The Effects of Prescribed Nitisinone (Orfadin™) on Cognition of both FAH +/+ and FAH -/- Mice using a Y-Maze Behavior Model

by

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Abstract

Background

Hereditary Tyrosinemia Type 1 (TT1) is an autosomal recessive disorder that is caused by the deficiency of the FAH enzyme, resulting in the elevation of tyrosine and its byproducts in the blood. This buildup of toxic metabolites in a child's bloodstream leads to catastrophic effects, which will eventually lead to death if not treated. However, the only treatments available for children with TT1 include pharmacological measures with Nitisinone (Orfadin) and a liver transplant. Unfortunately, both treatments carry major health risks along with their benefits. While liver transplants require life-long management and the risk of rejection, Nitisinone has also been linked to dangerous side effects. Studies have shown that Nitisinone leads to severe cognitive impairment while treating children with TT1, although the cause for the neurocognitive impairments is still unknown. With evidence that the only pharmacologic treatment for TT1 causes impaired cognition in children, increased awareness and the development of improved treatment is. This study will utilize the behavioral model, the Y-Maze, to test the effects of Nitisinone on neurocognitive function, particularly memory and learning. The results did not show differentiation among the carriers (FAH+/-) and the diseased (FAH-/-) mice.

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Acknowledgement

I would like to thank Dr. Beth Barnby for allowing us to join her research in order to help improve the treatment for children diagnosed with Tyrosinemia Type I. She has allowed us to be a part of this exciting and important research about this disease, current and possible future treatments. Her knowledge and passion about the subject is an inspiration. We would also like to thank Dr. Gordon MacGregor for allowing us to use the biology lab and Megan Hillgartner for carrying us through our entire research experiment. Thank you for giving us the opportunity to test the mice and work with the behavioral models. Finally, I would like to thank Dr. Ellise Adams for her guidance and encouragement throughout the entire research process.

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Introduction

Tyrosinemia type I (TT1) is an autosomal recessive disorder caused by the deficiency in fumarylacetoacetate hydrolase (FAH) in the catabolic metabolism of tyrosine. This disorder leads to elevated tyrosine and alpha-fetoprotein in the blood. If untreated, the buildup of tyrosine and its byproducts, fumarylacetoacetate and succinylacetone, lead to serious medical problems, including hepatic and renal disease. Tyrosinemia type I is treated with a low-tyrosine diet and prescribed Nitisinone. Nitisinone inhibits 4-hydroxyphenyl-pyruvate dioxygenase, an enzyme in the tyrosine catabolic pathway, as shown in Figure 1. By inhibiting the normal catabolism of tyrosine in patients with TT1, Nitisinone prevents the accumulation of catabolic intermediates, which would otherwise be converted to the toxic metabolites that are responsible for the observed liver and kidney toxicity seen in these patients. However, studies have shown that Nitisinone may lead to severe neurological deficits. Unfortunately, there are no other pharmacological treatments for children with TT1. The only other treatment option is a liver transplant, which includes life-long management and a high risk for rejection.

This study will test the neurocognitive deficits associated with Nitisinone in order to determine if these effects are caused by the prescribed medication or by the pathophysiology of Tyrosinemia Type 1 itself. This study may also contribute to knowledge of the severity of the neurocognitive effects of pharmacological treatment are for children with TT1 and if these effects outweigh the therapeutic measures of the drug. With evidence that Nitisinone causes impaired cognition, increased public awareness and the development of improved treatment is necessary for children with Tyrosinemia Type 1.

