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Control of alkaptonuria with nitisinone and gene therapy: A systematic review

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Abstract

Alkaptonuria (AKU) is a genetic disorder inherited in accordance with Mendel first law. Mutations in the *HGA* gene result in the AKU disorder. Three major features of this disorder: arthritis, ochronosis, and the presence of Homogentisic Acid (HGA) in the urine. The author searched the PubMed Databases at National Center for Biotechnology Information (NCBI) for articles on AKU published between 2014 and 2019. All articles were open access and in English. In this systematic review, the author included one's own references and other relevant publications. Search results showed that detection tools for people with AKU can include x-rays and genetic tests. No adequate treatment is available for AKU at present. However, counselors of genetic counseling may help patients with AKU and give counseling to them and their families. Candidate drugs of AKU are nitisinone and genetic manipulation techniques. Research results on the use of nitisinone on AKU have shown remarkable improvements. In the future, genetic manipulation techniques may be beneficial for treating AKU. These techniques are such as modified CRISPR/Cas9 (*Fok*I-dCas9), End-Joining Homology Techniques (EJHTs) and induced Pluripotent Stem Cells (iPSCs).

Keywords: *Alkaptonuria (AKU), Alcaptonuria, HGD, Homogentisic Acid (HGA)*

1. Introduction

Alkaptonuria (AKU) is a rare genetic disorder with a high level of circulating Homogentisic Acid (HGA) (Masoud *et al*., 2017; Hakim *et al*., 2018; and Nickavar and Azar, 2018), in urine (Griffin *et al*., 2018), blood, and tissues (Gupta *et al*., 2017). Inheritance of AKU follows an autosomal recessive pattern. However, autosomal dominant pattern occurred in AKU families in minority cases (Rana *et al*., 2015). AKU is the first disease that in accordance with the law of segregation. Mutations in the *HGD* gene result in this disorder ((Genetics Home Reference, 2019a; and Nelwan, 2013). AKU affects about one in 100,000 to 250,000 (Masoud *et al*., 2017) or one in 200,000 to one in 1,000,000 live births (Couto *et al*., 2018). Other names of AKU are alcaptonuria, HGA oxidase deficiency, and homogentisic aciduria (Genetics Home Reference, 2019b).

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Symptoms of AKU include arthropathy of major joints, calcifications of cartilaginous tissue with dark color, deterioration of cardiac valves (Gupta *et al.*, 2017; Karaoðlu *et al.*, 2016; and Nelwan, 2013), and pigmentation of ears, sclera (Rana *et al.*, 2015), eyes, and skin (Atalay *et al.*, 2015). No definite cure for AKU is available (Gupta *et al.*, 2017; Karaoğlu *et al.*, 2016; and Nelwan, 2013). Vitamin C may be used. However, it has been proved that vitamin C is not efficient for a number of reasons and dangerous for use in AKU (AKU Society, 2019). Rana *et al.* suggested that procedures such as joint surgeries and organ transplantations might be recommended for AKU patients (Rana *et al.*, 2015). Joint surgeries alleviate the symptoms of AKU. Organ transplant, especially the liver transplant, has been shown successful.

Modern trends such as genetic counseling, nitisinone, and genetic manipulation techniques hold potential for the elimination of AKU. Genetic counseling is beneficial to advise patients and their families and reduce the occurrence of AKU among people. If two carriers with no family history of AKU marriage, they can have children with AKU. This occurrence will lower than a couple with family history. Nitisinone may be useful for treating this disorder (Alajoulin *et al.*, 2015; Hakim *et al.*, 2018; Nickavar *et al.*, 2018; and Rathore *et al.*, 2016). The benefit of nitisinone for AKU patients is under evaluation. There have been studies regarding possible AKU treatment with nitisinone for humans. Nitisinone inhibits 4-hydroxyphenylpyruvate dioxygenase, a triketone (Phornphutkul *et al.*, 2002). It may be beneficial for treating AKU. Nitisinone reduces circulating of HGA (Milan *et al.*, 2019; and Nelwan, 2013). However, nitisinone is not yet licensed for use in AKU patients. Genetic manipulation techniques could be a potential tool for treating AKU patients in the future (India AKU Society). The ideas of genetic manipulation techniques are to use End-Joining Homology Techniques (EJHTs), *Fok*I-dCas9, induced Pluripotent Stem Cells (iPSCs), and virus's delivery such as Adeno-Associated Viruses (AAV) and HSV-1 for AKU treatment. For example, Pan *et al.* suggested that the *Fok*I-dCas9 can correct the major variant in the *PAH* gene. It suggests that this tool may be useful for correcting incorrect sequences of other inherited metabolic disorders (Pan *et al.*, 2016) such as AKU. It means that genetic manipulations techniques are useful tools for treating AKU.

Genetic manipulation techniques may cause health risks such as oncogenic transformation into the host genome. For example, the *c-Myc* retrovirus reactivation boosts tumorigenicity in chimeras. For these reasons, a wide array of delivery methods has been examined; safely integrating AAV vectors to non-integrating vectors as Sendai virus. In addition, non-viral and episomal reprogramming approaches have been developed. For example, Kamath et al has developed iPSCs with virus-free, *Myc*--free, and *Lin28*-free (Nelwan, 2017b). It suggests that genetic manipulation techniques are safe tools for treating metabolic disorders such as AKU.

