# Development of therapies for rare disorders due to GPX4 gene mutations: Roadmap and Opportunities

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# Introduction

Sedaghatian Type Spondylometaphyseal Dysplasia (SSMD) is an ultra-rare genetic disease caused by variants in the gene *GPX4* (glutathione peroxidase 4). It was first reported by Sedaghatian et al in 1978 and only nine cases have been reported since. Recently, we identified four additional living pediatric patients with this disease. Children with SSMD have severe hypotonia with lack of head control, are unable to sit or walk, are non-verbal, have significant physical and cognitive development delays, and are at a high risk of premature death due to respiratory or cardiac distress. The corresponding author of this article (S.K.R) is a parent of one of these patients, who started an organization, CureGPX4, aimed at developing cures for this disease (www.CureGPX4.org). The immediate goal is to find treatments that can improve the quality of life of SSMD patients within the next 6-12 months, in order to treat current patients, and ultimately finding modalities for treatment or even prevention of the disease in children with GPX4 mutations.

The goal of CureGPX4 is ambitious. There have been over 7,000 rare diseases described, ~5% of them have at least one treatment approved by the Food and Drug Administration (FDA) (Miyamoto et al). While there are initiatives aimed at speeding up therapeutic development for rare diseases, traditional small molecule drug discovery takes several years to complete, can cost billions of dollars, and identified therapeutic candidates have a low probability of clinical success (DiMasi et al). Emerging technologies such as gene therapy or antisense oligonucleotides (ASO) have a faster development timeline but can still be in the order of years, and in some cases are tailored for each patient (n=1 treatment), and in other cases, such as in mitochondrial encoded genes, such technologies have not been developed even in an experimental setting. In addition to the reducing time, CureGPX4 would need to raise several million dollars, produce relevant scientific discoveries, build a patient community, stimulate biotech industry investment, conduct clinical trials, and secure regulatory approvals to bring therapeutics to patients. Like other rare disease communities, CureGPX4 neither has the money, nor, more critically, do our children have the time to let this process play out.

As a critical first step, we (CureGPX4) have created a new roadmap for therapy development capable of meeting our lofty goal by applying a few guiding principles - namely - seek incremental therapies; prioritize saving time over money; and fail fast to maximize learning. We created the CureGPX4 roadmap by working backwards from patient needs, aiming for

incremental therapies, which may first slow, then stop, and finally reverse disease progression. In two weeks, we identified eight FDA-approved small-molecule drugs that could have benefits, by manually searching the literature. A few of the SSMD patients have begun courses of treatment using these drugs and some have even reported improvements albeit anecdotally. . We will next conduct a longitudinal natural history study, aim to identify reliable biomarkers for disease symptoms, invest in understanding the underlying disease biology, create disease models, and unify all the activities under a novel drug development pipeline ultimately aiming to identify and validate treatment protocols. The pipeline is open to repurposing existing drugs or drug combinations, novel small-molecules, and drugs based on emerging technologies like gene therapy, ASOs, and gene editing. We aim to test several drugs in multiple preclinical disease models at once to reduce selection bias. We will rapidly make all our results publicly available. This will allow us to leverage the broader scientific community, to identify lead drugs with maximum efficacy and facilitate novel discoveries with regards to disease mechanisms. By approaching the treatment using a network approach, we will break the silos and foster collaboration between our research, industry, and physician partners and encourage exchange of data and materials.

In this paper, we present our roadmap in greater detail. Typically, rare disease foundations have shared their success stories retroactively as roadmaps (Zuccato et al). However such roadmaps lack the high-resolution details and context to help an organization like ours tackling a new rare disease. We thus felt the overwhelming urgency to share our roadmap, however preliminary and optimistic, as a means to help other rare disease organizations in a similar position.

This article provides an overview of our current understanding of SSMD, which has not been summarized previously. The roadmap was created by collaboration between patient parents and advocates, scientists, and clinicians. It was created based on a newly cemented understanding of the genetic relationship between *GPX4* and SSMD, but with effectively no detailed knowledge of the underlying disease pathogenesis. The roadmap sets forth our suggested translational science principles and logistics that would be needed to enable breakthrough advancements necessary for treatment. The roadmap emerged from a virtual workshop held on March 19, 2020. Because CureGPX4 is a collaborative network and is open to feedback, we appreciate new ideas, help and guidance from the community. We are committed to periodically publishing updates to our roadmap. We hope that by openly sharing this roadmap and materials such as the IND Template, Roadmap Chart, Conference Guide, among others, we will facilitate other rare disease organizations increase their chances of success.

## SSMD Disease

Sedaghatian type Spondylometaphyseal Dysplasia (SSMD) is an extremely rare progressive disorder which is characterized by a multi-system presentation, including cupping/flaring of metaphyses, platyspondyly (flattening of the vertebrae), cardiac arrhythmia, and central nervous system (CNS) abnormalities, including hypogenesis of corpus callosum and cerebellar hypoplasia. The disease is congenital, and the majority of patients die in the first few days after birth due to respiratory distress. All reported cases of SSMD have been autosomal recessive in nature, caused by mutations in both alleles of the *glutathione peroxidase 4* (*GPX4*) gene (Smith et al.). GPX4 is a selenium-containing member of the family of antioxidant defense enzymes called glutathione peroxidases, which protects the cells against oxidative damage, and is involved in supporting mitochondrial function (Stockwell et al).