Review of Literature

The current pharmacological therapy for Tyrosinemia Type 1 (TT1) uses the drug 2-[2-nitro-4-trifluoromethylbenzoyl]-1, 3-cyclohexanedione (NTBC), commonly referred to as Nitisinone. Nitisinone prevents the toxic metabolic accumulations found in hereditary TT1 by blocking 4-hydroxypheylpyruvate dioxygenase, an upstream enzyme, in the tyrosine degradation pathway, as shown in Figure 1. Nitisinone effectively prevents the neurologic crisis, liver failure, and hepatocellular carcinoma usually found with untreated hereditary TT1. However, as Nitisinone biochemically switches the enzymatic defect found in TT1 to the defect found in Tyrosinemia type III, tyrosine concentrations are ultimately increased, leading to impaired cognitive function in patients taking the pharmacological therapy (Thimm et al., 2011). Higher tyrosine levels found in Tyrosinemia type II and III are considered to be the main contributing factor in the impaired cognitive functioning of these patients.

The neurocognitive impairment associated with Nitisinone treatment is still not proven to be an effect of the pharmacological treatment rather than the disease pathophysiology itself. Mild impaired cognitive function may have been an unobserved clinical manifestation of Tyrosinemia Type I, a symptom that failed to be recognized before the use of Nitisinone because of the short life span of untreated patients with Tyrosinemia Type I (Bendadi et al.,2013). Unfortunately, there are few human or animal studies to support or refute this hypothesis. Bendadi et al. (2013) and Laet et al. (2011) found a significant decrease in the Intelligence Quotient of a subset of patients in whom IQ was regularly tested, as shown in Figure 2. However, similarly low IQs in patients who had stopped taking Nitisinone after undergoing liver transplantation argues against the acute toxicity of Nitisinone. Most likely, Nitisinone affects cognitive function indirectly, by inducing profoundly elevated plasma tyrosine levels in patients. Inconclusive data could be

attributed to the limited sample size or other issues with regard to tyrosine measurement in the studies. The nature of the observed cognitive impairment remains unclear and the pathophysiology causing the impairment is still not fully understood.

Furthermore, neurocognitive defects could be increased by the diet necessary for children with TT1. Tyrosine levels can be reduced through a more restricted protein intake along with Nitisinone therapy. Since phenylalanine and tyrosine compete for transport to the brain, the combination for high tyrosine and low phenylalanine may lead to insufficient phenylalanine transport to the brain, decreasing the amount of phenylalanine available for protein and neurotransmitter synthesis. According to Bendadi et al. (2013) and Laet et al. (2011), this shortage may cause a deviant cognitive development that can be expressed later in life with mildly impaired cognitive function. Low phenylalanine is known to be associated with a decrease in IQ in patients with Phenylketonuria (PKU). Laet et al. (2011) observed patients with the lowest IQ had mean phenylalanine concentrations below 40 umol/L during the first two years of Nitisinone treatment.

Low phenylalanine levels in the brain also affect other amino acids in the brain. Neutral amino acids cross the blood brain barrier with a common carrier thus any change in amino acid concentrations will affect the uptake of other amino acid groups in the brain. Therefore, as tyrosine levels rise, phenylalanine levels are lowered. Low phenylalanine levels affect serotonin metabolism by causing an inhibition of tryptophan hydroxylase, the rate-limiting enzyme of serotonin synthesis. Behavioral alterations have been observed in humans and mice with disrupted tryptophan hydroxylase genes. Thimm, Richter-Werkle, & Kamp, (2012) found, for the first time, a significant tyrosine elevation in the cerebrospinal fluid of patients with TT1 under Nitisinone treatment. They also found that serotonin synthesis and release of these patients

was impaired. Although this research is fairly recent and not yet corroborated with other research it could help explain the cognitive impairment in TT1 patients.

With an increasing frequency of schooling difficulties, social issues and cognitive deficits in TT1 patients, it is necessary to further examine the exact pathogenic mechanism of the disease and the effects of Nitisinone therapy. The disruption of neurotransmitter synthesis, specifically serotonin, caused by the therapeutic treatment of TT1 can manifest as cognitive dysfunction and behavioral changes that can be studied using mazes. The Y-maze uses spontaneous alteration to test learning and memory function. Behavior models have also been utilized as mutant mouse models of human genetic diseases. Transgenic technology and knockout technology, the ability to remove a specific gene, make it possible to induce genetic diseases and test hypotheses about the importance of the gene for the neurotransmitter (Crawley, 1997). Through the use of the Y maze, this study seeks to answer the research question, Are there differences in the memory of FAH -/- (diseased) mice prescribed Nitisinone and FAH+/- (disease carrying) mice?