In this study, the author describes the progress in a study of AKU that focused on the genetic aspects, and treatments of AKU. The genetic aspects include the *HGD* gene, mutations in the *HGD* gene, and treatments: genetics counseling, nitisinone, and genetic manipulation techniques.

2. Methods

2.1. Systematic review

The present report follows the guidelines of the PRISMA extension statement for systematic review (Nelwan, 2018b). These guidelines also correspond to PROSPERO guidelines for such as review question, searches, and primary outcomes (Liberati *et al.*, 2009).

2.2. Searches

The author searched the PubMed Databases at National Center for Biotechnology Information (NCBI) for AKU articles. These included free PMC articles for CC-BY-4.0 and CC-BY-NC-ND licenses in English published from 2014 to 2019. Each search consisted of the first 40 articles. Other articles on the list were not considered. Keywords included "alkaptonuria," "alkaptonuria history," "alkaptonuria and heart disease," "alkaptonuria and kidney diseases," "alkaptonuria and arthrities," "alkaptonuria and diagnosis," "alkaptonuria and nitisinone," and "alkaptonuria and gene therapy." In some cases articles published before 2014 were also included. In addition, the author included own articles and other relevant publications in this study.

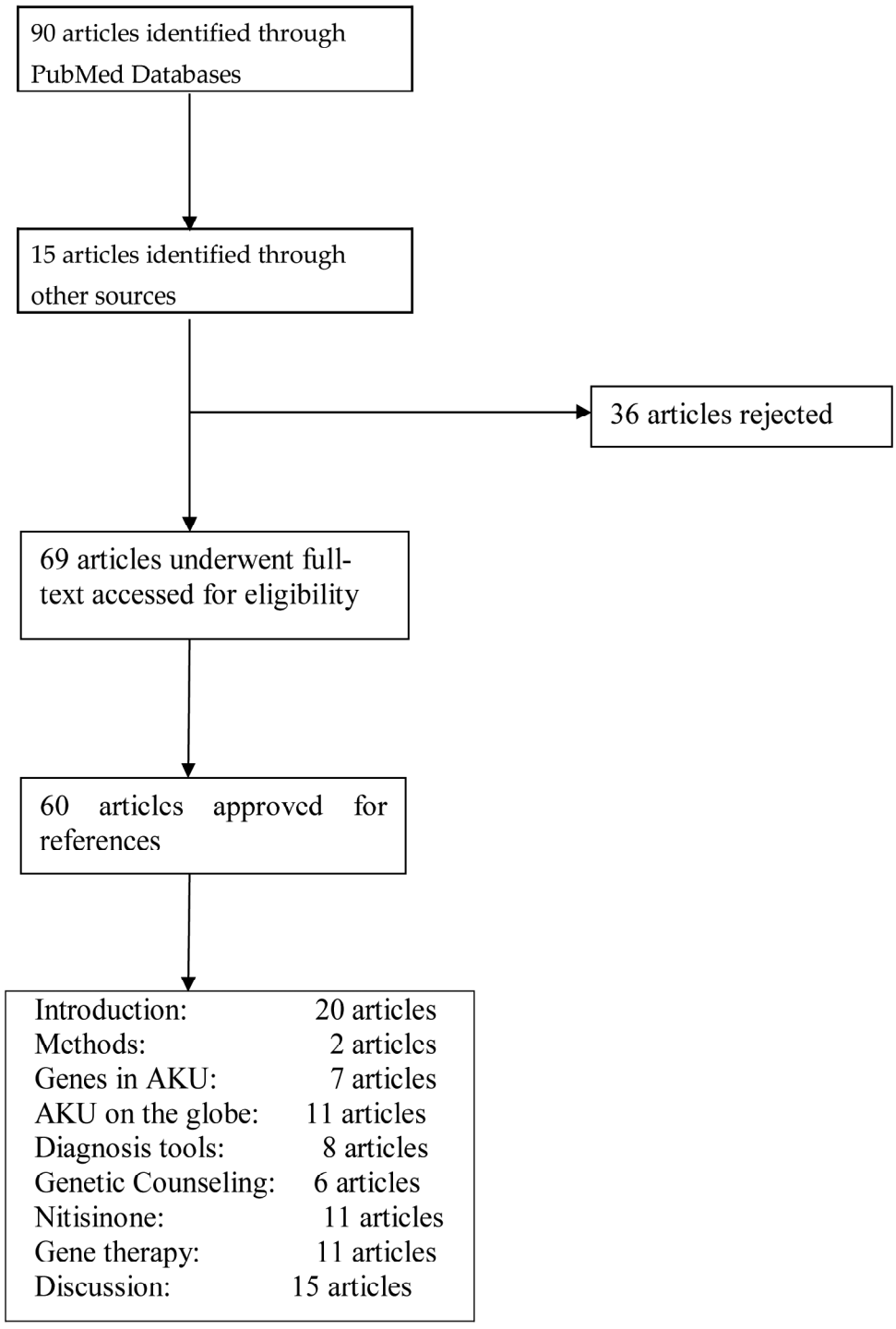

Figure 1: A flowchart articles selected in the systematic review

2.3. Exclusion criteria

Criteria for the exclusion of the literature included analysis of subgroups or subsets, conference proceedings, letter to the editor, and opinions publications, and publications other than English.