SSMD was first reported by Sedaghatian (after which the disorder is eponymously named) in 1978, reporting two brothers in Iran who each died within the first week of birth, and finding ‘severe congenital metaphyseal involvement, mild rhizomelic shortness of upper limbs, and mild platyspondyly (Sedaghatian et al). Since that time, a small number of further reports have been published describing patients with presumed SSMD (Table 1). In 2014, Smith et al. used whole exome sequencing of a child affected with SSMD (and unaffected parents) and found that mutations in *GPX4* are likely

responsible for SSMD. A small number of patients' *GPX4* sequences have been reported (shown in Table 2), including both point mutations and short deletion. The  predicted impact of these genetic lesions on GPX4 function are discussed below.

When we started writing this paper, we knew of four pediatric patients (3 male, 1 female, median age 31 months) currently living with this condition, but sadly one patient has passed away since.. Based on natural history data from these four patients, additional symptoms of SSMD can include severe hypotonia, global development delays, auditory neuropathy, cortical visual impairment, scoliosis, and hypertonia. The oldest patient developed intractable seizures at the age of 3 and continues to be treated with anticonvulsants to reduce the occurrence of breakthrough seizures. Currently, there are no treatments for SSMD, except for physical and occupational therapies. Without any treatment, babies born with this condition can never sit up or walk, have feeding difficulties, and display significantly delayed physical and cognitive development. They are at a high risk for premature death by cardiovascular, cerebrovascular, neuromuscular, or renal complications.

| | Sedaghatian | | | Opitz | Campell | | Peeden | Elçioglu | | Kerr | | | Koutouby | Foulds | English | Mahendran | Witters | Aygun | Smith | | This report | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Individual/Case | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
| Sex | M | M | F | M | M | M | M | M | M | M | M | M | M | F | F | M | F | M | F | M | M | M | M | F | M |
| Race | IR | IR | IR | IR | BC | BC | BA | PA | ME | PA | PA | PA | YE | BC | BC | BC | | TU | NE/IR | TU | | IN | | | IN |
| Consanguinity | - | - | - | + | - | - | - | - | - | + | + | + | - | - | - | + | - | + | - | + | | - | | | |
| Sibling occurance | + | + | + | - | + | + | - | - | - | + | + | + | - | - | - | - | - | - | - | | | - | + | + | |
| Birth Weight (kg) | | 3.3 | 3.8 | 3.28 | | | 3.12 | 2.9 | | 2.6 | 3 | 2.95 | 3.40 | 3.34 | 2.83 | 2.54 | 0.21 | 3.3 | | | | | | | |
| Gestation (weeks) | | | | | 30 | 41 | 40 | 36 | | 36 | 38 | 38 | 40 | 38 | 37 | 39 | 18 | 37 | | | | 38 | | | |
| Death (day) | 3 | 3 | 1 | 4 | SB | 1 | 2 | SB | 30 | SB | 1 | 1 | 161 | 30 | 17 | 3 | TP | 120 | 18 | 120 | 244 | NA 2 yr at pub | NA 11 yr at pub | NA 6 yr at pub | NA ?? yr at pub |
| Normal intrauterine growth | + | + | + | + | | - | - | - | - | - | + | - | + | + | + | + | - | | | | | | | | |
| Skeletal abnormalities | | | | | | | | | | | | | | | | | | | | | | | | | |
| Short neck | | | + | | + | + | + | | | - | - | - | + | + | - | | | | | | | | | | |
| Rhizomelic shorting of long bones | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | + | - | + | + | | + | | | |
| Metaphyseal cupping/ flaring/irregularity | + | + | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | + | | | |
| Epiphyseal ossification | + | + | | + | + | + | + | + | + | - | - | - | | + | | | | + | | | | | | | |
| Narrow/small chest | - | + | | - | - | - | - | + | + | - | - | - | + | - | - | | - | | | | | | | | |
| Cupping of rib ends | + | + | | + | + | + | + | + | + | - | - | - | + | + | + | + | + | | | | | | | | |
| Platyspondyly | + | + | | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | + | | | |
| Flared iliac wings | + | + | | + | + | + | + | + | + | + | + | + | | + | | + | | + | + | + | | | | | |
| Lacy/irregular iliac crest | + | + | | + | + | + | + | + | + | + | + | + | + | | | | | + | | + | | | | | |
| Facial abnormalities | | | | | | | | | | | | | | | | | | | | | | | | | |
| Telecanthus/ hypertelorism | | | | | | | | | | - | - | - | + | - | - | | | + | | | | | | | |
| Wide/flat nasal bridge | | + | + | + | | | | | | - | - | - | - | - | - | | + | | | | | | | | |
| Asymmetrical/abnormal ear placement | | + | + | + | | | + | | | - | + | - | - | + | - | | | | | | | | | | |
| Micrognathia | | | | + | + | + | | | | | | | + | + | + | | | + | | | | | | | |
| Nervous system abnormalities | | | | | | | | | | | | | | | | | | | | | | | | | |
| Simplified sulcal/gyral pattern | | | | | | - | + | | | | | | + | + | + | | + | + | + | + | | | | | |
| Seizures | | | | | | - | | | + | | | | + | | + | | | + | | + | | - | + | | |
| Other | | | | + | + | - | + | | + | | | | + | + | + | | + | + | + | + | | + | | | |
| Hypotonia | | + | | | | | | | | | | | + | | + | | | + | | | | + | | | |
| Delayed cognitive and physical development | | | | | | | | | | | | | | | | | | | | | + | + | + | + | + |

**Table 1**: Published reports of SSMD patients, with details captured that are disclosed in these studies. Note that first authors are described along top of table, cited as follows: XXX {ref}, YYY {ref2}