Methods

The sample consisted of: 13 total mice tested – seven males, six females, nine WT (wild type) (FAH +/-) – six males, three females, four Diseased (FAH-/-) – one male, three females. The sample of mice were bred from the FAH knockout mice ordered from The Jackson Laboratory. The original mice received were cryogenically frozen and then thawed and bred. This mutation was made at Oak Ridge National Laboratories by ENU mutagenesis of BALB/cRl males which were then bred to (C57BL/10Rl x C3Hf/Rl)F1 females. The offspring of that cross were bred to a stock bearing Del(Tyr)26DVT, which was generated in (101/Rl x C3H/Rl)F1 then bred through T Stock. Upon arrival to the biology lab the mice were again genotyped at three-

four weeks of age for confirmation of their status as either a diseased or a carrier of TT1, as shown in Figure, 3. All testing was conducted in Dr. MacGregor's UAH research laboratory.

Spontaneous alternation behavior in a Y-maze was used to test for neurocognitive changes in memory and learning. Testing was conducted according to previously established procedures. The mice were kept in the testing room overnight to become familiar to smells and sounds prior to the maze testing. The Y-maze consisted of three identical gray wooden arms (46cm long, 16cm wide) that were designed at a 120° angle from a center triangle platform, Figure 4. The Y-maze was placed on a table above floor level and white dividers were placed around the maze area to prevent distraction. A digital camera captured images and transmitted them to a computer using EthoVision XT; a video tracking software that tracks and analyzes the behavior, movement, and activity of each animal tested.

Spontaneous alternation behavior testing in the Y-maze began with a mouse being placed in the A arm of the maze. Randomization and order of the testing process is shown in Table 1. The recording of the five minute testing period began as the mouse passed the center of the maze. The subjects were allowed to freely move through the maze for the entire five minute period and their arm entries were manually recorded, Table 2. After each animal, the Y-maze was thoroughly cleaned with warm water and dried to insure the smell of each animal did not remain to distract the next animal tested. The spontaneous alternation behavior Y-maze is based off the tendency for a mouse to explore its environment. The premise being that a normal functioning rodent, intact memory, will explore each arm of the maze continuously without back tracking. An alternation is said to have happened when all three of the arms are visited without the back tracking to a previous arm. These alternations are indicative of memory deficits. For example, if the mouse travels to arm A, then B and then C followed by movement to arm B, then C, and then

A would equal one alternation. Each trial is calculated for the percentage of alternation behavior. The percentage of alternation behavior is figured via the formula:

$$\textit{Percentage of alternation behavior} = \frac{\textit{actual alternations}}{\textit{maximal alternations}} \times 100$$

Results

The highest percentage (86.95%) of spontaneous alternation in movement was achieved by #32 (wild type)(FAH+/-) mouse, as shown in Figure 6. The lowest spontaneous alternation (30.77%) was the score for #42 (wild type)(FAH+/-) mouse. Mouse #34 (FAH+/-) jumped out of the maze at the end; mouse #33(FAH+/-) jumped out of the maze at the end and also spent a majority of its time on the wall of the maze; and #43(FAH+/-) sat stationary in the center of the maze often. All three of the previous mentioned mice were wild type, carriers (FAH+/-). Also, mouse #46 (diseased) (FAH-/-) sat stationary in the center of the maze often during the trial.

The mean percentage for the spontaneous alternations achieved for the wild type mice, carriers (FAH+/-) was 59.32% and the mean percentage for the diseased mice (FAH -/-) was 60.18% as shown in Figure 7. Furthermore, the mean for the total number of arm entries for the diseased mice (FAH -/-) was 23 and 23.66 for the carrier mice (FAH +/-), as shown in Figure 5.