3. Results

The author took 90 articles from the PubMed Databases searches and took 15 articles from other relevant publication searches (Figure 1). After screening titles and abstracts, the author rejected 36 articles and took 69 articles for full-text review. These articles met the criteria for data extraction. After reviewing, the author put 60

articles (references) in the References section of this article. There were articles from MIM #203500, MIM #60747, and MIM #180380. Other articles were additional articles. Articles in the last box of Figure 1 show total articles put for each section. It did not describe all references in this article.

3.1. Genes in alkaptonuria

HGD is the gene formal symbol. Other names of *the HGD* gene consists of AKU, HGD_HUMAN, HGO, homogentisate 1,2-dioxygenase (homogentisate oxidase), HGA oxidase, and homogentisicase (Genetics Home Reference, 2019a). The *HGD* gene consists of 14 exons and encodes a 445 amino acid polypeptide with high homology to the Aspergillus hmgA (OMIM #60747) (Fernandez *et al.*, 1996). This gene occupies chromosome 3q in the chromosome map, 3q13.33 (OMIM #60747) (Groz, 2014). The *HGD* gene includes base pairs 120,628,168 to 120,682,571 (Genetics Home Reference, 2019a; and NCBI Gene). The gene provides instructions for making the HGD enzyme. The enzyme involves the catabolism of phenylalanine and tyrosine. HGD enzyme converts HGA to maleyacetoacetic acid during phenylalanine and tyrosine catabolism (Genetics Home Reference, 2019b; Hakim *et al.*, 2018; and Rana *et al.*, 2015).

A gene is the primary physical and functional unit genetic. Genes serve as instructions to construct molecules of protein and form DNA. Mutations can arise in a gene; a permanent change in the DNA. Gene mutations result in damage of protein. A genetic disorder is a condition caused by mutations in at least one gene (Nelwan, 2017a) such as Friedreich ataxia and AKU. Selvakumar *et al.* reported that there have been more than 80 mutations in the *HGD* gene in patients with AKU (Selvakumar *et al.*, 2018). Most of these mutations change single amino acids used to create the HGD enzyme. Substitution of the amino acid valine for the Met368Val is

Table 1: Mutations in the *HGD* Gene

Moleculae Consequence	**Total**	**References**
Frameshift	15	NCBI ClinVar
	3	Beltran-Valero
		de Bernabe *et al.* 1998
Intronic	2	Beltran-Valero
		de Bernabe *et al.* 1998
Missense	40	NCBI ClinVar
	16	Beltran-Valero
		de Bernabe *et al.* 1998
Nonsense	2	NCBI ClinVar
Splice site	10	NCBI ClinVar
	1	Beltran-Valero
		de Bernabe *et al.* 1998
Variation Type	**Total**	**References**
Deletion	23	NCBI ClinVar
Duplication	20	NCBI ClinVar
Indel	2	NCBI ClinVar
Insertion	7	NCBI ClinVar
Single nucleotide	94	NCBI ClinVar

the most common *HGD* gene mutation in European populations (Genetics Home Reference, 2019a). Mutations in the *HGD* gene can include duplication (NCBI ClinVar), missense (Beltran-Valero de Bernabe, 1998; and NCBI ClinVar) and the mutations are in a 54,363 bp gene (Rana *et al.*, 2015), frameshift (Beltran-Valero de Bernabe, 1998; and NCBI ClinVar), intronic (Beltran-Valero de Bernabe, 1998), nonsense (NCBI ClinVar), and splice site (Beltran-Valero de Bernabe, 1998; and NCBI ClinVar) (Table 1). These mutations damage the HGD enzyme's role to break down the amino acids phenylalanine and tyrosine (Genetics Home Reference, 2019b).

Table 2: Alkaptonuria in humans

No.	Old	References	Old	References
1	6	Karaoğlu *et al*. 2016	8	Nickavar *et al*. 2018
2	39	Damarla *et al*. 2017	48	Gupta *et al*. 2017
3	39	Alajoulin *et al*. 2015	50	Bhattar *et al*. 2015
4	39	Elcioglu *et al*. 2003	51	Phornphutkul *et al*. 2002
5	45	Alaya and Mzabi, 2017	57	Li *et et al*. 2016
6	46	Rana *et al*. 2015	59	Phornphutkul *et al*. 2002
7	48	Rathore *et al*. 2016	59	Biler *et al*. 2015
8	48	Mayatepek *et al*. 1998	60	Rathore *et al*. 2016
9	50	Karaoğlu *et al*. 2016	60	Harun *et al*. 2014
10	55	Karaoğlu *et al*. 2016	62	Mazoochy and Razi, 2018
11	60	Tekgöz *et al*. 2018	64	Azami and Maleki 2015
12	63	Chatzis *et al*. 2016	65	Couto *et al*. 2018
13	65	Hakim *et al*. 2018	65	Cunningham *et al*. 1989
14	70	Carrier and Harris, 1990	72	Isa *et al*. 2014
15	72	Atalay *et al*. 2015		
16	72	Selvakumar *et al*. 2018		

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	51.0625	54.64285714
Variance	281.129	225.1703297
Observations	16	14
Hypothesized Mean Difference	0	
df	28	
t-Stat	-0.6172	
P(*T*< = *t*) one-tail	0.27105	
t Critical one-tail	1.70113	
P(*T*< = *t*) two-tail	0.54211	
t Critical two-tail	2.04841	

Carrier and Harris *et al*; Cunningham *et al*; Elcioglu *et al*; Mayatepek *et al*;

Phornputkul el al were taken from MIM #203500 (Edit history: 07/09/2016).

