

There are several animal models that have been used in CLOVES research to date. Unfortunately, not all researchers are willing to share their animal models with others. While this is a problem, there are commercially relevant models that can be accessed by the research community. Some of the models with mutations in PIK3CA developed by researchers that are relevant for CLOVES take advantage of the H1047R or E545K PIK3CA mutations (the most common mutations in PIK3CA found in cancer) and many of these have already been identified by the CLOVES Syndrome Community. In addition to cancer, the E545K mutation is also found in patients with epilepsy and a mouse model has been used to study this mutation specifically in the brain (Roy et al., 2021). Unfortunately, this mouse model is not currently commercially available. This is not an insurmountable problem as many researchers may be willing to share their model or develop an assay that could benefit additional interests. If a model is identified that is not commercially available but would be perfect for study, we recommend speaking with the lead author to establish their willingness to share. If research has been published utilizing the model there may also be outreach through the publishing journal and University that can help access the mouse. On the other hand, there is a commercially available mouse model that has the H1047R mutation. Unfortunately, this colony is about to removed. This does not mean that the mice will no longer be available, just that they will have to generated from cryorecovery of embryos which takes longer than if the company is actively maintaining a mouse colony.

Currently, there are five commercially available mouse strains available through Jackson Laboratory for PIK3CA. While these strains were not specifically created to study CLOVES syndrome, they have mutations in the PIK3CA gene which may make them candidates as a model for CLOVES and other PIK3CA disorders. The table below gives information on the exact mutation or mutations in each model and the effects of each mutation.

Table 4. Commercially available mouse models of mutations in PIK3CA.

Strain Name	PIK3CA Mutation	Phenotype	Reference
1. B6.129S7(Cg)-Pik3ca^{tm1Jdo}/J (Pik3ca RBD)	2 point mutations (T208D, K227A) that effect RAS binding but not PI3K enzymatic activity	Mice demonstrate defects in growth factor signaling and lymphatic vasculature development	(Gupta et al., 2007)

2. B6N.129-Pik3ca^{tm1Jjz}/J (p110alphaflox)	Cre-lox for deletion of p110α	Loss of p110α allows for the study of insulin signaling, hepatic glucose and lipid metabolism, and oncogenicity	(Zhao et al., 2006)
3. B6.129S4-Pik3ca^{tm1Mawa}/J (Pik3ca:C420R)	Gain of function mutation (C420R)	C420R mutant which can be conditionally knocked-in	(di Blasio et al., 2018)
4. FVB.129S6-Gt(ROSA)26Sor$^{tm\ 1\ (Pik3ca*H1047R)\ Egan}$/J (R26-Pik3ca^{H1047R})	Breast cancer associated mutation H1047R	When crossed with a Cre-strain, mice develop mammary tumors as well as lymphoid and skin malignancies	(Adams et al., 2011)
5. C57BL/6-Gt(ROSA) 26 Sor$^{tm7\ (Pik3ca*,\ EGFP)Rsky}$/J (R26Stop FLP110*)	Constitutively active p110α allele	Inducible expression of activated PIK3 may be useful in studying cell survival, growth, and proliferation, and differentiation, regeneration, hypertension and cancer.	(Srinivasan et al., 2009)

Of these models, the strains most suited for research on CLOVES are strains 3 and 5. These strains are similar to CLOVES syndrome in that they increase the activity of p110α. One strain, the Pik3ca:C420R strain (strain number 3), has been used to test the efficacy of the drug alpelisib (Piqray, BYL719) on symptoms caused by a PIK3CA mutation (see *Current Treatment*) (Venot et al., 2018). ***Given that this drug has also been tested in CLOVES patients and proven to be effective provides further support to the utility of this mouse model in preclinical testing.*** Until alternative models are developed, this strain should be considered for future research.

Mosaic Mouse Models

CLOVES syndrome is complicated with regards to generating an animal model because it is a disease caused by mosaic mutations. Because the mutation in PIK3CA found in patients is not found in all cells of an individual, it can be very difficult to generate an animal with construct validity. Animals overexpressing a mutation in all cells can be useful, but it is important to remember that that might not be fully indicative of the human condition, especially in terms of treatment side effects in healthy, non-mutated cells. However, there are a few options already available and genetic tools are advancing to a point where there is hope of creating a mosaic model in the future.