## Role of GPX4 in Health and Disease

GPX4 is a selenoprotein antioxidant enzyme belonging to the family of mammalian isoenzymes called glutathione peroxidases The human *GPX4* gene contains seven exons and six introns, and can be expressed as three isoforms of the protein - mitochondrial (mGPX4 - UNIPROT P36969-1), cytosolic (cGPX4 - UNIPROT P36969-2) and nuclear (nGPX4). All three isoforms seem to be ubiquitously expressed in all tissues. The cytosolic isoform is known to be essential in somatic cells including neurons of the developing brain (Schneider et al, Liang et al) while the nuclear isoform is predominantly synthesized during late spermatogenesis (Pfeifr et al). As a selenoprotein, GPX4 contains the rare amino acid selenocysteine (U, Sec) in its active site (position 73 of 197), often termed the '21st amino acid'. The catalytic activity of Sec is indispensable for normal enzyme activity of GPX4 using Glutathione (GSH) as a substrate *[Ref: PMID: 29522794; PMID: 29290465; PMID: 28709976]*. Mouse models have shown that the enzyme is important for normal embryogenesis, maintaining mitochondrial oxidative phosphorylation, preventing lipid peroxidation, and playing a part in combating increased oxidative damage due to injury or chemotherapy *[Ref: PMID: PMID: 30082768; 29522794; PMID: 29290465; PMID: 28709976]*..

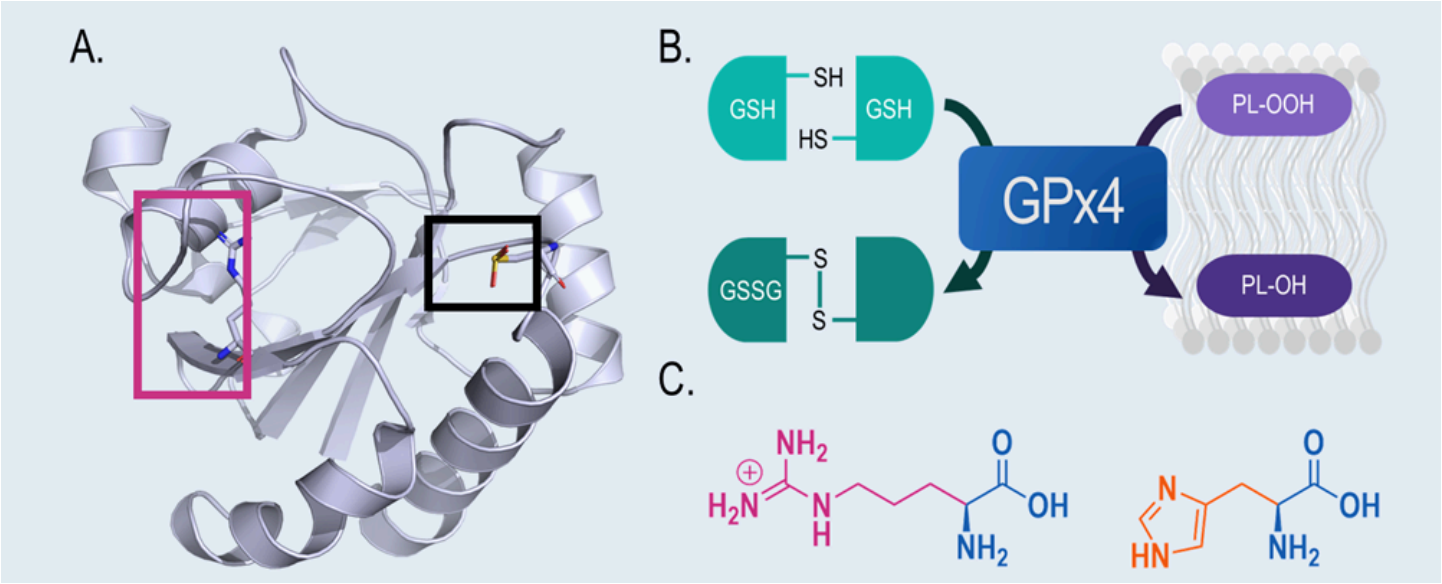


**Figure 1.** asdf

### Maintains mitochondrial oxidative phosphorylation

GPX4 has been shown to protect mitochondrial ATP generation by preventing oxidative damage to mitochondrial structures (Liang et al). Knockdown studies of *GPX4* results in a reduction in expression of genes encoding components of Complex I, IV, and V (Cole-Ezea et al), while overexpression of mGPX4 prevents release of the proapoptotic molecule cytochrome C from mitochondria, thus playing a key role as an anti-apoptotic agent in mitochondrial death pathways (Nomura et al). mGPX4 protects cardiac contractile function and preserves electron transport chain activities following ischemia/reperfusion (Dabkowski et al.)

## Prevents lipid peroxidation

The cytosolic isoform of GPX4 is capable of reducing complex lipid peroxides such as those present in lipid membrane bilayer of cells. Polyunsaturated-fatty-acid-containing phospholipids (PL-PUFAs) have been shown to be the lipids species most susceptible to peroxidation, with the bis-allylic carbons being most susceptible hydrogen atom abstraction (Feng et al). GPX4 localizes to lipid membranes where it accesses hydrophobic membrane lipids and reduces PL-PUFA hydroperoxides using reduced glutathione (GSH) as electron donor for the reaction *[PMID: 20649470]*.