Limitations

How this stock of mice was subsequently maintained prior to the Jackson Lab is uncertain thus the mutant line for mapping is ambiguous. The stock of mice included contributions from several inbred mice and therefore the exact origins are unknown. Due to the limited number of diseased mice available at the time of the study the results should not be generalized. Also, the mice were only 8-10 weeks of age at the time of testing therefore the

amount of time the mice have been treated with Nitisinone may not have been long enough to see significant differentiation between the carrier and diseased mice behavior.

Discussion

The diseased mice (FAH -/-) did not show a lower percentage of alternation (i.e. an altered memory) when tested in the Y-maze but actually scored slightly higher than the carriers. However, due to the small sample size of only four diseased mice (FAH -/-) the results should not be interpreted as the diseased mice (FAH -/-) on Nitisinone would perform as well if not better than the carrier (FAH+/-). Also, because three of the carrier mice (FAH +/-) spent most of their testing time attempting to escape the maze, most of their activity was not recorded in the alternation calculation thus lowering the mean score of the carrier mice (FAH+/-). It could be argued that a more cognitively functioning mouse would most likely attempt to escape the maze.

Furthermore, the amount of time the mice had been treated with Nitisinone should be taken into consideration. Eight to ten weeks may not be long enough to see cognitive changes. However, the findings could be used for comparison in future testing using older mice with a longer duration on the treatment. As the treatment is prolonged and tyrosine levels are elevated and neurotransmitters are affected the performance in the behavior model could yield a different result which would provide a significant finding with regards to the effects of both dosage as well as the duration of time on Nitisinone.

Implications for Future Research

Further research needs to be conducted using a larger sample of at least $n=30$ to obtain more accurate results when comparing the carrier to the diseased mouse. More research with a control group of mice not on the drug as well as wild type mice on the drug would provide a

more significant finding when comparing Nitisinone as a drug causing neurocognitive decline. Also, future studies that vary the dosage of Nitisinone within the diseased population of mice could provide another useful comparison of the negative effects of the drug.

Implications for Nursing Practice

Currently Nitisinone is the only pharmacological treatment for Tyrosinemia Type 1 and as such its effects on the neurocognitive functioning of patients is of great importance. The pathophysiological effects of altered amino acids and their direct relationship on neurotransmitters, particularly serotonin and dopamine, are crucial to the understanding of patients with Tyrosinemia Type 1 who are being treated with Nitisinone. Neurocognitive and behavioral changes in patients can influence all aspects of life. Therefore, it is imperative that health care providers be aware of these possible changes so that the most effective treatment is pursued and proper education of the patient and family is achieved.

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Table #1: Y-Maze Spontaneous Alternation Test Randomization
December 20, 2013