Traditional mouse models can serve a purpose; however, they are not the only animal model nor are they necessarily the best model to answer all questions relevant to CLOVES. Recently, great strides have been made towards creating more complex transgenic animal models. Since CLOVES Syndrome is caused by mosaic mutations in PIK3CA, we recommend exploring the creation of a mosaic mouse model to provide greater construct, and face, validity. Using a mosaic mouse model would allow researchers to understand the effect a treatment has, not only on mutated cells, but also on healthy cells. It also opens the possibility of trying targeted delivery to determine if that can have beneficial effects without off-target side-effects.

Table 5. Different approaches to generation of mosaic mouse models.

Technique	Summary	Pros	Cons	References
ifgMosaic	This technology enables the examination of multiple and combinatorial gene function with high temporal and cellular resolution	- Can be used for multiple genes - Can look at any gene of interest - Fluorescent markers allow for tracing of which cells contain the mutation	- Might get low rate of cells expressing transgene	(Pontes-Quero et al., 2017)
MADM (mosaic analysis with double markers)	Mosaic analysis with double markers (MADM) offers one approach to visualize and concomitantly manipulate genetically defined cells in mice with single-cell resolution	- Fluorescent markers allow for tracing of which cells contain the mutation	- Doesn't work for all genes - Generates homozygous mutant cells for a candidate gene of interest and wild-type cells in an otherwise heterozygous background	(Contreras et al., 2021)
CRiSPR-Cas9 mediated	CRiSPR-Cas9-mediated, site-directed mutagenesis in mice generates mosaic founder mice	- Can look at any gene of interest	- Would need to choose a representative mutation - Varied efficiency of desired point mutation, and other non-homologous end-joined variants - No fluorescent marker	(Vasu and Fox, 2021)

Additional Animal Models

While mice continue to be the most widely used small mammal model of disease, there are many additional animal models that may also be useful to consider. When deciding on which

animal model to use, it is best to keep in mind the question that is being asked and whether a chosen animal is best suited to answer that question. For example, if you want to know how a particular genetic mutation effects spinal cord development you would not want to use a fly or nematode since neither of these animals have a spinal column. However, a fly may be a good choice if you are interested in understanding the effect of genetic mosaicism on development since flies are a good model of mosaicism and have been used since the early 20th century (Germani et al., 2018) to study mosaicism in detail. In short, there is no one animal model that is best for all questions and it is prudent to think through the aims and goals of a particular question before deciding on the best animal model to use.

For CLOVES syndrome, there may be benefits to using animal models in addition to mice. One animal that is worth considering, especially for research into vascular development, is the zebrafish, or *Danio rerio*. Zebrafish share physiological similarity with mammals, are cheap to breed, develop quickly, have transparent bodies that allow for visualization of internal organs while the animal is still alive, and are amenable to many genome engineering approaches (Gut et al., 2017). They also may be a good model for early drug testing since they are responsive to water-soluble drug delivery. While there are transgenic lines available, so far none exist that would be good for studying CLOVES. The website zfin.org is a good place to start to see what data exists in zebrafish for a particular gene or mutant line. In addition to zebrafish, flies may also be a useful animal model for the CLOVES community. The fruit fly, or *Drosophila melanogaster*, is a model that has been used by geneticists for decades. Because of this, many techniques have been developed which make it a useful model for studying a genetic disorder such as CLOVES. There are many methods that exist for generating mosaic flies (Germani et al., 2018).

Cell Lines

Cell lines are very useful for exploring the biology of disease pathways and understanding treatment effects on biology. Cell lines, or immortalized cell lines, are an important tool in research. They are grown in the lab and can be useful for studying proteins. Unlike induced pluripotent stem cells (IPSCs), they do not share the same genetic background as a patient with a rare disease. However, they are much easier and faster to work with than IPSCs and mutations can be introduced into cell lines that mirror patient mutations. While cell lines do not fully recapitulate the human condition they can serve as a quick and easy screening tool to identify potential drug targets as well as serve as a helpful tool to screen drugs of interest. Initial screening of drugs done in cells can identify if the drug has an impact on the PI3K/AKT pathway. Screening for PI3K activity can be done by testing for the phosphorylation of Akt which indicates Akt activation. This type of screening could also be done in the presence of patient mutations to identify compounds that are able to overcome the disease-relevant dysfunction, or to better understand the effect of a mutation on specific function or molecular event.