## Loss of GPX4 can lead to ferroptosis

Ferroptosis is a distinct form of iron-dependent organized cell death *[REFs: PMID: 32413317; PMID: 32275866; PMID: 32165281]*. Loss of GPX4 results in higher levels of peroxidation of lipids in the cell membrane, triggering ferroptosis. Depletion of the cofactor of GPX4, glutathione, also leads to ferroptosis. Cell death with oxidized levels of phospholipids acylated with polyunsaturated fatty acids, involvement of redox-active iron, and a defective lipid peroxide repair, are the hallmark features of ferroptosis (Dixon et al). The antioxidant compound α-Tocopherol (Vitamin E) can stop lipid peroxidation and thereby half ferroptosis, as can iron chelators (Carlson, et al, 2016). Also the enzyme recently named ferroptosis-suppressing protein 1 (FSP1) can, in certain cells, act instead of and in parallel with GPX4 to reduce oxidized phospholipids and thereby also suppress ferroptosis works in parallel and can compensate for the loss of GPX4 *[PMID: 31634900; PMID: 31634899]*.

Ferroptosis has emerged as a mechanism of cell death relevant to multiple diseases including cardiovascular diseases (Kobayashi et al), acute kidney failure (Muller et al) and CNS disorders (Weiland et al, Yang et al.). Ferroptosis can, at least in certain cell types, be driven by loss of activity of lipid repair enzyme GPX4 and subsequent accumulation of lipid hydroperoxides. Depletion of Gpx4 in mice is known to induce ferroptotic cell death in embryo, testis, brain, liver, heart, and photoreceptor cells (Imai et al.), cause rapid motor neuron degeneration and paralysis (Chen L), promotes cognitive impairment (Hambright et al), triggers acute renal failure (Angeli et al), and results in impaired T-cell-mediated immune response (Matsushita et al). Mice with depleted GPX4 showed hallmarks of ferroptosis including an increase in lipid peroxidation in various cell types (Hambright et al).

## Mutation of GPX4 causes SSMD disease

Smith et al. have established the pathogenic role of three different variants in *GPX4* in causing the disease Sedaghatian type Spondylometaphyseal Skeletal Dysplasia (SSMD). The variants result in a loss-of-function of GPX4 through deletion or duplication resulting in a frameshift and premature truncation of the protein. Of the four patients reported in this paper, three patients have the same homozygous missense variant and one has a different (missense and duplication) genotype. Importantly, no cases of SSMD have been reported that are not caused by homozygous mutations in GPX4.

**Table 2**: Genetically characterized mutations in GPX4 in patients with SSMD.

| Individual | Sex | GPx4 Variant | Predicted molecular consequence | Amino acid substitution |
|---|---|---|---|---|
| 19 | F | GPx4(NM_001039848.1):c.587+5G>A heterozygous maternal; GPx4(NM_001039848.1):c.588-8_588-4del de novo | Nonsense | 5 bp deletion |
| 20 | M | Not available for affected child GPx4(NM_001039848.1):c.381C>A In both parents | Nonsense | p.Tyr127* |
| 21 | M | GPX4(NM_001039848.1):c.441dup heterozygous maternal; GPX4(NM_001039848.1):c.413C>G heterozygous paternal | Nonsense | (p.(Gly148ArgfsTer?)); (p.(Pro138Arg)) |
| 22 | M | | Missense | p.Arg216His |
| 23 | F | | Missense | p.Arg216His |
| 24 | M | | Missense | p.Arg216His |
| 25 | M | GPx4(NM_001039848.2):c.647G>A | Missense | p.Arg216His |

# Establishing the CureGPX4 Organization

The CureGPX4 organization was started by the parents of one of the children with SSMD disease. Our goal is to find a treatment to improve the quality of life of patients in the short term (6 to 12 months). In the long term (3 - 5yrs), we wanted to develop treatments to address the underlying disease pathology. As the founders lack formal biomedical research training, we have relied on inputs from other patient groups, academic researchers, and physicians to establish a structure and roadmap for the organization. Through these efforts, we have created a team of researchers, set clear directions, removed blockers to collaboration and created a roadmap towards reaching the goal.

## Science Team

We gathered the CureGPX4 Science Team, a cross-functional team of highly-collaborative experts sharing knowledge and working together to find treatments for this SSMD disease. The latest list of team members is available at curegpx4.org/team (and the current CureGPX4 Science Team is listed in the Supplementary Information). We included experts from the following functional areas necessary to build therapeutic hypotheses and successfully advise on a therapy development pipeline (described later in the paper):

- Basic science understanding of GPX4 structure and function
- Development & characterization of animal models
- Development & characterization of patient-derived induced pluripotent stem cell (iPSCs) models and organoids
- Drug screening & repurposing
- Oxidative stress biomarkers
- Physicians: Neurology, Genetics, Orthopedics

## Guiding Principles

Similar to other patient organizations, we are focused on finding treatments, but our choice of guiding principles dictate the activities we prioritize. The peculiarity in CureGPX4's roadmap is a direct result of our choices of guiding principles.

- **Incremental over Big-Bang**: Instead of investing several years creating one highly effective drug, we want to quickly discover a repurposing opportunity with reasonable efficacy, treat our patients, and buy us more time as we iterate to improve the drug's efficacy.
- **Treatment over Intellectual Property:** We are not looking for patentable novel technologies or molecules. To find treatments, we are focusing on repurposing approved drugs, using naturally occurring substances, testing non-pharmaceutical interventions (ie. chronic hypoxia), utilizing existing patented technology, and adapting generics.
- **N-of-1**: Well-designed clinical trials are not practical with just four patients. We are willing to consider treating patients with experimental drugs under a compassionate use program (FDA Program reference) as long as the drug is legal and safe.
- **Reduce Time over Money**: Given two choices of activities, we prefer to execute the faster, more expensive activity over the cheaper one. It does not necessarily mean our organization spends more money. On the contrary, activities that are quick to execute tend to be small and cheap. By choosing to reduce time over money, we not only hope to find treatment faster but also potentially cheaper.
- **Early Failures over Early Success**: We don't know enough about the disease to design the perfect set of experiments that will lead us to treatments. Instead of trying to prevent failures, we assume failures are inevitable in all our activities. We choose to fail fast,, maximize learnings from failures, and fail often enough until we learn to do it right.