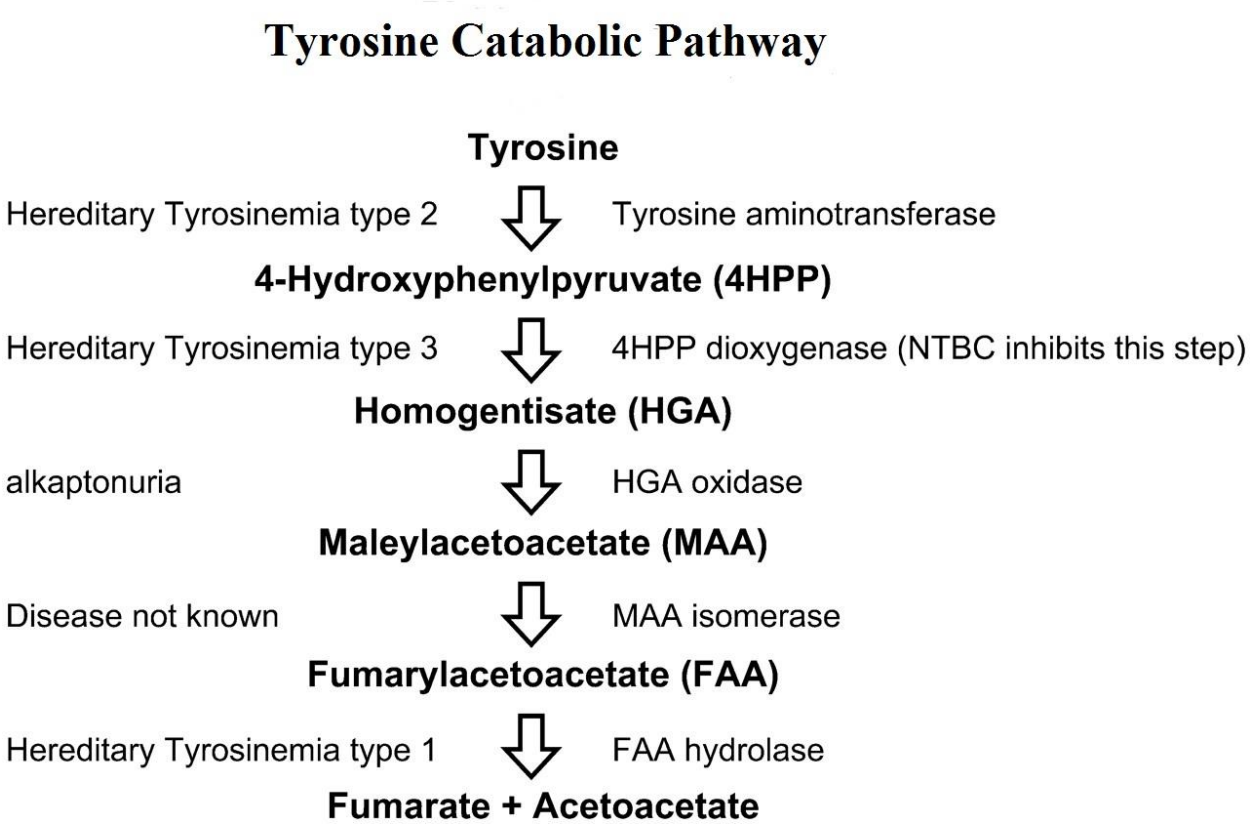
Identity	FAH	Sex	Color	Cage
34	WT (+/-)	M	brown	(33, 34, 36)
26	WT (+/-)	M	black	individual
44	WT (+/-)	M	brown	(43, 44, 46)
33	WT (+/-)	M	brown	(33, 34, 36)
46	D (-/-)	M	light brown	(43, 44, 46)
17	WT (+/-)	M	brown	individual
43	WT (+/-)	M	brown	(43, 44, 46)
38	D (-/-)	F	white	(38, 42, 45, 47)
18	D (-/-)	F	white	(18, 20, 21)
32	WT (+/-)	F	brown	individual
21	WT (+/-)	F	brown	(18, 20, 21)
42	WT (+/-)	F	light brown	(38, 42, 45, 47)
20	D (-/-)	F	white	(18, 20, 21)

TOTALS:
13 total mice tested – 7 males, 6 females
9 WT(Wild Type Carriers) – 6 males, 3 females
4 Diseased – 1 male, 3 females