Additionally, cell lines may be useful in identifying the dynamics of p110α and p85 interactions or used to explore the upstream regulation of PI3K by tyrosine kinase receptors, including the insulin receptor. These types of studies could be done in wildtype cell lines which reduces the cost of creation of mutant lines or generation of induced pluripotent stem cells. That being said, cell lines are not a perfect model, and the development of patient derived cell lines (such as IPSCs) or patient specific mutations can provide additional insight into disease mechanisms and heterogeneity in drug response across different PIK3CA mutations. Both options can be explored simultaneously and can be used to address different questions.

Organizational Strategy

Academic Groups

Investigating new therapeutics and advancing knowledge of the pathways disrupted in CLOVES patients requires dedicated clinicians, researchers, and patients/patient's families working together. Below is a compiled list of researchers who work in related fields or whose research interests are in alignment with the CLOVES syndrome community. We always recommend expanding your research network whenever possible to encourage fresh ideas and critical feedback on current approaches. These investigators have expertise that could be helpful in continuing progress towards solving some of the predominant concerns of CLOVES patients and their families. All information on the following researchers was gleaned from their websites and is current on December 17th, 2021.

Leslie G. Biesecker, MD
National Human Genome Research Institute, National Institutes of Health
Website: https://www.genome.gov/staff/Leslie-G-Biesecker-MD

Klaus Rajewsky, PhD
Max Delbruck Center for Molecular Medicine, Berlin
Website: https://www.mdc-berlin.de/k-rajewsky

Rui Benedito, PhD
CNIC, Spanish Center for Cardiovascular Research
Website: https://www.cnic.es/en/rui-benedito

Phillip Hawkins, PhD
Babraham Institute, UK
Website: https://www.babraham.ac.uk/our-research/signalling/phillip-hawkins

Len Stephens, PhD
Babraham Institute, UK
Website: https://www.babraham.ac.uk/our-research/signalling/len-stephens

Jeroen den Hertog
Hubrecht Institute, Netherlands

Website: https://www.hubrecht.eu/research-groups/den-hertog-group/

Randall T. Peterson
University of Utah Health
Website: https://pharmacy.utah.edu/pharmtox/randall/randall-peterson-lab

Tian Xu
Yale School of Medicine
Website: https://medicine.yale.edu/lab/xu/research/

Patient Groups and Umbrella Organizations

CLOVES Syndrome patients have access to patient groups not only in the US but worldwide. Odylia recommends partnering with other overgrowth disease patient groups, within and outside of PROS. An advantage of partnering with other overgrowth communities is their experience with clinical trials and drug development programs. The overgrowth organizations also face similar challenges with their disorders. Therefore, partnerships could help all parties move forward with identifying common researchers, clinicians, centers of excellence that specialize in overgrowth disorders as well as drugs that could potentially be used across different disorders. All of the following information is current on December 17th, 2021.

The CLOVES Syndrome Community has listed medical providers worldwide who have experience working with CLOVES and other PROS syndrome disorders on their website. The list below includes experts from other overgrowth disorders who may provide additional resources for collaboration.

Beckwith-Wiedemann Syndrome (BWS)

- BWS is the most commonly diagnosed overgrowth syndrome (Ko, 2013) and can be caused by dysregulation of genes on chromosome 11
- Though symptoms vary from person to person, the most common include large tongue, overgrowth of one side of the body, increased risk of Wilms tumor (a specific type of kidney tumor), abdominal wall defects, and abnormal levels of insulin in the blood.
- There is an ongoing clinical trial that includes patients with BWS that is investigating the efficacy of a combination of chemotherapy and surgery to treat young patients with Wilms tumors. This clinical trial may have implications for CLOVES patients with Wilms tumors.
    - https://www.clinicaltrials.gov/ct2/show/results/NCT00945009?cond=Beckwith-Wiedemann+Syndrome&draw=2&rank=5&view=results
- Because BWS is the most common overgrowth syndrome, it would be an advantage to work in collaboration with them to both increase awareness of overgrowth disorders and potentially model their Centers of Excellence model.
- Website: https://www.beckwithwiedemann.org

Proteus Syndrome

- Proteus Syndrome is characterized by progressive overgrowth and caused by mosaic mutations of the AKT1 gene.
- Symptoms vary but may include abnormal fat tissue distribution below the skin, skeletal deformities, increased risk of tumors and blood clots, and neurological abnormalities see Table 2 for more details.
- An advantage of working with the Proteus Syndrome community is the possibility for crossover treatments since many therapeutics used in Proteus target the PI3K/AKT pathway.
- Website: https://www.proteus-syndrome.org