### Collaboration Network

Our Science Team members are geographically distributed around the world, work at different institutions, motivated by different goals, speak different languages, and several were strangers to each other until CureGPX4 brought them together. To find a treatment, however, a team must collaborate with trust, integrity, shared goals, and a sense of urgency.

We therefore created *CureGPX4 Collaboration Network*, a safe and trusted space for the science team to collaborate. We are in the process of signing Confidentiality Disclosure Agreements (CDAs, Supplementary material contains the CDA template used for the research network) with all institutions in the network in order to facilitate free exchange of ideas, information, results and protocols without the reservations linked to potential intellectual property. Institutions participating in the network use the standard Uniform Biological Materials Transfer Agreement (UBMTA) template to freely exchange reagents, cells, biological samples, and other materials with each other for the purpose of finding a treatment.

## Roadmap for Therapy Development

To create a roadmap for therapy development, a *CureGPX4 Research Conference* was held on March 19, 2020, a one day conference aimed at bringing a diverse group of researchers from the science team, clinicians and industry partners who are working on finding a treatment for SSMD disease (Supplementary material X (link) contains all the materials used to run the conference including format, agenda, invitations and others.). The primary goal of the conference was to create a Roadmap for Therapy Development by the end of the day. The Roadmap for a rare disease should identify experiments necessary to understand the disease, identify drug repurposing opportunities, and explore the use of emerging technologies like gene therapy, ASOs, CRISPR/Cas9 and others to treat this disease. The conference was structured with the goal of making decisions to build the roadmap, in addition to sharing information. With 30 participants over 8 hours of meeting, we made 20+ decisions to build the roadmap. Fig 2 presents the roadmap chart created at the meeting. The following section explains the roadmap in more detail.

Five critical areas were identified for SSMD: i) the disease, ii) the target, iii) drug candidates (repurposing and new), iv) testing and lead identification, and v) clinical trials and compassionate use. For each of these areas, three points were addressed that provide clarity and clearly define next steps for CureGPX4:

- What do we know?
- What do we have?
- What do we need?

As information was gathered, priority goals were decided based on the (seemingly) simple question - "*What is the most important activity we can do right now to improve a patient's quality of life?*"

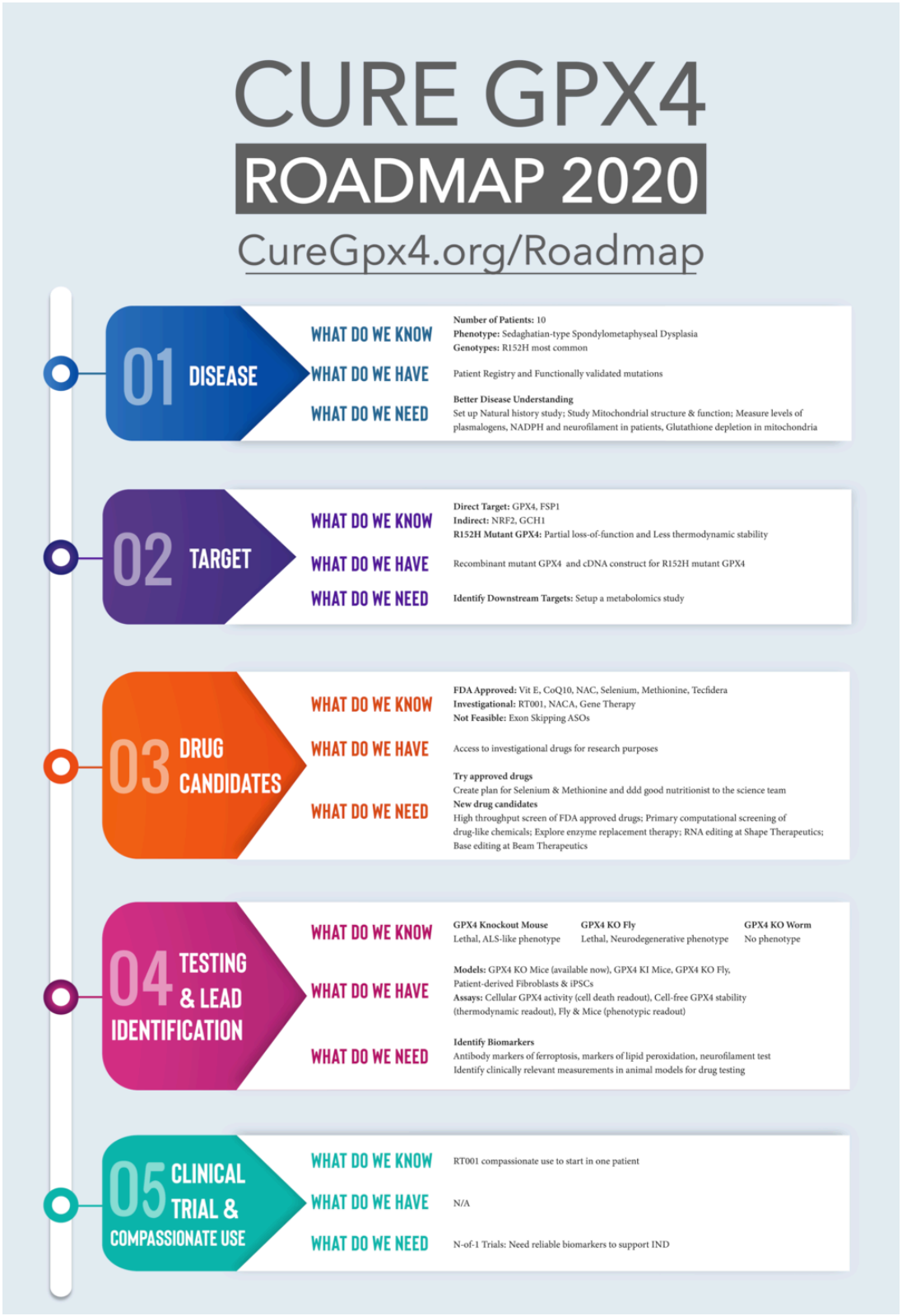


**Figure 2.** asdf

The following sections briefly describe the research activities that emerged from our roadmap, the rationale for the activities, current status and results.