Table #2: Alternation Pattern in Y-Maze

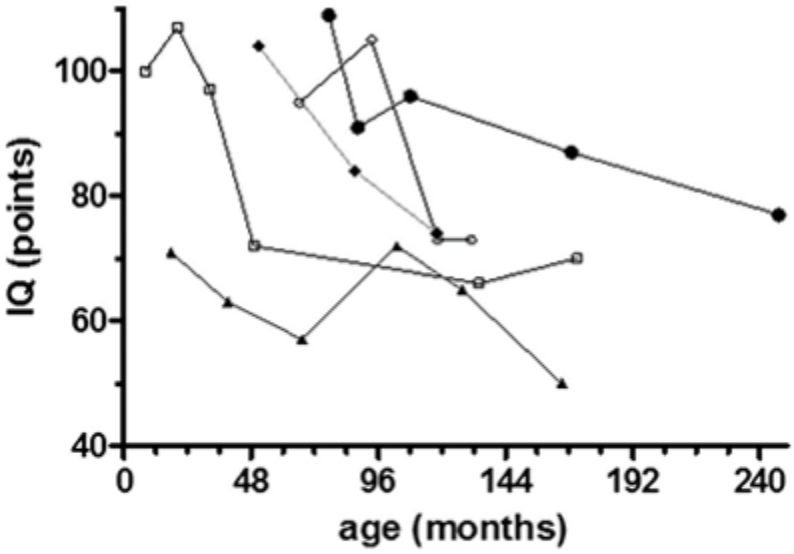
Identity	FAH	Sex	Alternation Pattern in Y-Maze
34	WT (+/+)	M	CACBABACBABCABABACBA
26	WT (+/+)	M	CBACBACABCBABABABCABCBABACA
44	WT (+/+)	M	BCBACBCABACBCBACBCABCACAB
33	WT (+/+)	M	CBACABACABACBACABACABABCACABACA
46	D (-/-)	M	CBABCBACACBACCBABCACB
17	WT (+/+)	M	ABCABACACACACBACBCBACB
43	WT (+/+)	M	BACBCBACBACBCABCACACB
38	D (-/-)	F	CBACBACABCBAACACBABCABABCACBA
18	D (-/-)	F	CBABCACABCACAB
32	WT (+/+)	F	ACBCABCABABCABCABCABCABCA
21	WT (+/+)	F	CACBABCABACACABCACACABCACABC
42	WT (+/+)	F	CBCACCBACBCBCBC
20	D (-/-)	F	BCACBACBCACBABCABABCACACBACA

Figure 1: Catabolic Pathway of Tyrosine



***Nitisinone is labeled NTBC**

Figure 2: Longitudinal IQ levels of patients on Nitisinone



Longitudinal IQ levels in patients with TT1 receiving Nitisinone. A significant drop of total IQ(average of 27 IQ points) (Bendadi et al., 2013)