PTEN Hamartoma Syndrome (PHTS)

- PHTS is caused by mutations in the PTEN gene. It is a spectrum of conditions including Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, and PTEN-related Proteus/Proteus-like syndrome.
- It would be advantageous to work with the PHTS community because it is caused by a mutation in another gene in the PI3K/AKT pathway. Treatments that work for this disorder may also work for CLOVES syndrome patients.
- Currently there is a clinical trial set to complete in December 2021 which is studying the effects of the drug RAD001 (everolimus), an mTor inhibitor, on PHTS patients. It's main goals are to look at safety and neurocognition, but it may be an interesting treatment option for CLOVES since it is similar to sirolimus.
    - https://www.clinicaltrials.gov/ct2/show/NCT02991807
- Website: https://ptenfoundation.org/

Parkes Weber Syndrome

- Parkes Weber syndrome is a rare, congenital disorder that presents with vascular anomalies, especially abnormally large vessels. These patients also often present with arteriovenous malformations in their limbs.
- This syndrome is caused by a mutation in the RASA1 gene, which encodes a protein called p120-RasGAP. While this is an understudied protein, it is known to influence the RAS/MAPK signaling pathway which is involved in cell differentiation and proliferation.
- A potential advantage of working with this community is in the treatments of both abnormally large vessels and AVMs which are symptoms found in CLOVES patients as well.
- Website: https://k-t.org/about-kt/similar-conditions

Castleman Disease

- Castleman disease includes three different immune system disorders: Unicentric Castleman disease, HHV-8-associate multicentric Castleman disease, and HHV-8-negative/idiopathic multicentric Castleman disease (iMCD).
- This disorder is difficult to diagnose, often involves multiple organ systems, and the cause is currently unknown

- The Castleman Disease Collaborative Network (CDCN) has extensive experience building data collection mechanisms for tracking medication use and outcomes of off-label use of drugs to treat CD. The CDCN founder, a doctor, research, and patient with CD, has experience in the off-lab use of sirolimus to treat CD and can advise on how best to navigate this landscape when your organization is initiating these efforts.
- Website: https://cdcn.org/

Centers of Excellence

A Center of Excellence (COE) is defined as a healthcare institution that offers resources based on a high concentration of diverse expertise relevant to a disease, group of diseases, or common presentations/symptoms. COEs utilize an interdisciplinary model approach in delivering treatment to patients with a disorder or related disorders and are often recognized as providing an exceptionally high level of care (Elrod and Fortenberry, 2017). A range of specialty areas are usually housed at the centers. The specialties in the CLOVES centers include: cardiology, orthopedics, radiology, ophthalmology, dermatology, neurology, neurosurgery, general surgery, and endocrinology, just to name a few. These specialties are also seen in vascular anomalies centers of excellence, which affords patients the opportunity to work with clinicians who have more than the average experience with overgrowth syndromes, which is important to access whenever working with rare diseases. This interdisciplinary approach allows for the best patient outcomes possible (Elrod and Fortenberry, 2017) due to quicker diagnosis, specialized standard of care, and combination treatment approaches. All of the following information is current on December 17th, 2021.

Recognizing that the CLOVES Syndrome Community has 6 COEs specifically recommended for CLOVES patients, we suggest consideration of additional partnerships with related communities that have additional experience with clinical trials, patient registries, and working with the PI3K/AKT pathway. A partnership may allow both organizations to leverage resources and funding, as well as, increasing accessibility for patients that live far from current CLOVES COEs.

Table 6: Contact information for the Centers of Excellence of the Umbrella Organizations listed above, as well as the benefits of contacting each center.

Center	Disease	Contact Name	Why this center?	Website
Children's Hospital of Philadelphia	Beckwith-Wiedemann Syndrome	Jennifer M Kalish, MD/PhD	The BWS foundation collaborates with this center of excellence on research, education, treatment and care for patients with BWS, their patients and care team. There is also a BWS registry based at CHOP.	https://www.research.chop.edu/beckwith-wiedemann-syndrome-program-of-excellence/bws-resources