3.2. Alkaptonuria on the globe

AKU occurs worldwide and the prevalence is one in 250,000 to 1,000,000 births (Genetics Home Reference, 2019b). This disorder has a very low prevalence. However, AKU has a high prevalence in China (Bhattar *et al.*, 2015), the Dominican Republic, Jordan, parts of South India (Nelwan, 2013), Singapore (Bhattar *et al.*, 2015), Slovakia (Nelwan, 2013), and Thailand (Bhattar *et al.*, 2015). In Slovakia, the prevalence is one in 19,000 (Genetics Home Reference, 2019b; and Nelwan, 2013). The prevalence in China, Jordan, the Dominican Republic, India, Singapore, and Thailand are unknown. Either male or female has the same chances of inheriting AKU (Table 2). In this systematic review, of the 30 case reports, 15 (50%) were adult males, 13 (43.33%) were adult females, 1 (3.33%) was a boy, and 1 (3.33%) was a girl. Of the 30 case reports of AKU, 16 (53.33%) were males and 14 (46.67%) were females. Ages of male and female were not significantly different (Table 2). The author did not find any reference for the prevalence of AKU with autosomal dominant.

Phornphutkul *et al.* reviewed 58 patients with AKU aged four to 80 years (MIM #203500) (Phornphutkul, 2002). The authors found that joint replacement was at a mean age of 55 years and the development of the renal stone at 64 years. In addition, cardiac-valve involvement was at 54 years and coronary artery calcification at 59 years. Linear regression analysis showed that the radiographic score for the severity of AKU commenced increasing after the age of 30 years. Males increased more rapidly than females. Phornphutkul *et al*. found that kidney stones occurred in 13 male and three female patients. Of the 27 males who were 31 to 36 years, eight were prostate stones. The development of prostate stones did not have an association with the development of kidney stones (NCBI ClinVar; Nelwan, 2013; and Phornphutkul, 2002). Kidney stones occur by 64 years old in 50% of individuals with AKU (Introne and Gahl, 2019). Three patients, each greater than 50 years old, were aortic valve replacement (NCBI ClinVar; Nelwan 2013; and Phornphutkul, 2002). Aortic valve stenosis occurs at a high frequency in the sixth and seventh decades of life (Introne and Gahl, 2019).

AKU causes urine to turn black (Hakim *et al.*, 2018; Genetics Home Reference, 2019a; and Masoud *et al.*, 2017) on alkalization or when exposed to air (Hakim *et al.*, 2018; Genetics Home Reference, 2019a; Introne and Gahl, 2019; and Li *et al.*, 2016). In children, there are no symptoms of AKU other than the urine turning black. Pain associated with AKU starts around the second decade of life (Nelwan, 2013). Ochronosis builds up the blackish blue pigment in cartilage, connective tissues, and skin (Genetics Home Reference, 2019b). Ochronosis occurs after 30 years old (Genetics Home Reference, 2019; and Introne and Gahl, 2019b). Patients with AKU typically develop arthritis, particularly in the spine and large joints, beginning in early adulthood (Nelwan, 2013). Arthritis begins in the third decade. Joint symptoms involving the spine appear in that decade. In one large series, low back pain occurred prior to 30 years old in 49% and prior to 40 years old in 94% (Introne and Gahl, 2019). Selvakumar *et al.* introduced the idea that a patient with arthritis reached 72 years old without being detected as an AKU sufferer. Ochronosis occurs in the fifth decade for the pigment to accumulate in valvular tissue (Selvakumar *et al.*, 2018). HGA and its oxidation products accumulate in arteries and pancreas, heart valves, hyaline cartilage, ligaments, renal tubule epithelial cells, skin, tendons, the cartilage of the ears and nose, and the sclera (Karaoğlu, 2016).

3.3. Diagnosis tools for alkaptonuria

Diagnosis of AKU can be at any age; early childhood and older people. In early childhood, the urine is turning black after exposure to the air. Diagnosis in later in life can include such as back pain and joint paint (Nelwan, 2013). Several methods are available for diagnosis of AKU. These can include computed tomography (CT), gas chromatography-mass spectrometry (GCMS), liquid chromatography tandem mass spectrometry (LC-MS/MS), magnetic resonance imaging (MRI), radiographic examinations (X-rays), and genetic tests (Table 3).

Li *et al.* used CT scans and MRI to a 62-year-old woman to indicate AKU. With this method, the authors found multilevel degenerative disc disease from cervical to lumbar spine, including calcification, osteophytosis, and vacuum phenomenon. MRI showed the spinal cord compression at multi-level. It occurred primarily from the unecessary ligamentum flavum posteriorly. The patient's urine turned to dark after exposure to the air (Li *et al.*, 2016). Mazoochy and Razi examine radiographic a 57-year-old woman to indicate ochronosis. The right hip X-ray in the patient showed progression in ochronotic arthropathy (Mazoochy and Razi, 2018). Hakim *et al.* (2018) used radiographic evaluation to display degenerative arthritis in both hips and knees with the left side being more affected than the right in a 65-year-old man. To confirm this, the authors used DNA sequencing. The authors detected an uncommon genomic deletion of 69 bp of exon 2 and surrounding DNA sequences in flanking introns (Hakim *et al.*, 2018). The diagnosis of AKU could base on the detection of a significant amount of HGA in the urine by GCMS analysis (Introne and Gahl, 2019; and Nickavar *et al.*, 2018) (Table 3). The

amount of HGA excreted per day was between one and eight grams (Introne and Gahl, 2019). However, GCMS is not routine approach for diagnosis of AKU. LC-MS/MS is largely used. Davison *et al.* used LC-MS/MS to confirm the elevated HGA in patients with AKU (Davison *et al.*, 2019). Introne and Gahl suggested that identification of biallelic pathogenic variants in the *HGD* gene on molecular genetic testing confirmed the diagnosis and allowed family studies. MRI method can detect abnormalities in the tendon in patients with AKU (Introne and Gahl, 2019).