Figure 3: Genotyping

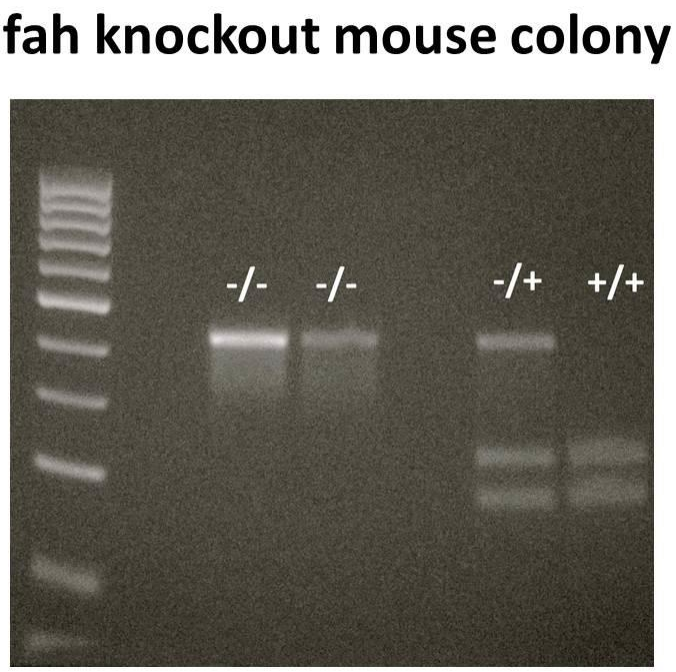


Figure 4: Y-Maze

Figure 5: Averages for total arm entries in Y-Maze

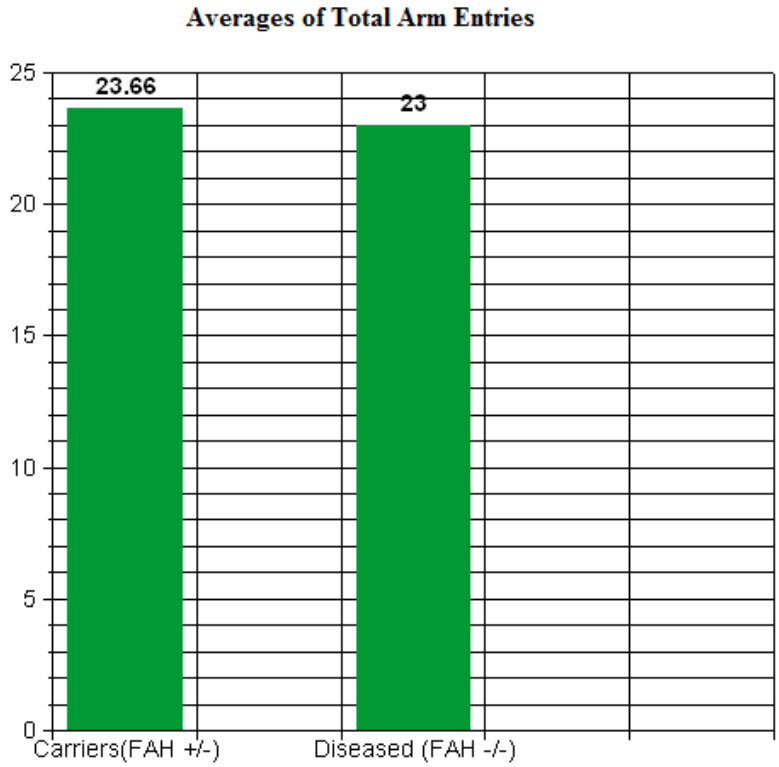


Figure 6: Percentage of Alternation Behavior

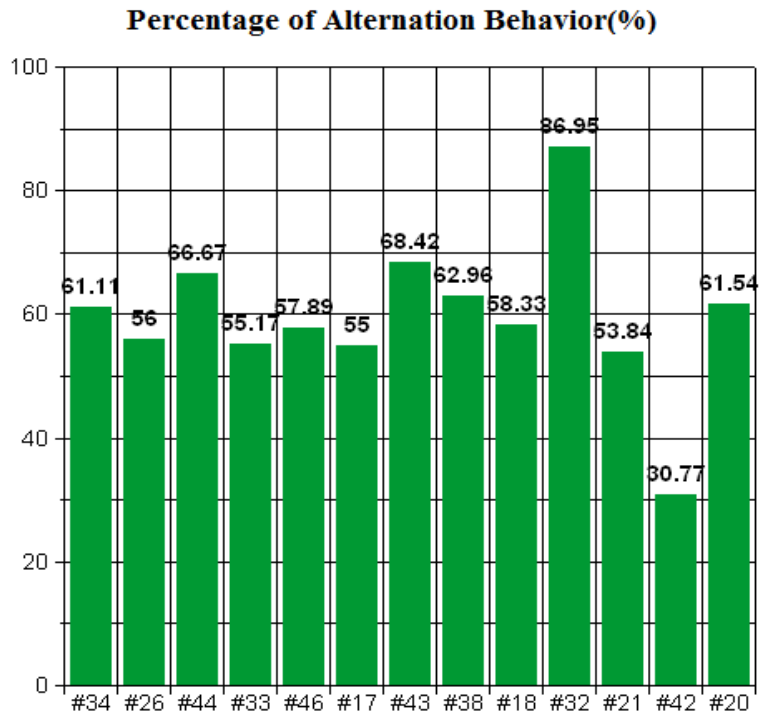


Figure 7: Diseased vs Carrier Comparison Graph