Children's National	Proteus Syndrome	Laura L. Tosi, MD	Children's National was the pediatric hospital that worked with the NIH to identify the gene variant of Proteus Syndrome. The site also has extensive experience with working with AKT1 mutations.	https://childrensnational.org
Cleveland Clinic	PTEN HTS	Charis Eng, MD/PhD	The Cleveland Clinic is one the designated PTEN/PHTS Centers of Excellence. Their PTEN Multidisciplinary clinic provides services to both adults and children. Evidence-based care provided, based on the expertise of the team.	https://my.clevelandclinic.org/departments/genomics/specialties/pten-clinic
Vascular Anomalies Center at Boston Children's	Parkes Weber Syndrome	Ahmad Alomari, MD, MSc, FSIR	This center collaborates with clinicians from many different specialties with expertise in treating vascular anomalies and related symptoms.	https://www.childrenshospital.org/centers-and-services/programs/o-_-z/vac#

Exploring the Clinical Landscape

Current Treatment

So far, the current drug treatments for CLOVES mainly target the PI3K pathway which inhibits tumor progression, inhibit cellular proliferation, and increase cellular death (Yang et al., 2019). Treatment with sirolimus (or rapamycin), an mTor inhibitor, can aid in the treatment of vascular anomalies, decrease the size of lipomatous and lymphatic masses, improve physical capacity, autonomy and overall quality of life (de Grazia et al., 2019), however it does not work for all patients. In addition to sirolimus, a recent study was conducted in mice and humans to test the impact of alpelisib (Piqray) on severely affected CLOVES patients. This study found reduction in tumor sizes, renal function improvement and reduction in spinal venous malformations (Venot et al., 2018) which is very promising for movement into additional patients.

Recently, Novartis conducted a real world, non-interventional retrospective study (EPIK-P1) by reviewing the charts of 57 patients (39 pediatric and 18 adult) who were treated with alpelisib daily for two weeks (Novartis, 2021). For primary analysis, only 32 of the cases had completed the treatment and were included in the analysis. Similar to the earlier report by Venot et al., Novartis found improvements with 38% of patients showing a 20% reduction in lesion size, with 74% of patients showing a mean of 13.7% reduction in lesion size. Unlike the previous study however, all patients did not have a reduction in lesion size, but, no patients experienced disease progression or death at time of primary endpoint analysis. Unfortunately, there were also more reported adverse events in the EPIK-P1 study than in the previous report by Venot et

al. While these differences are important to note, it is difficult to interpret them since it is not clear what dose of alpelisib patients received in the retrospective study nor if there was a consistent dose given across patients. In the Venot et al. study patients received 250mg/day, which is the lowest dose used in clinical trials, for at least 18 months (Venot et al., 2018). Patients in the retrospective study were treated for only 2 weeks. This could limit the potential for beneficial effects and, if the dose is high, could also account for an increase in adverse effects. Higher levels of variability are expected in retrospective studies in general because it is not possible to control for consistency across all patients in the same way an interventional study is controlled. Although the reduction in lesion size in response to alpelisib was lower and there was an increase in adverse effects in the patients of the EPIK-P1 study in comparison to the Venot et al. study, the findings in both show promise due to observed improvements in quality of life and the potential for treatment without surgery. It is clear from these two studies that additional work needs to be done to determine the best course of treatment.

The CLOVES Syndrome Community has developed an extensive list of current or planned clinical trials for CLOVES involving PI3K/AKT/mTor inhibitors. Because this list is so well developed, we only have a few things to add to it. All of the following information is current on December 17th, 2021.

- VT30-(BBP 681): this is a PI3Kα inhibitor developed by BridgeBio Pharma and Venthura. There is currently an ongoing, first in human clinical trial which includes patients with venous malformation, lymphatic malformations and/or venolymphatic malformations associated with PIK3CA mutations.
    - https://www.clinicaltrials.gov/ct2/show/NCT04409145
- Sonolisib (PX – 866): this is a PI3K inhibitor developed by Oncothyreon currently in clinical trials for glioblastoma and prostate cancer among other cancers.
- Taselisib (GDC-0032): this is a PI3K inhibitor that recently terminated a clinical trial in patients with PI3KCA mutation related overgrowths. The trial was terminated due to suspected unexpected serious adverse reaction (SUSAR).
- Miransertib (ARQ 092, ArQule, MK-7075): is an Akt-inhibitor that has ongoing clinical trials in Proteus syndrome (Not being offered to new CLOVES patients currently, but in trials in PROS syndromes) See *Clinical Trials* section