Table 3: Diagnosis tools for alkaptonuria

Tools	Diagnose	References
CT	Degeneration disc	Introne and Gahl, 2019
	Coronary artery clas.	Damarla *et al.* 2017
	Prostate stones	
GCMS	Detection of HGA	Introne and Gahl, 2019
GS	Exom sequencing	Damarla *et al.* 2017
	Genome sequencing	
	Mitoch. Sequencing	
LC-MS/MS	Detection of HGA,	Davison *et al.* 2019
	tyrosine	
MRI	Arthritis	Damarla *et al.* 2017
	Ochrronosis	
MSI	Detection of	Davison *et al.* 2019
	nitisinone	
SG	Sequence of HGD	Damarla *et al.* 2017
X-rays	Arthritis	Hakim *et al.* 2018
	Ochrronosis	Li *et al.* 2016
	Prostate stones	

Note: CT = computed tomography; GCMS = gas; cromatography-mass spectrometry; GS = genome; sequencing; LC-MS/MS = liquid chromatography tandem; mass spectrometry; MRI = magnetic resonance imaging; and MSI = mass spectrometry imaging

Introne and Gahl suggested molecular genetics methods to detect AKU; single-gene testing (SG) and genome sequencing (GS). In SG, it first performs sequence analysis of *HGD* gene, followed by gene-targeted deletion/duplication analysis, if only one or no pathogenic variant is found. This testing may be performed first in individuals of high-risk ancestry such as the Dominican Republic, Jordan, and Slovakia. The author stated that GS consists of exom sequencing, genome sequencing, and mitochondrial sequencing. These testing may be used if serial SG testing (and/or use of multigene panel) fails to confirm a diagnosis in an individual with features of AKU (Introne and Gahl, 2019).

Other methods for diagnosis of AKU are physical examinations and laboratory tests. These methods can be used for aortic valve replacement. Physical examinations for identification of blackish blue pigment can include head (nose, ear, and jaw), knees, and shoulder. Laboratory examinations can include blood counts and blood pressure. Selvakumar suggested that symptoms of an AKU patient could include such as dyslipidemia and hypertension (Selvakumar *et al.*, 2018).

3.4. Genetic counseling

Related marriages increase the expression of a genetic disorder incidence. Unrelated marriages can help to hide a genetic disorder. It shows that it is important to plan an unrelated marriage to hide a genetic disorder. This requires contacting a genetic professional, or a counselor in a genetic disorder for getting information in details (Nelwan, 2017a) regarding disorders such as AKU and Friedreich ataxia. A healthy married couple maybe has questions whether or not they have chances of getting an AKU child. A genetic counselor should have their answers to those questions. Counselors may also answer questions for testing of AKU. It relies on a family whether or not they would like to use genetic counseling suggestions. A genetic counselor can help to provide ideas about how a genetic disorder such as AKU can be avoided its appearance in a family.

AKU may be inherited in an autosomal recessive pattern (Damarla *et al.*, 2017; and Rana *et al.*, 2015). Autosomal dominant inheritance in AKU are uncommon cases (Rana *et al.*, 2015). Damarla *et al.* suggested that AKU is in either atosomal recessive or autosomal dominant trait (Damarla *et al.*, 2017). At the conception of autosomal recessive, each sib of an affected individual has a 25% chance of being affected (recessive homozygote), a 50% of being asymptomatic carrier (heterozygote), and a 25% of being unaffected and not a carrier (dominant homozygote). Once an at-risk sib is known to be unaffected, the chance of his/her being a carrier is 2/3. At the conception of autosomal dominant, the chance of being affected is 75%. It includes a 25% of dominant homozygote and a 50% of being carrier or heterozygote. The chance of being an unaffected is 25%.

In the autosomal recessive pattern, the incidence of AKU would be lower if the parents are unrelated. If the parents were relative, consanguinity, the incidence of alkaptonuria would be higher. The mathematical equation for a consanguinity marriage is

$(q^2 + pqF)/q^2$

F is the coefficient of consanguinity. The equation of F is $F = \Sigma(½)p + m + 1$(Kalmes and Huret, 2002; and Nelwan, 2013). If prevalence of AKU is one in 250,000, and if the parents are cousins, the chance of an expression of AKU would be as follows:

$F = (½)^{2+2+1} + (½)^{2+2+1} = 1/16$

$q^2 = 1/250{,}000 = 0.000004$

$q = \sqrt{0.000004} = 0.002$

$p = 1 - 0.002 = 0.998$

If the accurate equation is used, the result is:

$(q^2 + pqF)/q^2 = 32.1875 = 32.19$

Thus, the AKU would be about 32.19 times higher if the parents were consanguinity cousins than if the parents were unrelated (Nelwan, 2013).