## 1. Identify Patient Needs

**Status**: Completed

The first step is to understand the patient population and patient needs, to provide a clear vision to the team. More importantly, it allows us to measure progress towards the end goal. With only four known patients at the time, CureGPX4 could have face-to-face conversations with each family to understand their experience and needs. For a larger patient community, one might need to use scalable tools such as surveys (Slade et al). We sought to identify what patient families expect from the therapies, acceptable tradeoffs between treatment benefit and risk outcomes, and more broadly their dream for relief from the suffering of SSMD. Patient families expressed the the following:

- **Incremental therapies**: First slow down, then stop, and then reverse the disease
- **Goal is Independence**: We are okay with disabilities but hope our children can live independently, and take care of themselves
- **Sustainable therapies**: We want the therapy to be covered by health insurance and available to patients for as many years as they need

## 2. Low-Throughput Drug Repurposing

**Status**: Completed

There are currently no approved or experimental therapies for SSMD. Even well-funded biotech industry research would take years to develop a novel and efficacious drug for this condition. Guided by the immediate needs of our patients for incremental therapies, we seek to identify existing FDA-approved drugs that could be repurposed for this condition.

We evaluated setting up a high-throughput drug screen to test 4,000 FDA approved molecules on cell or animal models. Using a commercial vendor it would cost over $150,000 (USD), take 9 to 15 months, and would require disease models that could be screened. The CureGPX4 network will be creating disease models using fruit fly (*D. Melanogaster*), worms (*C. Elegans*) and zebrafish (*D. Rerio*), validating their phenotypes, and testing drugs similar to the model described in Iyer S et al. However, at this point, our understanding of the natural history of the disease limits our ability to design and interpret such screens, making the investment risky.

To move forward with drug discovery while our basic scientific understanding of this disease advances , we searched the literature to identify FDA approved drugs or supplements predicted to be capable of compensating for the loss of GPX4 function. Drugs capable of compensating for GPX4 loss would fall under one of the following, non-mutually exclusive, categories:

1. Increase GPX4 protein levels and/or increase residual GPX4 activity
2. Increase the activity GPX4 antioxidant pathways by modifying the quantity of other participating proteins
3. Increase the activity of alternate compensatory pathways
4. Reduce or scavenge the phospholipid oxidation damage due to reduced GPX4 activity (e.g. use of antioxidants)
5. Drugs that have been found to be effective in similar conditions

**Results**

Using this approach, we identified 36 FDA approved drugs and supplements with reasonable hypotheses. From the 36, we shortlisted the following based on data on efficacy and safety. (Full of list of drugs, mechanisms of action and rationale available in supplementary material)

| Name | Rationale | Availability | SSMD Status |
|---|---|---|---|
| Vitamin E | Potent antioxidant known to prevent ferroptosis | Over-the-counter | Administered to 2 of 4 patients<br><br>Dosage: 15mg twice a day<br><br>Product: Compounded at pharmacy |
| N-acetyl-cysteine | Increases Glutathione biosynthesis to boost residual GPX4 activity | Over-the-counter | Administered to 2 of 4 patients<br><br>Dosage: 300mg three times a day<br><br>Product: NDC:63323-695-04 |
| CoQ10 | Essential for repair of peroxidized lipids. Acts as an antioxidant. | Over-the-counter | Administered to 2 of 4 patients<br><br>Dosage: 50mg twice a day<br><br>Product: Compounded at pharmacy |
| Selenium | Production of GPX4 is limited by availability of selenium. Additional supplementation might enhance GPX4 quantities | Over-the-counter | Administered to 1 of 4 patients<br><br>Dosage: 75 mcg once a day<br><br>Product: AllergyResearchGroup |
| L-methionine | Increase glutathione synthesis through the transsulfuration pathway | Over-the-counter | Pending administration |
| RT001 | Protects lipid membranes against peroxidation. | In clinical trials for treating multiple indications | Administered to 1 of 4 patients under Expanded Access |
| Dimethyl fumarate | Activates NRF2 gene, master regulator of oxidative stress response | Approved in USA for treating multiple sclerosis | Evaluating safety of off-label use through in-vivo and in-vitro studies |

| | | | |
|---|---|---|---|
| Idebenone | Similar to CoQ10 in activity with better bioavailability | Approved in EU for treating Leber's hereditary optic neuropathy. In clinical trials in USA. | In conversation with company for Expanded Access |

## 3. Patient Registry and Longitudinal Natural History Study

**Status**: In-Progress

Natural history is a scientific and systematic study of the patients to understand clinical, biological, and social aspects of the disease. Qualitative and quantitative data from natural history studies is critical to understand the course of a disease and its impact on patients, and informs the design of clinical trials for therapy development. Natural history studies help physicians recommend disease management strategies, identify unrecognized impacts of disease, give a voice to patients, and ensure regulators can perform an unbiased assessment of trial outcomes. Natural history data can also inform new hypotheses for translational science. For example, many patients with SSMD have optic nerve abnormalities, which suggests an important role of GPX4 in development of optic nerves and vision.