Surgical intervention is a more invasive approach to treatment and management of CLOVES. While these procedures can be effective, there are often downsides and risks to the intervention that highlight the need for development of new therapeutics and innovative approaches. The most commonly used surgical interventions include sclerotherapy, embolization, debulking and IVC filter. Surgical procedures used in the treatment of CLOVES include:

- Sclerotherapy: a treatment method that utilizes the injection of a solution directly into the veins, causing the blood vessels to swell up, stick together and eventually turn into scar tissue. Side effects can include prolonged hardening of the veins, brown lines or

spots at the infection site, bruising, revascularization, and allergic reactions (https://my.clevelandclinic.org/health/treatments/6763-sclerotherapy). *The downside of sclerotherapy is the possibility of recurrence of overgrowth in the treated area in PROS patients* (Pagliazzi et al., 2021).

- Embolization: defined as the use of tiny particles to block a blood vessel. Embolization is used to stop bleeding or blood flow to a tumor or abnormal area of tissue hopefully allowing for preservation of healthy surrounding. Embolic agents include spongy material, polymers, clot forming liquids, stainless steel, platinum coils, liquid glue and chemotherapy medications. Embolization is highly effective in cutting off blood supply to tumors and provides less damage to the body in comparison to sclerotherapy for example. *Risks and side effects include bruising at site of catheter insertion, allergic reaction to contrast fluid, damage to blood vessels from catheter, kidney damage from contract fluid, mild discomfort, and pain* (https://www.cvmus.com/vascular-procedures/embolization-procedure-what-expect-and-faq).

- Debulking surgery: consists of the surgical removal of tissue overgrowth in an effort to increase functionality and decrease deformity of the area. Although debulking surgery can increase the efficaciousness of other therapies such as drugs and radiation *recurrence of masses is common.* Debulking usually requires a drug therapy afterwards to reduce the likelihood of recurrence of the tumor (Alomar et al., 2019). Sclerotherapy and other non-invasive medical modalities are recommended before surgical resection (Weissler et al., 2017).

- Inferior Vena Cava (IVC) filter: The inferior vena cava is the primary vein carrying oxygen-poor blood from the lower body back to the heart. An IVC filter is a small device used to stop blood clots from moving to the lungs. The filter catches and stops blood clots from reaching the heart and lungs and in turn prevents pulmonary embolisms. An IVC filter can prevent pulmonary embolisms, life-threatening clots that can form and travel through the bloodstream and into the lungs of children with CLOVES.

Combination Treatment Surgery and Drug Therapy

Debulking surgery can be an effective tool in reducing masses, however this is not always an option for all patients and even after surgery there can be recurrence. To address this problem, clinicians and researchers have tried combining surgery with drug treatment. In a study that compared the use of sirolimus to the use Piqray in treatment of a mouse model, researchers found that Piqray could be a beneficial treatment in patient for which sirolimus is not an option (Venot et al., 2018). In future, additional PI3K/AKT pathway inhibitor drugs could also be explored for the same use. This type of treatment mimics what is often done in the treatment of cancer where an effort is made to reduce the size of tumors prior to surgery. It may be helpful to consider variations on the protocol- before, after, or both before and after surgery- to improve outcomes.

Surgery and Slow-Release Devices

In addition to encouraging research into treatments that target new mechanisms, we also suggest considering research into triggered or targeted drug release devices such as coated surgical meshes or slow-release implants. Surgical meshes are made of various biopolymers and polymers and are used to provide additional support to damaged tissue and can be used in combination with chemical coatings. So far, we have not identified examples of their use in CLOVES syndrome but potential benefits include strengthening the area where the tumor was removed and potentially reducing the likelihood of tumor recurrence if used with a coating that inhibits overactive PI3K. One example of the use of coated surgical meshes in glioblastoma found promise in a drug-eluting 3D-printed mesh and management of tumor growth (Hosseinzadeh et al., 2019) which gives hope that this type of device could be used for other conditions.

Slow-release implants may also be useful to consider applying in innovative ways because they can increase dosing compliance, deliver medication to a localized area (i.e. a tumor or specific vascular area), often minimize side effects, and usually lower doses of drugs can be used overall. Slow-release implants are advantageous in that they offer direct release of the treatment to the affected site over time (Stewart et al., 2018). This direct delivery method could potentially further reduce, if not eliminate recurrence of overgrowth areas all together but has not been test in CLOVES before. Biodegradable implants rely on passive release of drug and can be degraded by the body overtime. On the other hand, dynamic implants are often made of metallic components and act more as a pump which does not biodegrade (Stewart et al., 2018). Both tools have benefits that could be worth investigating for CLOVES patients.