Carrier detection and prenatal diagnosis are possible if the disease-causing mutations for AKU have existed in the family. The testing can use DNA extracted from chorionic villus sampling at 10-12 weeks gestation. The testing can also use DNA extracted from fetal cells through amniocentesis at 15-18 weeks gestation. Preimplantation genetic diagnosis for at-risk pregnancies is also possible (Introne and Gahl, 2019).

Prenatal testing for AKU, that does not affect intellect and life span, are uncommon. However, most centers would consider decisions for prenatal testing to be the choice of the parents. Discussion of these issues is appropriate (Introne and Gahl, 2019; and Nelwan, 2013).

3.5. Nitisinone

No treatment for AKU is available at present (Nelwan, 2013; and Selvakumar *et al.*, 2018). However, nitisinone is under research for treating AKU. It blocks the HGD enzyme and it should be suggested as an efficient drug for treating AKU (Alajoulin *et al.* 2015; and Nickavar and Azar, 2018). Patients treated with nitisinone have demonstrated reduction of plasma HGA levels (Azami and Maleki, 2015; Introne and Gahl, 2019; and Selvakumar *et al.*, 2018). Nickavar *et al* reported the use of nitisinone and low tyrosine diet to treat AKU (Nickavar and Azar, 2018). At present, use of nitisinone in patients with AKU is still in clinical trials. However, the FDA has not yet approved this drug for treating AKU. AKU society estimates that one percent of the AKU population is taking nitisinone off-label (Aku Society, 2019; and Nelwan, 2013). FDA has approved nitisinone for treating tyrosinemia type 1 (Nelwan, 2013; and Phornphutkul *et al.*, 2002), and its permission does not

include AKU. Montagutelli *et al.* discovered a murine model of AKU. The authors observed that nitisinone reduced HGA in the urine (Montagutelli *et al.*, 1994). However, no long-term studies in animals have been performed to evaluate the carcinogenic potential of nitisinone (Nelwan, 2013). Long-term studies are needed to confirm its helpfulness (Hakim *et al.*, 2018).

Phornputkul *et al.* reported that urinary HGA excretion decreases from 0.9 to 0.13 g per day after a 10-day treatmen of nitisinone in a 51-year old woman. The same reduction also occurred after a 9-day treatment of nitisinone in a 59-year old woman. Plasma tyrosine levels in these AKU patients increased with no clinical signs or symptoms. The authors suggested the long-term safety and efficacy of this nitisinone treatment requires further evaluation (Phornphutkul *et al.*, 2002).

Ranganath *et al.* suggested that nitisinone reduced HGA in AKU patients (Table 4). In their study, the authors found that the most efficacious dose was 8 mg/day. In this dose, the reduction of HGA was 98% (Ranganath *et al.*, 2016).

Table 4: Use of nitisinone in AKU patients

Group	Dose per day	u-HGA24
1	control	0.15 mmol
2	1 mg	0.57 mmol
3	2 mg	1.44 mmol
4	4 mg	3.26 mmol
5	8 mg	31.53 mmol

Note: u-HGA24 = once daily urinary excretion; mg = milligram; mmol = millimoles; and group 5 is the best.

Treatment with nitisinone is unlikely to cause depression in patients with AKU. Nitisinone does not have direct effect on monoamine neutrotransmitter metabolism in the central nervous system (CNS) of mice with AKU. It is unlikely to result altered mood or cognition in humans with AKU disorder (Davison *et al.*, 2019).

3.6. Genetic manipulation techniques

Nelwan introduced the idea that genetic manipulation techniques may be beneficial to treat monogenic recessive disorders (Nelwan, 2018a; Nelwan, 2017a; and Nelwan 2017c). Genetic manipulation techniques may include AAV, CRISPR/Cas9 system, EJHTs (Nelwan, 2017) and iPSCs (Nelwan, 2017b). Monogenic recessive disorders may include AKU, Friedreich ataxia, and oculocutaneous albinism. These techniques may help to fix incorrect sequences in a genetic disorder such as AKU. With this correction, it may help the *HGD* gene to work normally and increase the enzyme deficiency, for example.

Genetic manipulation using virus delivery tools may include AAV, adenoviruses, herpes simplex virus type 1 (HSV-1), retrovirus, and Sendai virus. AAV vectors are non-integrating vectors, safely integrating, or low risk of integrating into the host genome. These vectors have a genome of 4.7 kb. However, Nelwan(2017a). have created an AAV vector with a 5.2 kb genome, suggesting that AAV vectors may be developed for a bigger genome or even big genome as HSV-1 vectors. At least 12 vector serotypes have been available. These include such as AAV1, AAVrh, and AAV9 (Nelwan, 2017a). Adenoviruses are non-enveloped and non-integrating vectors that penetrate the cells. These vectors have the capacity of cargo up to 30 kb (Nelwan, 2017a; and Nelwan, 2017b). Lentiviruses belong to retroviruses family. Retroviruses are single-stranded RNA viruses. These viruses integrate into the host cells. Retroviruses have a genome of 7-10 kb. Sendai virus has a genome of about 15.4 kb. This vector is a non-integrating vector. An HSV-1 vector is a possible option to accommodate a large DNA molecule and is a non-integrating vector. HSV-1 vectors have a genome of 152 kb (Nelwan, 2017a).