Prior to collecting natural history data, a patient registry must be established. A registry is simply an up-to-date address book of every patient in a disease population, managed (in this case) by CureGPX4. To collect natural history data, we will design, create and send surveys to every patient in the registry periodically. A qualified individual must create a study protocol and get it approved by an Institutional Review Board (IRB) before starting the study. IRB approval is necessary to collect, store and act on data from human subjects. Surveys are created and sent using standard off-the-shelf software (Sanford CoRDS, NORD Registry) or created with a HIPAA compliant software such as RedCap. SSMD disease displays rapid progression in the first years of life, so CureGPX4 has decided to send out monthly surveys. We will use a custom RedCap installation to store the data in a compliant manner and retain complete ownership of the data. We will follow FDA's guidance on natural history study design (link) to collect data in a compliant, useful and stay relevant to drug development in the future.

The visibility of CureGPX4 as a Foundation is essential to ensure that clinicians and patient advocates can connect to. CureGPX4 has established a stand-alone web-page (curegpx4.org) and also worked with the National Center for Translational Science to create a SSMD page at the Genetic and Rare Disease (GARD) Information Center (https://rarediseases.info.nih.gov/diseases/4993/spondylometaphyseal-dysplasia-sedaghatian-type).

## 5. Understanding the disease biology

Answers to *"How do variants in GPX4 gene cause SSMD disease?*" will help us identify one or more components of the biological pathway involved that may be targeted therapeutically. Answering this question relies on an understanding of the function of GPX4, and the mechanistic cellular consequences of a total or partial loss of GPX4 function. Based on our current understanding of *GPX4*, oxidative stress response pathways, and the phenotype of SSMD disease, we have arrived at an initial set of primary research questions.

### How prevalent are GPX4 mutations in the community and are the variants pathogenic?

To define the relative pathogenicity of *GPX4* variants, we will analyze patient-derived fibroblasts for hallmarks of oxidative stress and ferroptosis. We will try to restore the  wild-type cellular phenotype by transfecting with cells with

wildtype *GPX4* gene, expressing the wildtype protein and silencing the mutant protein. We will also assess publicly available human genome sequences to study the range and extent of disease-causing and as-yet unknown GPX4 mutations in the human genome.

**Opportunities**:

- Validated variants documented in the disease allows clinical genetic testing companies to call this gene variant as pathogenic in their reports. It enables physicians to confidently diagnose patients with this disease
- De-risks other basic science and translational activities that assume pathogenicity of this gene variant
- Peer-reviewed publications on the validated variant and disease will raise awareness of SSMD

## How do individual variants impact the protein's structure and function?

Coding variants changing GPX4 protein sequence may lead to total, partial or no loss of catalytic function. In some cases, the mutant protein may be catalytically active, but less stable within the cell. To understand the impact of individual variants on protein's structure, localization, and expression levels we will study recombinant protein with specific variants, using recently developed techniques for production of selenoproteins *[Ref: PMID: 28193838]*. We will analyze thermodynamic stability and antioxidant activity using cell-free assays on recombinant protein. We will use computational modelling to predict the protein structure and validate it with X-ray crystallography.

To measure cellular activity of protein, we will use reference GPX4 null cell lines expressing disease-causing GPX4 mutations to assess markers of oxidative stress and sensitivity to ferroptosis, and determine whether it is possible to rescue any of these phenotypes with expression by expressing the wildtype protein. We will repeat the assays on patient-derived fibroblasts to get high confidence that the variant is indeed causing the functional changes and nothing else.

**Opportunities**

- Unlocks new therapeutic opportunities depending on the nature of the change in protein's function. For example, antisense oligonucleotide therapeutics would be good candidates for variants causing gain of function.
- Cell-free assays using recombinant protein will allow us to screen drugs for protein binding and modulation of GPX4 activity.
- In-vitro assays on fibroblast cells allow us to screen thousands of FDA approved drugs in a high-throughput fashion to identify drugs that could potentially restore cellular function.
- Understanding the impact of patient mutations of GPX4 will lead to a greater understanding of the basic biology of GPX4 function

## What cellular functions are impacted?

When observing cellular changes, we want to understand if and how there is a difference between "acute" versus "chronic" oxidative stress condition. A patient with mutated GPX4 since embryonic development could be considered to be under "chronic" oxidative stress whereas the oxidative stress in an in-vitro or in-vivo assay knocking down GPX4 could be considered "acute".

Cells adapt to the change in gene function by upregulating other pathways. GPX4 uses glutathione (GSH) to scavenge lipid hydroperoxides in the cell membrane. Loss of GPX4 activity might activate other compensatory genes or pathways in response to increased ROS, such as FSP1. We will use RNASeq to look at gene expression changes and metabolomics and lipidomics analyses to examine changes in pathways, networks, cellular lipids, and other metabolites. One isoform of GPX4 is trafficked to the mitochondria, and GPX4 has been shown to be critical for mitochondrial function (Tsz-Leung To et al), so mitochondrial activity in patient-derived fibroblasts will also be examined.

**Opportunities**

- With greater understanding of the cellular consequences of GPX4 loss, we improve our odds of finding new therapeutics to treat this disease
- Understanding the impact of GPX4 loss on mitochondrial structure and function will open the door for mitochondria-specific therapeutics already in drug development pipelines
- Any quantifiable impact of mitochondrial dysfunction in blood or urine samples could open the possibility to identify clinically significant biomarkers of disease

### How is the brain structure and function impacted?

SSMD disease causes developmental delays, and changes in brain structure as revealed through patient MRIs. We will advocate for the study of neurological changes using conditional complete GPX4 knockout mice, GPX4 mutant transgenic mice, and by differentiating patient-derived iPSC lines into brain organoids.