Clinical Trials

Current Clinical Trials in CLOVES

CLOVES Syndrome Community has an extensive list of ongoing clinical trials for CLOVES patients, the majority of which focus on the PI3K/AKT pathway. However, there is currently one additional clinical trial that includes CLOVES patients looking at the safety of Miransertib, an Akt inhibitor. This trial includes patients with PROS and Proteus syndromes. Additionally, there is also a trial on the long-term safety and efficacy of alpelisib (Piqray) in PROS patients. All of the following information is current on December 17th, 2021. Details are included here:

- A Study of the Safety and Tolerability in Participants with PIK3CA-related Overgrowth Spectrum or Proteus Syndrome Who Are Being Treated with Miransertib (MK-7075) in Other Studies (MK-7075-006)
    - https://clinicaltrials.gov/ct2/show/NCT04980872
- Study Assessing Long-term Safety and Efficacy of Alpelisib in Patients with PIK3CA-Related Overgrowth Spectrum (PROS) Who Previously Participated in Study CBYL719F12002 (EPIK-P1) (EPIK-P3)
    - https://www.clinicaltrials.gov/ct2/show/NCT04980833

Furthermore, there are two ongoing studies looking at the effects of Miransertib in patients with Proteus syndrome that don't include PROS patients. While these may not be immediately beneficial to CLOVES patients, it is worthwhile to track the outcomes of the trial as the outcomes may be useful for future CLOVES treatments as the protein mutated in Proteus syndrome is in the same biological pathway as PI3K.

- Dose Finding Trial of MK-7075 in Children and Adults with Proteus Syndrome
    - https://www.clinicaltrials.gov/ct2/show/NCT02594215
    - ARQ 092 (Miransertib) in Proteus Syndrome https://www.clinicaltrials.gov/ct2/show/NCT04316546

Repurposing Drugs through Clinical Trials

Drug repurposing usually refers to the research process whereby drugs are tested in a disease-relevant cellular or animal model to evaluate their suitability in treating a disease. That being said, there are a growing number of clinical cases, especially for rare diseases, that test experimental and approved compounds directly in patients before initial testing in animals. While this is not advised when there is a means to testing through translational models, there are instances where it can make sense to proceed directly with clinical testing. The methods to do so include off-label use, compassionate use (also known as Expanded Access), and treatment under the Right to Try Act. Given the similarities between different PROS and the common targeting of the PI3K/AKT pathway, these avenues may be worth exploring in more depth for current experimental and approved drugs as well as those that are developed in the future. Below is an overview of each access category:

Off label use refers the use of an approved drug for an unapproved indication (disease or symptom) or condition. Each drug is approved for use in specific indications and when a treating physician prescribes a drug for an indication that is not on the list of approved indications then that drug is being used 'off label.' This is a legal and common practice and is justified when scientific evidence suggests that the drug will be both effective and safe in the treatment of the un-approved indication and is in the best interest of the patient.

Compassionate use and treatment under the **Right to Try Act** refers to use of an investigational drug being tested for another indication. In this case, patients and their physicians can petition the drug manufacturer to access the drug for use when the patient has exhausted all other possible treatments and has a life-threatening or serious condition. Compassionate use applications must be reviewed by the FDA and an Institutional Review Board (IRB), while the FDA and IRB do not have to review applications under the Right to Try Act, only the treating physician and the drug manufacturer are required to approve of use under a Right to Try application.

Importantly, in the context of rare diseases like CLOVES, off label use of drugs or specialized use cases such as compassionate use can lead to the effective identification of new treatments, but

only when information is shared, and more than one patient is treated. For this reason, registries and natural history studies can be very effective in the tracking of treatment trends in a rare disease community and dissemination of that information to researchers, clinicians, and patient foundations. The inspiring story of Dr Fajgenbaum outlines this pathway well. Dr. Fajgenbaum is a clinician, researcher, and patient of his own disease that began using sirolimus off-label to treat his disease, Castleman Disease. He now leads the Castleman Disease Collaborative Network, a priority of which is data collection to identify effective treatments and research priorities more quickly. Data tracking in these specialized use cases is important to aiding the entire CLOVES community in identification of effective therapeutics. Future resources should be directed towards effective data collection and data mining efforts.

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