Gene delivery vectors such as AAV and HSV-1 may deliver gene-editing tools. Gene-editing tools include meganucleases (MNs), ZFNs, TALENs, and CRISPR/Cas9 system. MNs as gene editing tool has not been widely used. Both ZFNs and TALENs have the same techniques and use different DNA binding arrays: zinc finger arrays and TAL effectors repeats. Finally, the CRISPR/Cas9 system uses sgRNA to produce site-specific gene editing aim cells with great frequency (Nelwan, 2017b).

CRISPR/Cas9 systems NHEJ-mediated DSB repair leads to the introduction of small insertions or deletions at the targeted site. It results in the knockout of gene function through frameshift mutations (Shankar *et al.*, 2018). CRISPR/Cas9 system is the most popular method for editing mutated genes in various researches at present. However, Anuar *et al.* used TALENs to impair genetically the function of all three *H2A.B3* genes that used only one pair rather than multiple pairs. It could reduce the likelihood of mosaicism. Importantly, it reduces the possibility of off-target mutations (Anuar *et al.*, 2019). However, the CRISPR/Cas9 system and EJHTs may allow correct insertion in a large fragment.

Five methods are available for EJHTs: NHEJ, microhomology-mediated end-joining (MMEJ), HR, homology-mediated end-joining (HMEJ), and homology-independent targeted integration (HITI). HITI depends on NHEJ repair mechanism. NHEJ-based method presented random directions in integration and various types of indels at the junctions. The NHEJ is active in the entire cell cycle. The MMEJ-based method display low efficiency in the cultured cell. The MMEJ is active in the early S/G1 phase. The HR-mediated method allows correct insertion in a large fragment (Nelwan, 2018a). HR is active during S/G2 phase (Nelwan, 2018a; Chu *et al.*, 2019; and Yoshino *et al.*, 2019), using the sister chromatid as recombination template. Finally, the HMEJ-based method achieves transgenic integration in mouse and monkey embryos, as well as in hepatocytes and neurons *in vivo*. All methods may be useful for generating animal models and for targeted gene therapies (Nelwan, 2018a). Programmable nucleases assisted EJHTs are a feasible approach to generate knock-in non-human primate models of human diseases.

The iPSC is a genetic engineering technique for obtaining the same stage as embryonic growth phase and embryonic properties through reprogramming factors. These reprogramming factors include *c-Myc*, *Klf4*, *Oct4*, *Sox2 Lin28*, and *Nanog*. The iPSCs, which are free of virus, can be *l-Myc*, *c-Myc*, and *Lin28*. The iPSCs can produce large numbers of diseased cells for drug screening and can differentiate into various target cells in appropriate culture cell conditions (Nelwan, 2017b; and Nelwan, 2018a).

The author did not find any reference relating to genetic manipulation tools for treating AKU. However, Nelwan and Pan *et al* suggested that genetic manipulation techniques such as CRISPR/Cas9 system (Nelwan, 2017b; and Pan *et al.*, 2016) and iPSCs might be useful for treating monogenic recessive disorders (Nelwan, 2017b) such as AKU and PKU.

4. Discussion

Stenn *et al.* discovered that the Egyptian mummy Harwa, dating from 1500 B.C, was AKU. Harwa was the first known patient with AKU. Phornphutkul *et al.* (2002) provided a review of the natural history of AKU. The author suggested that Garrod described AKU as the first disorder in humans inherited according to the Law of Segregation (MIM #203500) (Stenn *et al.*, 1977).

To slow down AKU development, once it is suspected, both medical history and diagnosis are relevant. For patients who plan to have a child and come from a family or community with a high frequency of AKU, they require genetic counseling (Cieszyński *et al.*, 2016). Genetic counselors may provide information regarding the diagnosis and treatment of AKU.

Several strategies are available to diagnose AKU. These include CT, genetic tests, GCMS, LC-MS/MS, X-rays (Table 3), and physical examination. In addition, CT can be used for diagnosing cervical and lumbar spine; calcification, osteophytosis, and vacuum phenomenon. X-rays could be beneficial for diagnosing such as renal stones, arthritis, and ochronosis in the conjunctiva and cornea. Use of X-rays may be beneficial for diagnosing calcification of the ear cartilage. Physical examination may find ochronosis signs: purple or black discoloration in on the skin of the hands (Introne and Gahl, 2019). In this study, the author only has two children with AKU (Table 2). It indicates the need for an important diagnostic tool for diagnosing AKU for children. It may be genetic tests: single-gene testing and genome sequencing. Use of genetic tests in children may be useful for treating AKU as early as possible. Treatments may include the use of vitamin C and nitisinone. In addition, genetic manipulation techniques may be useful to treat AKU in the future.

Azami and Maleki showed that use of vitamin C and nitisinone in an AKU patient reduced complaint of mild back pain (Azami and Maleki, 2015). It suggests that vitamin C along with nitisinone is beneficial to help patients with AKU (Phornphutkul *et al.*, 2002). However, Rathore *et al.* stated that there are concerns about the side effects using nitisinone. These side effects can be ameliorated by reducing the dietary intake of tyrosine (Rathore *et al.*, 2016). Nitisinone reduces urinary HGA excretion by nearly 70% (Cieszyński *et al.*, 2016) to 95%.

Use of nitisinone requires dietary restriction as its side effect is hypertyrosinemia. To delay the onset and progression of AKU, a combination of protein restriction diet, vitamin C, and nitisinone are required. Vitamin C in doses of 1 g/day represents a rational treatment in older children and adults. However, benefit of vitamin C in AKU patients is doubtful. Vitamin C serves as a cofactor for 4-hydroxyphenylpyruvat dioxygenase (HPPD). HPPD results in increase of HGA production (Alajoulin *et al.*, 2015). The use of vitamin C is not recommended (India AKU Society). Long-term safety and efficacy of nitisinone treatment are unknown (Cieszyński *et al.*, 2016).