**Opportunities**

- iPSC differentiated into brain cells are potentially valuable models for drug screening (Adams et al.)
- Similarities to other neurological conditions will allow us to repurpose drugs available for that condition
- Insight into the impact of ROS regulation of normal neuronal cell function can be gained
- Insight into the impact of GPX4 mutations during human neurodevelopment (Trujillo et al.)

### How is metaphyseal bone development impacted?

Patients with SSMD disease are born with skeletal changes that progresses with age. To our knowledge, there has been no prior work to characterize skeletal morphology in model organisms. We will study the skeletal changes using conditional complete GPX4 knockout mice and GPX4 mutant transgenic mice. We will dive deep into the development of bones and chondrocytes by differentiating patient-derived iPSCs.

**Opportunities**

- Insights on the impact of oxidative stress on bone development could lead to fundamental understanding of biological processes opening up opportunities to develop therapies for other rare or common conditions
- Understanding the skeletal progression could open the possibility of using patient's bone X-rays as one of the endpoints for clinical trials in the future

## 6. Disease Models

The purpose of a disease model is to predict a drug's impact on the quality of a patient's life without giving it to humans. Models should be developed to accurately recapitulate the human disease within the biological system or mimic the process they represent ex: biochemical, cellular, whole organism etc. We also want models to be sensitive enough to show a measurable difference when intervened with a drug. In the context of SSMD, ensuring that scientists have identified and can agree on the appropriate ortholog to human GPX4 for manipulation is critical, and as a selenoprotein GPX4 presents further challenges across other species. For example, drosophila and worm (C. elegans) do not express a selenocysteine-containing ortholog of GPX4, and zebrafish appear to have two selenocysteine-containing orthologs of GPX4. On the other hand, mice (and other mammals) have a single selenocysteine-containing ortholog of GPX4.

The following models are considered to be worth pursuing with the goal of covering as many biological systems as possible to study oxidation effects:

- Human GPX4 recombinant protein, both wildtype and mutant (available now at Karolinska Institute)

- CRISPR edited GPX4 variant in reference cell lines (available now at Columbia University)
- SSMD patient-derived fibroblasts (available now at RUCDR Biorepository)
- SSMD patient-derived iPSCs lines (work-in-progress, ETA 1-Oct-2020)
- Brain organoids derived from patient-derived iPSCs (not started)
- GPX4 conditional/complete knockout mice (available now at JAX)
- GPX4 conditional knock-in patient point-mutant mice (work-in-progress, ETA 1-Feb-2021)

For genetic conditions, animal models are built by recreating the genetic variation in the animal's genome or silencing the gene entirely. These are good approximations of the human condition but seldom sufficient to predict the clinical outcome of a drug. Some might argue that patient derived cells, fibroblasts or iPSCs are good predictors of clinical outcome, however may not mimic the effect one would expect in a whole organism. The clinical relevance of a model might be obvious in hindsight, but there is no way to determine the ideal model a priori. Based on this observation, we will use multiple models to evaluate a drug to get higher confidence.

## 7. Emerging Technologies

Emerging technologies such as Antisense Oligonucleotides (ASOs), Gene Replacement Therapy, and CRISPR-Cas9 gene editing and others can precisely correct the genetic defect. ASOs are designed to skip the exon where mutations occur, in the hopes of restoring the protein's function, albeit partially. We analyzed GPX4 protein structure in-silico with exons skipped and noticed a destabilization of the structure (data not shown). This ruled out exon-skipping ASOs as a possible therapeutic candidate for SSMD disease.

Gene replacement therapies are attractive, especially to deliver a functional copy of GPX4 to neurons that are most susceptible to loss of GPX4. At 2.8 kilobases long, GPX4 fits within AAV9, one of the most common AAV serotypes used in gene-therapy strategies for neurological diseases. However, the *GPX4* gene is complicated by a 3' non-coded region that is required to ensure that the selenocysteine is added into the GPX4 protein, and this requires design considerations of a gene therapy vector. With an investment of $5-7 million and 2-3 years timeframe, we will be creating a gene therapy treatment for this disease.

# Conclusion

Children born with SSMD suffer throughout their short lives from multiple comorbidities. They do not have the luxury to wait for the development of the most efficacious treatments. CureGPX4's unique roadmap and guiding principles reflect this sense of urgency and patient need. However, we had to overcome several biases and challenges to create this roadmap. Firstly, the common wisdom among patient organizations is to start investing basic science activities and, when mature enough, progress towards translational activities. Secondly, the promise of gene replacement therapies to deliver a "cure" lured us away (temporarily) from investing in drug repurposing or basic science. Thirdly, the difficulty of obtaining grants to work on ultra-rare conditions meant that crowdfunding was the only option to bootstrap the initiative, leaving us with little money to invest in parallelizing efforts.

Ultimately, CureGPX4 aims to raise awareness and find treatments for kids affected by SSMD. Through our scientific and clinical network, we aim to ensure that a translational science approach to GPX4 function and biology can lead to new therapeutics for SSMD. However, we consider it our responsibility to help other patient organizations in similar situations to learn from our mistakes and help them with tools we have built to advance our journey. We encourage others to copy our roadmap, improvise based on their needs, and share publicly to help others in similar situations.

## Supplementary Materials

1. IND template for compassionate use - Single-Patient-IND-Template-Packet.docx
2. Conference format & Guide: Supplement: Conference Format & Guide
3. Roadmap Chart template: Supplement: Roadmap Chart Template
4. Low Throughput drug repurposing chart: Supplement: Low-Throughput Drug Repurposing for GPX4
5. Weekly status report:

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