Introne and Gahl reported that nitisinone reduced urinary HGA excretion by at least 69% in two individuals. However, it elevated plasma tyrosine concentrations, triggering photophobia. The other side effect is cornea crystals. Theoretically, neurologic complications associated with tyrosinemia type III may develop. Additionally, low-dose nitisinone reduced urinary HGA by up to 95% in nine individuals with AKU. In the same study, the author informed the use of nitisinone in seven individuals for up to 15 weeks. These patients received normal protein intake. All had elevated plasma tyrosine concentrations. No ophthalmic, neurologic, or severe dermatologic complications were observed. Two individuals had transient elevations in liver transaminase levels. Liver transaminase levels returned to normal after stopping nitisinone. Introne introduced the idea that in a three-year therapeutically trial, 2 mg of nitisinone reduced urine and plasma HGA by 95%. Plasma tyrosine averaged 800 μM without dietary restriction. Side effects were minimal. One affected individual developed corneal crystals that required discontinuation of nitisinone (Introne and Gahl, 2019).

Use of nitisinone requires long-term studies for further evaluation (Hakim *et al.*, 2018; and Phornphutkul *et al.*, 2002). A murine model for research of AKU disorder have been available (MIM #203500) (Montagutelli, 1994). Davison *et al.* concluded that nitisinone does not have effect on metabolism of monoamine neutransmitter in mice CNS. Nitisinone is unlikely result in changed mood or cognition in AKU patients (Davison *et al.*, 2019).

Many strategies are available for treating AKU; genetic manipulation techniques. These include such as AAV, *Fok*I-dCas9, iPSCs, EJHTs. The EJHTs may include MMEJ-mediated technique and HMEJ-mediated technique. Manipulation genetic techniques may be very effective for treating both AKU with autosomal recessive and AKU with autosomal dominant.

In animal models, programmable nucleases and EJHTs may be useful tools for treating recessive disorders such as hemophilia A and AKU. Nelwan suggested that the CRISPR/Cas9 system in combination with HMEJ-mediated or iPSCs technique may be beneficial to treat monogenic recessive disorder such as hemophilia A, antagonistic disorder, and Friedreich ataxia. The author suggested that the CRISPR/Cas9 system might correct the mutated gene, constructed DSBs, and EJHTs (HMEJ) would correct the nick. It might correct the mutated segments or removed copies in the string. The antagonistic disorder is X-linked recessive inheritance pattern (Nelwan, 2018a). CRISPR/Cas9 system in combination with HMEJ-mediated or iPSCs may be beneficial for treating AKU with autosomal dominant as well.

The use of AAV-mediated RNA interference (RNAi) therapeutic may be beneficial for treating both recessive genetic disorders and dominant genetic disorders. Malerba *et al.* used this strategy to knock down the endogenous wild type and mutant forms of *PAPBN1* allele. The authors also used gene addition to replace the transcript with a compensatory normal human codon-optimized mRNA resistant to RNAi-induced cleavage. A similar pre-clinical strategy has been used to restore the functionality of rhodopsin in photoreceptors of the animal model of Retinitis pigmentosa. Those researchers used this strategy for treating oculopharyngeal and Retinitis pigmentosa, respectively. Oculopharyngeal is an autosomal dominant genetic disorder (Malerba *et al.*, 2017). Rhodopsin is an autosomal recessive or dominant genetic disorder (MIM #180380) (Kartasasmita *et al.*, 2011; and Vaithinathan *et al.*, 1994). It shows that this strategy may be effective for treating AKU with either autosomal recessive, including dominant inheritance, if any.

Pan *et al* developed a modified CRISPR system: *Fok*I-dCas 9. *Fok*I-dCas9 corrects p.Arg408Trp in the *PAH* gene. The p.Arg408Trp is the most common variant in penylketonuria (PKU). PKU as AKU is a rare autosomal recessive disorder inherited accordance with the law of segregation. Pan *et al* suggested that the *Fok*I-dCas9 system could be used for precession medicine for other metabolism diseases (Pan *et al.*, 2016) such as AKU.

5. Conclusion

AKU has an autosomal recessive inheritance pattern. However, the autosomal dominant pattern occur quietly rare and uncommon cases in AKU families. To control AKU, the diagnosis has a significant role. Diagnosis techniques include CT, genetic tests, GCMS, LC-MS/MS, and X-rays. Genetics tests include single-gene testing

and genome sequencing. No efficient drugs are available at present. Use of vitamin C does not have a positive result to elevate patients with AKU. Vitamin C is doubtful for its beneficial for AKU patients. It is not recommended for AKU patients. Genetic counseling may help to direct patients with AKU and their families about how to face or slow down this disorder. Drug candidate to control AKU is nitisinone. Nitisinone has shown remarkable advantage as a drug for treating patients with AKU. Nitisinone does not trigger depression in patients with AKU. Genetic manipulation techniques are potential tools and useful tools to control AKU in the future. These tools may include AAV-mediated RNAi, *Fok*I-dCas9, EJHTs, and iPSCs.

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Conflicts of interest

The author has indicated that he has no conflicts of interests regarding the content of this article.

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